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Mechanistic Insights from Substrate Preference in Unsaturated Glucuronyl Hydrolase

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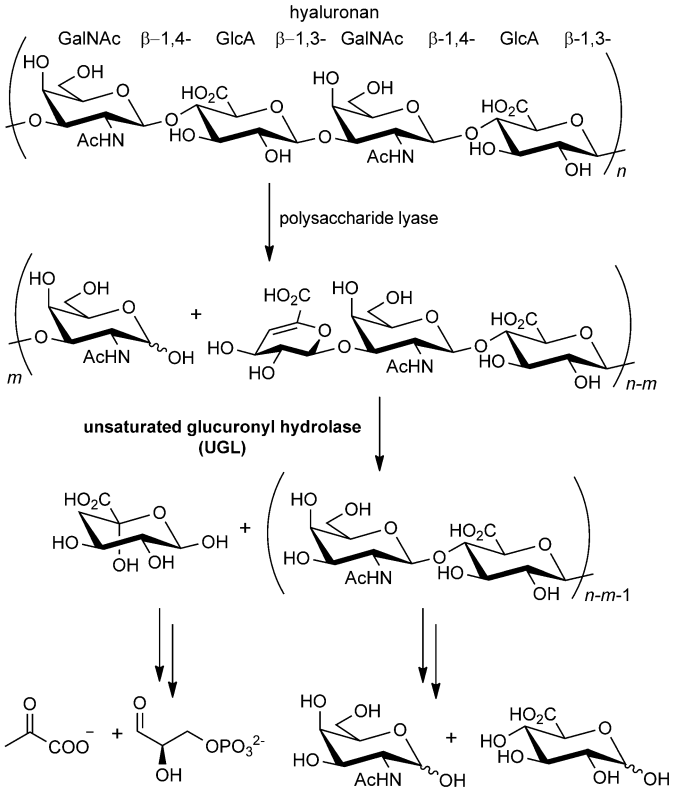
Natural and synthetic unsaturated glucuronides were tested as substrates for *Clostridium perfringens* unsaturated glucuronyl hydrolase to probe its mechanism and to guide inhibitor design. Of the natural substrates, a chondroitin disaccharide substrate with sulfation of the primary alcohol on carbon 6 of its *N*-acetylgalactosamine moiety was found to have the highest turnover number of any substrate reported for an unsaturated glucuronyl hydrolase, with $k_{cat} = 112\ s^{-1}$. Synthetic aryl glycoside substrates with electron-withdrawing aglycone substituents were cleaved more slowly than those with electron-donating substituents. Similarly, an unsaturated glucuronyl fluoride was found to be a particularly poor substrate, with $k_{cat}/K_m = 44\ nM^{-1}s^{-1}$—a very unusual result for a glycoside-cleaving enzyme. These results are consistent with a transition state with positive charge at carbon 5 and the endocyclic oxygen, as anticipated in the hydration mechanism proposed. However, several analogues designed to take advantage of strong enzyme binding to such a transition state showed little to no inhibition. This result suggests that further work is required to understand the true nature of the transition state stabilised by this enzyme.

Introduction

Mammalian tissues are generally composed of cells embedded in a matrix of structural proteins and carbohydrates.[1] The structural elements of this matrix present a significant barrier to the spread of extracellular bacterial infection. To overcome this barrier, many bacteria that cause extracellular infection have evolved a metabolic pathway to degrade glycosaminoglycans, the carbohydrate components of this matrix, and this pathway is a virulence factor.[2] Degradation of glycosaminoglycans removes a physical barrier to the spread of infection while also providing a useful food source for growth. The pathway employed by bacteria for this purpose is composed of enzymes that are mechanistically very different from the native mammalian enzymes responsible for degradation or remodelling of glycosaminoglycans. Because of the unusual mechanistic features of the enzymes in this pathway, and its importance in infectivity, it is surmised that chemical agents able to inhibit or to inactivate this pathway would probably be selective, and that these agents would have potential as therapeutic agents to treat extracellular bacterial infection, particularly as bacteriostatic agents to prevent infection following surgery.

The bacterial pathway for degradation of glycosaminoglycans by bacteria is composed of two key reactions,[3] as shown in Scheme 1. In the first reaction, a polysaccharide lyase cleaves endolytically and generates oligosaccharides with unsaturated uronic acid units at their non-reducing termini. This reaction, achieved through an E1cb mechanism, has already been the subject of much study, and the interested reader is referred to several recent reviews.[4] After endolytic lyase cleav-



Scheme 1. The metabolic pathway for degradation of glycosaminoglycans by bacteria.

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age the oligosaccharides are imported, and a second class of enzymes called unsaturated glucuronyl hydrolases (UGLs) acts on the unsaturated uronic-acid-containing oligosaccharides to remove the unusual non-reducing terminal monomer units.[5] The liberated sugar then enters primary metabolism through a pathway that yields pyruvate and D-glyceraldehyde 3-phosphate,[6] whereas action of glycoside hydrolases on the liberated oligosaccharide affords free monosaccharides that are available to the cell.

UGLs have been much less intensely studied than polysaccharide lyases, but appear also to follow a non-hydrolytic mechanism for glycoside cleavage. The evidence to date[7] shows that UGLs achieve cleavage of the glycosidic bond by hydration of the double bond between carbons 4 and 5 of the substrate non-reducing unsaturated moiety. This generates a hemi-ketal intermediate, which undergoes rearrangement to open the pyranose ring and to form a hemi-acetal, which then further rearranges to cleave the glycosidic bond. This mechanism is shown in Scheme 2; the rate-determining step is assumed to be the hydration reaction, and its transition state is assumed to resemble the high-energy oxocarbenium ion shown in the centre top structure.

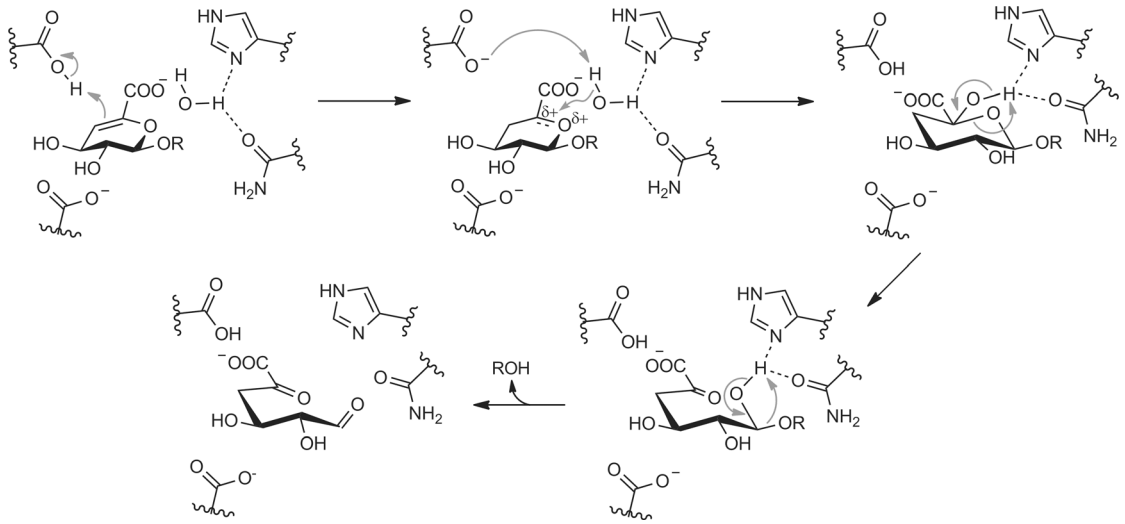
The current understanding of the UGL chemical mechanism is based on a series of X-ray crystal structures as well as on one mechanistic study. In the substrate-bound X-ray crystal structure of *Bacillus* sp. GL1 UGL the positioning of potential catalytic residues was observed to be unsuitable for a conventional Koshland-type glycosidase mechanism.[7a] On the basis of this observation the hydration-initiated mechanism was proposed, but limited evidence was presented. A subsequent study[7b] demonstrated that the sites of proton and nucleophile addition are indeed at carbons 4 and 5, respectively, as required by this hydration-initiated mechanism. Hydration of substrates with carbon- and sulfur-linked groups at the anomeric carbon—an outcome only possible under such a mechanism—was also observed. For the 4-deutero substrate a kinetic isotope effect of $k_H/k_D = 1.06$ on k_{cat}/K_m was measured; this was consistent with the hydration-initiated mechanism and provided insight into the transition-state structure for the first irreversible step of this mechanism.

In an attempt to inform both mechanistic understanding of the UGL hydration-initiated glycoside cleavage reaction and eventual design of UGL inhibitors or inactivators, the effect of variations in the UGL substrate on the reaction rate with *Clostridium perfringens* UGL was investigated in this work. These substrates were natural disaccharides sourced from polysaccharide-lyase-cleaved glycosaminoglycans, as well as synthetic aryl pseudo-disaccharides and unsaturated glucuronyl fluorides. Several candidate inhibitor leads, designed on the basis of current understanding of the UGL mechanism, were also tested, but were found to bind poorly. These results suggest that the current understanding of the UGL mechanism remains incomplete, particularly in the details of the hydration reaction itself.

Results and Discussion

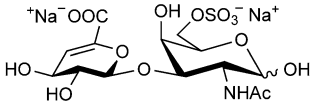
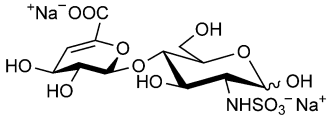
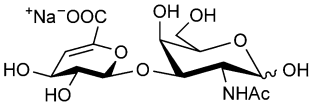
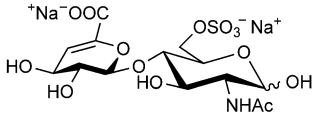
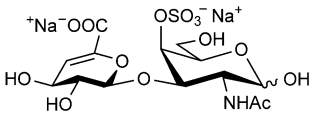
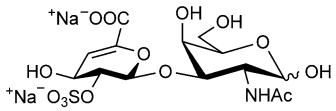
Natural substrates

The natural substrates for *C perfringens* UGL are the products of the action of polysaccharide lyases on glycosaminoglycans. As such, these substrates can vary significantly in their glycosidic linkages and sulfation patterns, depending on the source glycosaminoglycan. Other UGLs that have previously been studied, from both pathogenic and non-pathogenic source organisms, have shown substantial variability in their preferred substrates.[3,8] In order to probe the natural substrate preferences of *C. perfringens* UGL, the Michaelis–Menten kinetic parameters for the cleavage of a variety of unsaturated disaccharides of glycosaminoglycan origin were determined, as shown in Table 1, and compared with those of equivalent enzymes from other species (Table S1 in the Supporting Information). Activity was highly variable with these substrates, ranging from the highest k_{cat} reported for an UGL with any substrate, at 112 s^{-1}



Scheme 2. The currently accepted mechanism for UGLs.

Table 1. Kinetic parameters for *C. perfringens* UGL with natural substrates.

Substrate structure	#	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [s^{-1} mM^{-1}]
	**1**	112 ± 7	3.7 ± 0.8	30 ± 8
	**2**	14 ± 2	1.2 ± 0.4	12 ± 6
	**3**[7b]	8.5 ± 0.3	3.0 ± 0.2	2.8 ± 0.3
	**4**	–	–	0.03 ± 0.003
	**5**	0.09 ± 0.006	4.5 ± 0.6	0.020 ± 0.004
	**6**	–	–	n.d.[a]

[a] No activity detected up to 7.5 mM.

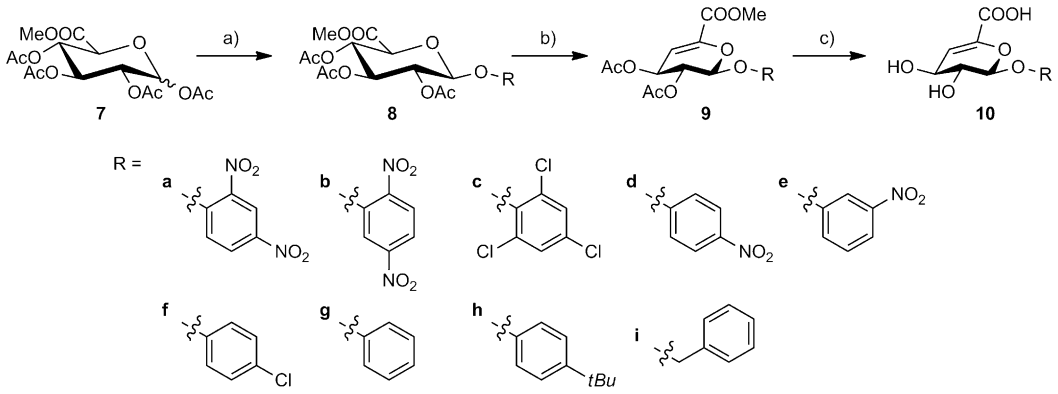
(**1**), to no detectable activity (**6**). The reason for this particularly high k_{cat} is unclear, but *C. perfringens* UGL does contain the 6-sulfate binding domain identified by crystallography in *Streptococcus agalactiae* UGL.[9] It is possible that in *C. perfringens* UGL this domain is acting to improve turnover by optimising placement of catalytic residues, and thus turnover, rather than substrate binding, as was seen for *S. agalactiae* UGL. K_m was seen to be less variable among the better substrates. For one substrate (**4**), K_m could not be determined, because substrate saturation could not be achieved within the limitations of the assay and compound availability. Substrate disaccharides derived both from chondroitin and from heparin sulfate were accepted by *C. perfringens* UGL, with no clear a priori determinant of the level of activity.

The lack of activity with a 2′-sulfated substrate is not particularly surprising because the negatively charged sulfate is placed in close proximity to Asp88 (*Bacillus* sp. GL1 numbering), one of the active-site residues shown by mutagenesis to be crucial for catalytic activity,[10] although its role remains unclear. The interaction of these two negative charges could very plausibly interfere with that residue's (currently unknown) catalytic function. However, it is notable that UGLs from some bacterial strains were able to cleave this substrate. The Streptococcal strains (see the Supporting Information) all showed low levels of hydrolysis for such substrates, and for *Bacillus* this is a close third-best substrate. Clearly, it is possible for UGL to accommodate a sulfate in this position, but how this occurs and what this means for the catalytic role of this residue is unclear. It is possible that the sulfate group itself takes over the role of Asp88 in these cases because it has a similar charge and probably occupies a similar location.

Linear free-energy relationship

Measurement of a leaving group free-energy relationship for the UGL-catalysed hydrolysis of aryl glycosides was undertaken as a way of probing the magnitude and nature of charge development on the phenol oxygen in the transition state. To measure such a free-energy relationship, a series of aryl unsaturated glycosides was synthesised chemically, by the methods summarised in Scheme 3.

Kinetic parameters for the hydrolysis of these substrates by UGL are summarised in Table 2. The best substrates were found to be those with higher pK_a values, largely as a result of higher k_{cat} values rather than of higher k_{cat}/K_m values. Log plots of k_{cat} and k_{cat}/K_m against pK_a are given in Figure 1 and reveal



Scheme 3. Synthesis of aryl unsaturated glucuronides. Conditions: a) for **8a**–**e** i: HBr/AcOH, Ac_2O, CH_2Cl_2, 4 °C, ii: substituted phenol, Ag_2O, MeCN; for **8f**–**h** i: hydrazine acetate, CH_2Cl_2/MeOH, 0 °C to RT, ii: trichloroacetonitrile, DBU, CH_2Cl_2, −78 °C to RT, iii: substituted phenol, BF_3·EtO_2, CH_2Cl_2, −78 °C to RT; for **8i** i: hydrazine acetate, CH_2Cl_2/MeOH, 0 °C to RT, ii: benzyl bromide, Ag_2CO_3, toluene; b) DBU, CH_2Cl_2; c) for **10a** and **10b** HCl_{aq}; for **10c** and **10f**–**i** $NaOH_{aq}$; for **10d** i: NaOMe (cat.), MeOH, 0 °C, ii: LiOH, THF/H_2O, 0 °C; for **10e** i: NaOMe, MeOH, 0 °C, ii: H_2O.

Table 2. Kinetic parameters for *C. perfringens* UGL with synthetic aryl glycoside substrates.

#	pK_a	k_{cat} [s^{-1}]	K_m [mM]	k_{cat}/K_m [$mM^{-1}s^{-1}$]
10a	3.96	0.140 ± 0.005	0.16 ± 0.02	0.9 ± 0.1
10b	5.15	0.64 ± 0.04	0.59 ± 0.06	1.1 ± 0.2
10c[7b]	6.39	0.312 ± 0.007	0.58 ± 0.04	0.55 ± 0.05
10d[7b]	7.18	2.05 ± 0.06	0.26 ± 0.02	7.9 ± 0.8
10e	8.39	14.7 ± 0.5	0.88 ± 0.03	16 ± 1
10f	9.38	3.9 ± 0.1	0.84 ± 0.09	4.6 ± 0.6
10g[7b]	9.99	4.3 ± 0.2	3.2 ± 0.4	1.3 ± 0.2
10h	10.37	3.27 ± 0.02	0.88 ± 0.02	3.7 ± 0.1
10i	15.40	18.6 ± 0.6	9.0 ± 0.5	2.1 ± 0.2

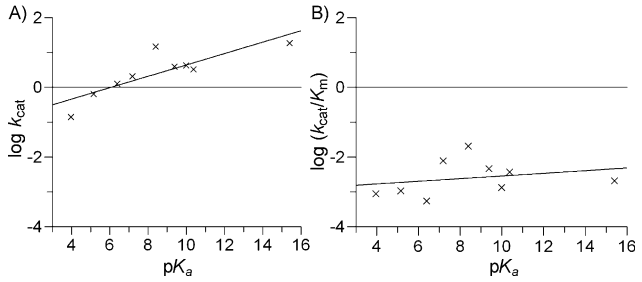


Figure 1. Plots of A) log k_{cat}, and B) log (k_{cat}/K_m) against pK_a for hydrolysis of aryl unsaturated glucuronides by *C. perfringens* UGL.

slopes of $\beta lg = 0.16 \pm 0.04$ and $\beta lg = 0.04 \pm 0.05$, respectively. The error range for the plot of log (k_{cat}/K_m) includes zero, and so it is considered to be a flat line, showing negligible influence from electron-withdrawing effects of the leaving group on the first irreversible step. The slope of log k_{cat} is low and positive, and the error range excludes zero, showing that more strongly electron withdrawing groups make the overall rate-determining step slower. This is the opposite trend to what is typically observed with glycoside hydrolases,[11] as well as with polysaccharide lyases[12] and α-1,4-glucan lyases.[13]

The trend observed in Figure 1 is consistent with a transition state involving a partial positive charge that is destabilised by electron withdrawing groups—either a small amount of charge close to the leaving group or a larger charge at a greater distance. This second situation matches what would be expected from the currently accepted hydration-initiated mechanism shown in Scheme 2. The observation of a positive slope is also consistent with the kinetic isotope effect on k_{cat}/K_m resulting from deuterium-for-hydrogen substitution at carbon 4, which was previously measured to be small and normal.[7b] This normal value was interpreted as arising from substantial positive charge development at carbon 5 during the first irreversible step, with the effect from hyperconjugation being large enough to outweigh the effect from change in hybridisation.

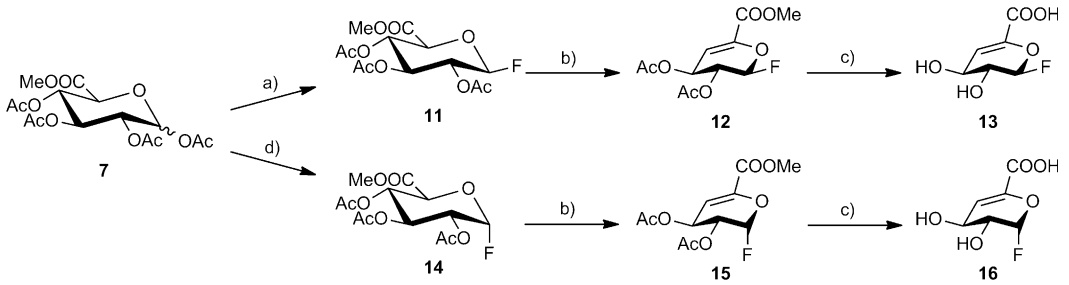
Glycosyl fluoride substrate

To investigate this trend further, a substrate with an even more activated leaving group, and thus an even more strongly electron withdrawing effect, was sought in pseudo-equatorially configured unsaturated glucuronyl fluoride **13** (Scheme 4). The high electronegativity of fluorine generally makes glycosyl fluorides very good substrates for carbohydrate-active enzymes; indeed, they are good substrates for essentially all previously studied glycoside hydrolases,[14] including those with eliminative mechanisms such as glycoside hydrolases from family GH31 (α-1,4-glucan lyase),[13] whereas 4-fluoro-substituted glucuronides are good substrates for polysaccharide lyases.[12] In the hydration-initiated mechanism currently accepted for UGL, however, this unsaturated glucuronyl fluoride was expected to be a very poor substrate.

Because fluorine is an almost isosteric substitution with hydrogen, and hydrolysis of both anomers of phenyl unsaturated glucuronide **10g** has previously been observed,[7b] pseudo-axially configured unsaturated glucuronyl fluoride **16** (Scheme 4), the other anomer of **13**, was also synthesised as a potential substrate.

Testing of these two substrates revealed that the pseudo-equatorial unsaturated glucuronyl fluoride **13** was a very poor substrate for UGL, as predicted, with a k_{cat}/K_m value of 44 ± 2 $nM^{-1}s^{-1}$ (over five orders of magnitude lower than the worst aryl substrates in Table 2). This matches the trend seen with increasingly electron-withdrawing substituents in the previous section, but is a stronger effect than would be predicted solely on the basis of electronegativity. Such a low relative activity for a glycoside-cleaving enzyme with a fluorinated substrate is highly unusual.

Hydrolysis of the pseudo-axial unsaturated glucuronyl fluoride **16** by UGL was not detectable at up to 15 mM substrate. This lack of any activity with **16** is somewhat surprising given



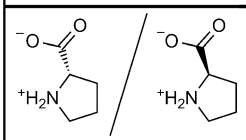
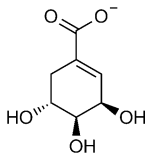
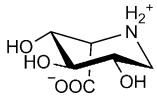
Scheme 4. Synthesis of unsaturated glucuronyl fluorides. Conditions: a) i: HBr/AcOH, Ac_2O, CH_2Cl_2, 4 °C, ii: AgF, MeCN; b) DBU, CH_2Cl_2; c) i: NaOMe (cat.), MeOH, 0 °C, ii: LiOH, THF/H_2O, 0 °C; d) HF/pyridine, 4 °C.

that **13** is cleaved by UGL, that both isomers of phenyl unsaturated glucuronide are cleaved by this enzyme, and that the anomeric substituent is not involved in the rate-determining step. Lack of binding is unlikely to be the cause, given the small size of fluorine. Indeed, compound **16** was shown to bind to the UGL active site as a competitive inhibitor with a K_i of 6.4 ± 1.2 mM, similar to the K_i of 10.7 ± 0.9 mM determined for **13** (see the Supporting Information). Most probably the apparent inactivity has its origin in inherent reactivity differences for the α and β anomers. β-Glucosyl fluoride hydrolyses some 40 times faster that its α anomer,[15] largely as a consequence of its higher ground state energy. On the presumption that a similar effect is in play in this case, a k_{cat} value 40 times lower than that of **13** would be at the limit of detection.

Inhibition

Compounds that mimic the transition state of a reaction are often very potent competitive inhibitors, because they recruit the very strong interactions normally developed between an enzyme and its substrate at the transition state. Development of transition state analogue inhibitors can therefore serve as a useful test of our understanding of the enzymatic transition state, as well as an entry into potential therapeutics.[16] Several candidates for UGL transition state mimics, based on geometry and charge placement, were tested as competitive inhibitors. The data for these inhibitors are shown in Table 3.

Table 3. Candidate inhibitors for *C. perfringens* UGL.

Inhibitor structure	#	K_i [mM]	Type of inhibition
	**17a**, **17b**	n.d.[a]	none
	**18**	3.0 ± 0.4	competitive
	**19**	7.2 ± 0.9	competitive

[a] No activity detected up to 10 mM.

The simplest potential inhibitors tested were the L and D isomers of proline (**17a** and **17b**, respectively) which were anticipated to be improved versions of the previously reported inhibitor glycine ($K_i = 6.5$ mM for *Bacillus* sp. GL1 UGL)[10] because the ring structure more closely resembles the substrate. The positively charged nitrogen was anticipated to occupy the same position as the endocyclic oxygen, in the hope that it might take advantage of binding interactions that stabilise the partial positive charge developing at the transition state. Disappointingly, compounds **17a** and **17b** were non-inhibitory. Closer examination of the crystal structure of the *Bacillus* sp GL1 UGL·glycine complex revealed that glycine binds with its amino group between the two catalytically important aspartate residues,[10] and not in the same location as the substrate endocyclic oxygen.[7a] Such a binding mode appears to be unavailable to proline because of its longer carbon chain.

Shikimic acid (**18**), a precursor to the aromatic amino acids, was tested as an inhibitor on the basis of potential mimicry of the geometry either of the transition state or of the substrate. The three free hydroxy groups in **18** allow binding of the double bond in the positions either of the substrate carbons 4 and 5 or of the transition state carbon 5 and endocyclic oxygen, although there are some stereochemical mismatches. Mimicry of transition state geometry and conformation is one of the contributing factors to the strong inhibition of influenza neuraminidase by inhibitors such as 2′-deoxy-3′-dehydro-*N*-acetyl-α-D-neuraminic acid ("DANA"), zanamivir ("Relenza") and oseltamivir ("Tamiflu").[17] Although **18** was observed to bind and act as a competitive inhibitor, the affinity is very similar to that of the substrates reported here; this thus suggests shikimic acid is not a transition state geometry mimic for UGL. The ring double bond of shikimic acid was also observed not to be hydrated by UGL, unlike that in the *C*-glycoside analogue Kdn2en;[7b] this is consistent with an important role for the endocyclic oxygen in turnover. However, as mentioned above, the stereochemistry of the hydroxy groups in shikimic acid also does not match that of UGL substrates, which prevents any strong conclusions from being drawn from this observation.

A closer mimic of the transition state charge pattern for the hydration reaction shown in Scheme 2 was sought in L-idurono-deoxynojirimycin (**19**). This structure mimics the positioning of the carboxylate group of the hydrated product,[7b] as well as placing a positively charged amino group in the same position as the endocyclic oxygen. Although this compound contains a hydroxy group at carbon 4, this is expected to be accommodated by the enzyme active site because D-glucuronic acid has previously been shown to inhibit the enzyme (although the type of inhibition was not demonstrated, and no K_i value was determined).[8a] L-Idurono-deoxynojirimycin was indeed observed to bind to *C. perfringens* UGL as a competitive inhibitor, but the affinity is low and it is clearly not acting as a transition state mimic.

Conclusions

Presented here are kinetic data for a series of natural and synthetic substrates of *Clostridium perfringens* UGL, with the aim of improving our understanding of the catalytic mechanism of this unusual enzyme, as well as testing of several inhibitor candidates based on this information. The best natural substrate found in this work is the unsaturated chondroitin-derived disaccharide with sulfation at carbon 6 of the *N*-acetylgalactosamine moiety, with the highest k_{cat} reported for any UGL to date. However, the pattern of optimal substrate activity clearly varies with species, meaning that design of an inhibitor mim-

icking the charge pattern of substrate sulfates is of limited use. Synthetic aryl glycoside substrates were seen to bind more strongly than the natural substrates to *C. perfringens* UGL, but with slightly lower turnover numbers. A trend with a positive slope was clearly observed in a plot of $\log k_{cat}$ for these aryl substrates against the pK_a values of their aglycones. This is consistent with a positively charged transition state, with this charge being located far from the anomeric centre. This charge is presumed, on the basis of the currently accepted mechanism, to be on carbon 5 and the endocyclic oxygen. A similar plot of $\log(k_{cat}/K_m)$ against pK_a showed no clear trend, with the slope error range including zero. In stark contrast to what is found for glycosidases that follow "Koshland" mechanisms, the glycosyl fluorides showed little or no turnover, consistent with the notion that highly electronegative anomeric substituents deactivate the protonation step.

The inhibition results presented here suggest that UGL does not strongly bind to, and stabilise, a transition state with substantial positive charge on the endocyclic oxygen within a pyranose ring. This suggests that the positive charge arising from proton donation remains localised on carbon 5, perhaps stabilised by means other than electron donation from the adjacent endocyclic oxygen. Candidates for this stabilisation include the carbon 6 carboxylate group or some through-space effect. Notably, Asp88 (*Bacillus* sp. GL1 numbering), the residue shown by mutagenesis to be important for catalysis but with unknown function, is not appropriately placed to stabilise such a charge directly. Design of a transition state mimic with positive charge at an analogous position to carbon 5 is more difficult because of steric limitations at this centre and the adjacent negative charge on the carbon 6 carboxylate group. Further work on understanding the mechanism of UGL and its transition state is clearly needed if strong-binding inhibitors are to be designed on the basis of transition state mimicry.

Experimental Section

General materials and methods: Chemicals were of reagent grade or higher, purchased from Sigma–Aldrich unless otherwise stated, and used without further purification. Natural substrates **1**, **2**, **3**, **4**, **5** and **6** were purchased from Carbosynth, UK (http://www.carbosynth.com/). L-Idurono-deoxynojirimycin (**19**) was a kind gift from Dr. Ethan Goddard-Borger and was synthesised essentially according to Bashyal et al.[18] *Clostridium perfringens* UGL was expressed and purified as previously reported,[7b] and hydrolysis reaction conditions were optimised. TLC was performed on pre-coated 60F 254 silica plates (Merck), with visualisation by use of UV light followed by charring with ammonium molybdate (10%) in H_2SO_4 (2 M). Flash column chromatography was performed with 230–400 mesh silica gel and an in-house compressed air system. For anhydrous reactions solvents were freshly distilled (CH_2Cl_2 over CaH_2, MeOH over Mg), and glassware was dried in an oven. NMR spectra were recorded with Bruker Avance 300 and 400 (with either an inverse or a direct probe) spectrometers at 300 and 400 MHz, respectively. Chemical shifts are reported in δ scale in parts per million from tetramethylsilane (TMS) with internal reference to solvent for ^1H and ^{13}C shifts, whereas ^{19}F shifts are reported relative to an external standard of $CFCl_3$ at 0 ppm. All couplings are reciprocal. Low-resolution mass spectra were recorded with a Waters ZQ instrument fitted with ESCI ion source and Waters 2695 HPLC for sample delivery, whereas high-resolution mass spectra were submitted to the University of British Columbia mass spectrometry facility for analysis with a Waters/Micromass LCT instrument with electrospray ionisation and time of flight detection in either positive or negative mode. HPLC was carried out with an Agilent eclipse XD-C18 column (5 μm pore size, 9.4×250 mm) on a Waters 600 instrument at 4 mL min^{-1} and monitored by a Waters 2996 photodiode-array detector.

Kinetics: Kinetic measurements were performed in matched reduced-volume quartz cuvettes (path length 1 cm, or 0.1 cm for above 1 mM of non-chromogenic substrates) with a Varian Cary 4000 spectrophotometer, an automatic cell changer and a Peltier temperature controller, at 37 °C unless otherwise specified. Reaction mixtures were allowed to pre-incubate for 5 min before addition of enzyme to start the reaction. All nonlinear regression was performed with GraFit 5.0 (Erithacus Software Limited; http://www.erithacus.com/grafit). Michaelis–Menten kinetic parameters were determined by measurement of initial rates in MES·NaOH buffer (pH 6.6, 50 mM) with BSA (1 mg mL^{-1}) and UGL (around 1 μM), varied to give accurate initial rates at all concentrations of a given substrate (MES·NaOH buffer was subsequently found to be a competitive inhibitor—$K_i = 57 \pm 5$ mM—but its use was continued at the same concentration to allow comparison with previously published results). Extinction coefficients (see the Supporting Information) were determined by allowing a 1 mM reaction to run to completion overnight, and wavelength was selected on the basis of largest difference in absorbance within the linear range of the spectrophotometer (total Abs ≤ 3.5). Initial rates were measured over a range of at least five different substrate concentrations, ranging from at least $K_m/5$ to $5\times K_m$ ($K_m/7$ to $7\times K_m$ where possible), and the Michaelis–Menten equation was fitted by non-linear regression. Where reactions were linear over a sufficiently long time, several rates were measured simultaneously with the aid of an automated cell changer.

With the heparin- and chondroitin-derived natural substrates (**1**, **2**, **3**, **4**, **5** and **6**) saturation kinetics could not be attained because rates for substrate concentrations over 5 mM could not be reliably measured due to high initial absorbance, as the reactions were monitored through decreases in absorbance. The particularly poorly binding substrates heparan 6-sulfate disaccharide (**4**) and pseudo-equatorial unsaturated glucuronyl fluoride **13** showed very little deviation from linear dependence of rate on substrate concentration over this range, so initial rates were fitted with a linear model in GraFit 5.0 to determine k_{cat}/K_m, with K_m approximated through estimation of K_i for **13**. Plots of rate against substrate concentration are provided in the Supporting Information.

To estimate K_i for the pseudo-equatorial unsaturated glucuronyl fluoride **13**, which had been shown in other assays to act as a substrate, it was assumed that inhibition was competitive. With this compound, the inhibition constant was approximated by measurement of seven rates with varied inhibitor concentrations and a fixed substrate (**10d**) concentration slightly above K_m. Plotting of the reciprocals of these rates against the inhibitor concentration allowed determination of an intercept with the inverse of V_{max}, defined as $-1/K_i$.

All other inhibitors were assayed with a matrix of substrate and inhibitor concentrations bracketing K_m and a previously estimated K_i value (by the method outlined in the previous paragraph with the assumption of competitive inhibition), respectively. If inhibition was observed, these rates were then fitted to modified Michaelis–

Menten equations describing the reaction in the presence of competitive, non-competitive and mixed-type inhibition by non-linear regression. The equation giving the lowest errors was deemed to be the most appropriate; its selection was corroborated by plotting 1/rate against inhibitor concentration (a Dixon plot) and observing the intersection of all plots at $X=-1/K_i$ and $Y=1/V_{max}$.

General method for Koenigs–Knorr glycosylation

Bromination: Globally protected glucuronic acid **7** (1.18 g, 3.13 mmol) was dissolved in dichloromethane (4.5 mL). Acetic anhydride (0.5 mL) and HBr in acetic acid (33%, 20 mL) were then added at 0 °C, and the mixture was stirred at 4 °C until the reaction was complete as determined by TLC (petroleum ether/ethyl acetate 2:1). The reaction was quenched in ice/water, the aqueous layer was extracted thrice with dichloromethane, and this pooled organic phase was then extracted quickly with cold water, cold sat. $NaHCO_3$ (twice) and brine. After drying over $MgSO_4$, concentration, and co-evaporation from toluene, the colourless syrup (1.20 g, 3.02 mmol, 97%) was used without further purification. A sample for analysis was purified by flash column chromatography (petroleum ether/ethyl acetate 3:1), yielding a white powder. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.67$ (d, $J_{1,2}=4.0$ Hz, 1H; H-1), 5.64 (t, $J_{2,3}=J_{3,4}=9.8$ Hz, 1H; H-3), 5.27 (dd, $J_{4,5}=10.3$ Hz, 1H; H-4), 4.88 (dd, 1H; H-2), 4.61 (d, 1H; H-5), 3.79 (s, 3H; OMe), 2.13 (s, 3H; OAc), 2.09 (s, 3H; OAc), 2.08 ppm (s, 3H; OAc); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=169.63$ (2OAc), 169.43 (OAc), 166.6 (C-6), 85.5 (C-1), 72.1 (C-3), 70.3 (C-2), 69.3 (C-4), 68.5 (C-5), 53.1 (OMe), 20.60 (2OAc), 20.45 ppm (OAc); MS: *m/z* calcd: 419.0/421.0 $[M+Na]^+$; found: 419.2/421.2.

Glycosylation: The relevant acceptor phenol (≈1.3 equiv) was dried by co-evaporation from acetonitrile where necessary and then dissolved along with the above-formed glycosyl bromide in dry acetonitrile (to give approximately 0.1 M sugar). Ag_2O (2–3 equiv) was added, and the reaction mixture was stirred vigorously overnight at ambient temperature in the dark. The reaction mixture was then filtered through a plug of Celite and concentrated before being dissolved in ethyl acetate and washed extensively with sat. $NaHCO_3$, water and brine. The organic phase was dried over $MgSO_4$, and the solvent was then evaporated in vacuo before further purification as necessary.

General method for glycosylation by Schmidt donor

Anomeric deprotection: Globally protected glucuronic acid **7** (1.902 g, 5.05 mmol) was dissolved in dichloromethane/methanol (1:1, 90 mL), hydrazine acetate was added (0.719 g, 1.6 equiv), and the reaction mixture was stirred at 0 °C and then at ambient temperature (2 h each). The solvent was subsequently evaporated in vacuo, the reaction mixture was dissolved in ethyl acetate, and the product was washed with water, HCl (1 M), and brine and then dried over $MgSO_4$. The product was purified by flash column chromatography (petroleum ether/ethyl acetate 3:2 to 1:1) to give a white foam (1.09 g, 3.26 mmol, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=5.50$ (t, $J_{2,3}=J_{3,4}=9.4$ Hz, 1H; H-3), 5.46 (d, $J_{1,2}=3.0$ Hz, 1H; H-1), 5.09 (t, $J_{4,5}=10.1$ Hz, 1H; H-4), 4.82 (dd, 1H; H-2), 4.52 (d, 1H; H-5), 3.66 (s, 3H; OMe), 2.01 (s, 3H; OAc), 1.96 (s, 3H; OAc), 1.95 ppm (s, 3H; OAc); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=170.2$ (OAc), 170.0 (OAc), 169.7 (OAc), 168.5 (C-6), 89.9 (C-1), 70.6 (C-3), 69.4 (C-2), 69.0 (C-4), 67.7 (C-5), 52.7 (OMe), 20.5 (2OAc), 20.3 ppm (OAc); MS: *m/z* calcd 357.1 $[M+Na]^+$; found: 357.3.

Glycosylation: The above globally protected glucuronyl hemiacetal (0.4 g, 1.20 mmol) was dissolved in dichloromethane (12 mL), molecular sieves (4 Å) were added, and the reaction vessel was flushed with argon and then cooled in a dry ice/acetone bath. Trichloroacetonitrile (1.2 mL, 10 equiv) and then 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU, 0.25 mL, 1.5 equiv) were added, and the reaction mixture was stirred for 30 min at −78 °C and then allowed to warm to ambient temperature. The reaction mixture was concentrated and filtered through a plug of silica gel (elution with petroleum ether/ethyl acetate 2:1).

The resulting crude trichloroacetimidate intermediate and the relevant acceptor phenol (≈1.3 equiv) were dissolved in dichloromethane (25 mL), and the mixture was dried over molecular sieves (4 Å) under argon and then cooled in a dry ice/acetone bath. Boron trifluoride diethyl etherate (0.05 mL, 0.3 equiv) was added, and the reaction mixture was stirred for 30 min at −78 °C before being allowed to warm to ambient temperature with stirring overnight. The mixture was subsequently diluted with an equal volume of dichloromethane and washed with sat. $NaHCO_3$ (3×) and brine. The organic phase was dried over $MgSO_4$, and the solvent was then evaporated in vacuo.

General method for DBU-promoted elimination: Protected aryl glucuronide was dissolved in dichloromethane (to give approximately 0.1 M of sugar), DBU (1.3 equiv) was added slowly through a septum, and the reaction mixture was stirred at room temperature overnight. When found to be necessary, molecular sieves (4 Å) were used and the solution was flushed to dryness with argon or nitrogen before addition of reagent. Once the reaction was complete (as judged by TLC), the reaction mixture was concentrated and filtered through a plug of silica (washing with ethyl acetate/petroleum ether 2:1) before further purification as necessary.

General method for ester saponification: The protected substrate was dissolved in acetone to approximately 0.1 M and cooled to 0 °C. An equal volume of NaOH (1 M) was added. The reaction mixture was stirred for 5 min and then quenched with a slight excess of HCl (1 M) before further purification as necessary.

General method for Zemplén deprotection: The acetylated compound was dissolved in dichloromethane/methanol (1:1, to approximately 0.1 M) and cooled to 0 °C. Either sodium methoxide in methanol (stock at 5.4 M) or a small piece of sodium metal was then added to give a final concentration of between 5 and 50 mM. Deacetylation was monitored by TLC, and upon completion the reaction was quenched with Sephadex ion exchange resin (H^+ form) and then filtered, before further purification as necessary.

General method for hydrolysis with aqueous lithium hydroxide: The methyl-ester-protected compound was dissolved in THF and water (1.75:1, to approximately 0.1 M), the mixture was cooled to 0 °C, and lithium hydroxide (1 M, 2 equiv) was added. The reaction was allowed to proceed for 5 min before quenching with Sephadex ion exchange resin (H^+ form) and was then filtered, before further purification as necessary.

Protected glucuronide 8a: 2,4-Dinitrophenol (0.983 g, 5.34 mmol, 3.75 equiv) was used as acceptor in the general method for Koenigs–Knorr glycosylation with globally protected glucuronyl bromide (0.585 g, 1.47 mmol). The resulting off-white powder (0.580 g, 1.16 mmol, 79%) was used without further purification. ^{1}H NMR (300 MHz, $[D_6]$DMSO): $\delta=8.79$ (d, $J_{3',5'}=2.8$ Hz, 1H; H-5'), 8.54 (dd, $J_{2',3'}=9.3$ Hz, 1H; H-3'), 7.65 (d, 1H; H-2'), 5.97 (d, $J_{1,2}=7.4$ Hz, 1H; H-1), 5.45 (t, $J_{2,3}=J_{3,4}=9.3$ Hz, 1H; H-3), 5.16 (t, 1H; H-2), 5.13 (t, $J_{4,5}=9.9$ Hz, 1H; H-4), 4.80 (d, 1H; H-5), 3.62 (s, 3H; OMe), 2.02 (s, 3H; OAc), 2.01 (s, 3H; OAc), 2.00 ppm (s, 3H; OAc).

Protected glucuronide 8b: 2,5-Dinitrophenol (0.245 g, 1.33 mmol, 1.3 equiv) was used as acceptor in the general method for Koe-

nigs–Knorr glycosylation with globally protected glucuronyl bromide (0.403 g, 1.02 mmol). The resulting off-white powder (0.442 g, 0.883 mmol, 85%) was used without further purification. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.22–8.11 (m, 3H; H-3′,4′,6′), 5.98 (d, $J_{1,2}$ = 7.4 Hz, 1H; H-1), 5.41 (t, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, 1H; H-3), 5.16 (t, $J_{4,5}$ = 9.5 Hz, 1H; H-4), 5.14 (dd, 1H; H-2), 4.83 (d, 1H; H-5), 3.64 (s, 3H; OMe), 2.02 (s, 6H; 2OAc), 1.99 ppm (s, 3H; OAc).

Protected glucuronide 8e: 3-Nitrophenol (0.220 g, 1.58 mmol, 1.25 equiv) was used as acceptor in the general method for Koenigs–Knorr glycosylation with globally protected glucuronyl bromide (0.505 g, 1.27 mmol). Purification by flash column chromatography (petroleum ether/ethyl acetate 2:1) yielded an off-white powder (0.304 g, 0.668 mmol, 53%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 7.95 (ddd, $J_{4',5'}$ = 8.2, $J_{2',4'}$ = 2.2, $J_{4',6'}$ = 0.8 Hz, 1H; H-4′), 7.80 (t, $J_{2',6'}$ = 2.2 Hz, 1H; H-2′), 7.65 (t, $J_{5',6'}$ = 8.2 Hz, 1H; H-5′), 7.48 (ddd, 1H; H-6′), 5.87 (d, $J_{1,2}$ = 7.8 Hz, 1H; H-1), 5.46 (t, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, 1H; H-3), 5.14 (dd, 1H; H-2), 5.09 (t, $J_{4,5}$ = 9.9 Hz, 1H; H-4), 4.76 (d, 1H; H-5), 3.62 (s, 3H; OMe), 2.02 (s, 3H; OAc), 2.01 (s, 3H; OAc), 1.99 ppm (s, 3H; OAc).

Protected glucuronide 8f: 4-Chlorophenol (0.19 g, 1.48 mmol, 1.2 equiv) was used as acceptor in the general method for glycosylation by Schmidt donor with globally protected glucuronyl hemiacetal (0.4 g, 1.20 mmol). Purification by flash column chromatography (petroleum ether/ethyl acetate 3:1) yielded a white powder (0.103 g, 0.232 mmol, 20%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.25 (d, $J_{2',3'}$ = $J_{5',6'}$ = 9.0 Hz, 2H; H-3′,5′), 6.94 (d, 2H; H-2′,6′), 5.36–5.23 (m, 3H; H-2,3,4), 5.11 (d, $J_{1,2}$ = 7.2 Hz, 1H; H-1), 4.19 (d, $J_{4,5}$ = 9.5 Hz, 1H; H-5), 3.73 (s, 3H; OMe), 2.06 (s, 3H; OAc), 2.05 (s, 3H; OAc), 2.04 ppm (s, 3H; OAc).

Protected glucuronide 8h: 4-*tert*-Butylphenol (0.25 g, 1.66 mmol, 1.4 equiv) was used as acceptor in the general method for glycosylation by Schmidt donor with globally protected glucuronyl hemiacetal (0.4 g, 1.20 mmol). Purification by flash column chromatography (petroleum ether/ethyl acetate 3:1) yielded an amorphous solid (0.158 g, 0.339 mmol, 28%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.31 (d, $J_{2',3'}$ = $J_{5',6'}$ = 8.9 Hz, 2H; H-3′,5′), 6.93 (d, 2H; H-2′,6′), 5.36–5.25 (m, 3H; H-2,3,4), 5.14 (d, $J_{1,2}$ = 7.2 Hz, 1H; H-1), 4.19 (d, $J_{4,5}$ = 9.5 Hz, 1H; H-5), 3.74(s, 3H; OMe), 2.06 (s, 3H; OAc), 2.05 (s, 3H; OAc), 2.05 (s, 3H; OAc), 1.30 ppm (s, 9H; *tert*-butyl).

Protected glucuronide 8i: Globally protected glucuronyl hemiacetal (see first half of general method for glycosylation by Schmidt donor, 0.343 g, 1.03 mmol) was dissolved in toluene (2 mL), and the solution was flushed with nitrogen. Benzyl bromide (0.245 mL, 2 equiv) and silver(I) carbonate (0.885 g, 3 equiv) were added, and the reaction mixture was stirred overnight at ambient temperature in the dark. The reaction was subsequently quenched with triethylamine, and the mixture was diluted with dichloromethane and filtered through Celite, after which the filtrate was washed with HCl (1 M), saturated $NaHCO_3$, water and brine and then dried over $MgSO_4$. Purification by flash column chromatography (petroleum ether/ethyl acetate 7:2) followed by recrystallisation from toluene/petroleum ether yielded white plates (0.135 g, 0.318 mmol, 31%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.36–7.29 (m, 5H; H-2′,3′,4′,5′,6′), 5.28–5.18 (m, 2H; H-7′a,7′b), 5.08 (br t, $J_{3,4}$ = 8.3, $J_{4,5}$ = 9.2 Hz, 1H; H-4), 4.92 (d, $J_{1,2}$ = 12.3 Hz, 1H; H-1), 4.64–4.58 (m, 2H; H-2,3), 4.02 (d, 1H; H-5), 3.76 (s, 3H; OMe), 2.01 (s, 3H; OAc), 2.01 (s, 3H; OAc), 1.99 ppm (s, 3H; OAc); ^{13}C NMR (75 MHz, CDCl$_3$): δ = 170.3 (OAc), 169.51 (OAc), 169.35 (OAc), 167.4 (C-6), 136.6 (Ar), 128.6 (Ar), 128.2 (Ar), 127.9 (Ar), 99.4 (C-1), 72.8 (C-3), 72.2 (C-2), 71.3 (C-4), 71.0 (OCH_2Ar), 69.6 (C-5), 53.1 (OMe), 20.74 (2OAc), 20.64 ppm (OAc); MS: *m/z* calcd: 447.1 $[M+Na]^+$; found: 447.4.

Protected unsaturated glucuronide 9a: Protected glucuronide **8a** (0.275 g, 0.550 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (petroleum ether/ethyl acetate 5:2) gave a white powder (0.133 g, 0.302 mmol, 55%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 8.80 (d, $J_{3',5'}$ = 2.8 Hz, 1H; H-5′), 8.60 (dd, $J_{2',3'}$ = 9.3 Hz, 1H; H-3′), 7.83 (d, 1H; H-2′), 6.57 (dd, $J_{3,4}$ = 2.1, $J_{2,4}$ = 0.9 Hz, 1H; H-4), 6.25 (dd, $J_{1,2}$ = 4.8, $J_{1,3}$ = 1.3 Hz, 1H; H-1), 5.27–5.25 (td, $J_{2,3}$ = 2.1 Hz, 1H; H-3), 5.20 (ddd, 1H; H-2), 3.73 (s, 3H; OMe), 2.10 (s, 3H; OAc), 2.09 ppm (s, 3H; OAc).

Protected unsaturated glucuronide 9b: Protected glucuronide **8b** (0.222 g, 0.444 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (petroleum ether/ethyl acetate 3:1) gave a white powder (0.098 g, 0.223 mmol, 50%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 8.40 (d, $J_{4',6'}$ = 2.2 Hz, 1H; H-6′), 8.04 (dd, $J_{3',4'}$ = 8.8, 1H; H-4′), 7.90 (d, 1H; H-3′), 6.35 (dd, $J_{3,4}$ = 4.9, $J_{2,4}$ = 1.4 Hz, 1H; H-4), 6.05 (dd, $J_{1,2}$ = 1.9, $J_{1,3}$ = 1.1 Hz, 1H; H-1), 5.28 (ddd, $J_{2,3}$ = 1.1 Hz, 1H; H-2), 5.24 (dt, 1H; H-3), 3.80 (s, 3H; OMe), 2.14 (s, 3H; OAc), 2.13 ppm (s, 3H; OAc).

Protected unsaturated glucuronide 9e: Protected glucuronide **8e** (0.103 g, 0.232 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (hexanes/ethyl acetate 3:1) gave a white powder (0.064 g, 0.166 mmol, 72%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.93–7.90 (m, 2H; H-2′,4′), 7.50–7.41 (m, 2H; H-5′,6′), 6.30 (dd, $J_{3,4}$ = 4.0, $J_{2,4}$ = 1.9 Hz, 1H; H-4), 5.88 (dd, $J_{1,2}$ = 1.9, $J_{1,3}$ = 1.1 Hz, 1H; H-1), 5.30–5.26 (m, 2H; H-2,3), 3.77 (s, 3H; OMe), 2.12 (s, 3H; OAc), 2.10 ppm (s, 3H; OAc).

Protected unsaturated glucuronide 9f: Protected glucuronide **8f** (0.103 g, 0.232 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (hexanes/ethyl acetate 3:1) gave a white powder (0.064 g, 0.166 mmol, 72%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.26 (d, $J_{2',3'}$ = $J_{5',6'}$ = 9.0 Hz, 2H; H-3′,5′), 7.03 (d, 2H; H-2′,6′), 6.29 (dd, $J_{3,4}$ = 4.5, $J_{2,4}$ = 1.5 Hz, 1H; H-4), 5.77 (dd, $J_{1,2}$ = 2.7, $J_{1,3}$ = 1.0 Hz, 1H; H-1), 5.29–5.26 (m, 2H; H-2,3), 3.80 (s, 3H; OMe), 2.13 (s, 3H; OAc), 2.11 ppm (s, 3H; OAc); ^{13}C NMR (101 MHz, CD$_3$OD): δ = 170.4 (OAc), 169.7 (OAc), 162.1 (C-6), 154.9 (C-5), 142.1 (Ar), 129.4 (Ar), 128.1 (Ar), 118.5 (Ar), 107.7 (C-4), 95.1 (C-1), 68.2 (C-3), 64.9 (C-2), 51.9 (OMe), 19.47 (OAc), 19.32 ppm (OAc).

Protected unsaturated glucuronide 9h: Protected glucuronide **8h** (0.158 g, 0.338 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (hexanes/ethyl acetate 4:1) gave a white powder (0.061 g, 0.150 mmol, 44%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.31 (d, $J_{2',3'}$ = $J_{5',6'}$ = 8.9 Hz, 2H; H-3′,5′), 7.02 (d, 2H; H-2′,6′), 6.28 (dd, $J_{3,4}$ = 4.2, $J_{2,4}$ = 1.6 Hz, 1H; H-4), 5.78 (dd, $J_{1,2}$ = 2.6, $J_{1,3}$ = 1.4 Hz, 1H; H-1), 5.31–5.26 (m, 2H; H-2,3), 3.81 (s, 3H; OMe), 2.13 (s, 3H; OAc), 2.11 (s, 3H; OAc), 1.29 ppm (s, 9H; *tert*-butyl); ^{13}C NMR (101 MHz, CD$_3$OD): δ = 171.7 (OAc), 171.0 (OAc), 163.4 (C-6), 155.4 (C-5), 147.4 (Ar), 143.6 (Ar), 127.5 (Ar), 117.8 (Ar), 108.7 (C-4), 96.8 (C-1), 69.7 (C-3), 66.4 (C-2), 53.1 (OMe), 35.1 (*tert*-butyl), 31.9 (*tert*-butyl), 20.73 (OAc), 20.59 ppm (OAc).

Protected unsaturated glucuronide 9i: Protected glucuronide **8i** (32 mg, 0.075 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (petroleum ether/ethyl acetate 5:2) gave a colourless syrup (0.020 g, 0.055 mmol, 73%). ^{1}H NMR (300 MHz, CDCl$_3$): δ = 7.37–7.28 (m, 5H; H-2′,3′,4′,5′,6′), 6.23 (dd, $J_{3,4}$ = 4.5, $J_{2,4}$ = 1.1 Hz, 1H; H-4), 5.30 (d, $J_{1,2}$ = 2.5 Hz, 1H; H-1), 5.20 (dd, $J_{2,3}$ = 1.9 Hz, 1H; H-3), 5.14 (br q, 1H; H-2), 4.87 (d, $J_{7'a,7'b}$ = 12.3 Hz, 1H; H-7′a), 4.68

(d, 1H; H-7′b), 3.82 (s, 3H; OMe), 2.08 (s, 3H; OAc), 2.06 ppm (s, 3H; OAc); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.2 (OAc), 169.5 (OAc), 162.3 (C-6), 142.4 (C-5), 136.8 (Ar), 128.5 (Ar), 128.0 (Ar), 127.5 (Ar), 107.5 (C-4), 95.7 (C-1), 70.6 (OCH_2Ar), 68.5 (C-3), 64.4 (C-2), 52.7 (OMe), 21.03 (OAc), 20.88 ppm (OAc); MS: *m/z* calcd: 387.1 $[M+Na]^+$; found: 387.3.

Unsaturated glucuronide 10a: Protected unsaturated glucuronide **9a** (30 mg, 0.068 mmol) was dissolved in acetone (3.4 mL). Aqueous HCl (1 M, 3.4 mL) was added, and the reaction mixture with stirred at ambient temperature for 20 days, with monitoring by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1). The reaction was stopped by removing the organic solvent under vacuum and immediately purified by HPLC over a C-18 stationary phase (elution with water for 5 min, then a linear gradient to 100% acetonitrile over 1 hour). Product fractions were identified by UV/Vis absorbance on-line and confirmed by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1), and were then pooled and lyophilised to give an off-white powder (7.8 mg, 0.022 mmol, 32%). ^{1}H NMR (600 MHz, CD_3OD): δ = 8.78 (d, $J_{3',5'}$ = 2.8 Hz, 1H; H-5′), 8.53 (dd, $J_{2',3'}$ = 9.3 Hz, 1H; H-3′), 7.86 (d, 1H; H-2′), 6.30 (dd, $J_{3,4}$ = 4.2, J' = 0.7 Hz, 1H; H-4), 6.07 (d, $J_{1,2}$ = 4.1, $J_{1,3}$ = 0.7 Hz, 1H; H-1), 4.15 (brtd, $J_{2,3}$ = 3.8 Hz, 1H; H-3), 4.09–4.08 ppm (brtd, 1H; H-2); ^{13}C NMR (151 MHz, CD_3OD): δ = 164.67 (C-6), 155.14 (C-5), 143.26 (Ar), 141.15 (Ar), 141.07 (Ar), 130.16 (Ar), 122.67 (Ar), 119.74 (Ar), 114.11 (C-4), 99.78 (C-1), 70.56 (C-3), 66.89 ppm (C-2); HRMS: *m/z* calcd: 341.0257 $[M-H]^-$; found: 341.0260.

Unsaturated glucuronide 10b: Protected unsaturated glucuronide **9b** (24 mg, 0.050 mmol) was dissolved in acetone (2.5 mL). Aqueous HCl (1 M, 2.5 mL) was added, and the reaction mixture was stirred at ambient temperature for 16 days, with monitoring by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1). The reaction was stopped by removing the organic solvent under vacuum, and the mixture was immediately purified by HPLC over a C-18 stationary phase (elution with water for 5 min, then a linear gradient to 100% acetonitrile over 1 hour). Product fractions were identified by UV/Vis absorbance on-line and confirmed by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1), and were then pooled and lyophilised to give an off-white powder (9.9 mg, 0.028 mmol, 56%). ^{1}H NMR (600 MHz, CD_3OD): δ = 8.43 (d, $J_{4',6'}$ = 1.0 Hz, 1H; H-6′), 8.03–8.11 (m, 2H; H-3′,4′), 6.27 (d, $J_{3,4}$ = 4.1 Hz, 1H; H-4), 5.97 (d, $J_{1,2}$ = 4.1 Hz, 1H; H-1), 4.13 (brt, $J_{2,3}$ = 3.8 Hz, 1H; H-3), 4.05 ppm (brt, 1H; H-2); ^{13}C NMR (151 MHz, CD_3OD): δ = 163.4 (C-6), 150.4 (C-5), 149.5 (Ar), 144.6 (Ar), 139.9 (Ar), 126.1 (Ar), 118.0 (Ar), 114.5 (Ar), 112.9 (C-4), 99.2 (C-1), 69.4 (C-3), 65.6 ppm (C-2); HRMS: *m/z* calcd: 341.0257 $[M-H]^-$; found: 341.0251.

Unsaturated glucuronide 10e: Protected unsaturated glucuronide **9e** (44 mg, 0.111 mmol) was subjected to the general method for Zemplén deprotection, but with use of a slight excess of sodium methoxide instead of a catalytic amount. Deacetylation was complete within 5 min, at which point water (200 μL) was added, and the reaction mixture was stirred for a further 60 min at 0 °C. The reaction was quenched with Sephadex ion exchange resin (H^+ form), and the mixture was then filtered. Purification by flash column chromatography (ethyl acetate/methanol/water 7:2:1) and then with C-18 Sep-pak (5 g, washed with water, 40% acetonitrile in water and 100% acetonitrile) gave a slightly yellow powder after lyophilisation (28.1 mg, 0.095 mmol, 85%). ^{1}H NMR (600 MHz, D_2O): δ = 7.96–7.88 (m, 2H; H-2′,4′), 7.55–7.45 (m, 2H; H-5′,6′), 6.24 (d, $J_{3,4}$ = 4.1 Hz, 1H; H-4), 5.84 (d, $J_{1,2}$ = 4.6 Hz, 1H; H-1), 4.27 (t, $J_{2,3}$ = 4.1 Hz, 1H; H-3), 4.10 ppm (brt, 1H; H-2); ^{13}C NMR (151 MHz, D_2O): δ = 164.7 (C-6), 155.6 (C-5), 148.1 (Ar), 139.9 (Ar), 130.1 (Ar), 123.7 (Ar), 118.0 (Ar), 111.9 (Ar), 111.8 (C-4), 97.4 (C-1), 68.6 (C-3), 64.9 ppm (C-2); HRMS: *m/z* calcd: 296.0406 $[M-H]^-$; found: 296.0411.

Unsaturated glucuronide 10f: Protected unsaturated glucuronide **9f** (32 mg, 0.083 mmol) was deprotected by the general method for ester saponification. The product was purified by HPLC over a C-18 stationary phase (elution with water for 5 min, then a linear gradient to 100% acetonitrile over 1 hour). Product fractions were identified by UV/Vis absorbance on-line and confirmed by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1), and were then pooled and lyophilised to give an off-white powder (10.8 mg, 0.038 mmol, 46%). ^{1}H NMR (400 MHz, D_2O): δ = 8.09 (d, $J_{2',3'} = J_{5',6'}$ = 9.2 Hz, 2H; H-3′,5′), 7.17 (d, 2H; H-2′,6′), 6.22 (d, $J_{3,4}$ = 4.1 Hz, 1H; H-4), 5.84 (d, $J_{1,2}$ = 4.8 Hz, 1H; H-1), 4.29 (brt, $J_{2,3}$ = 4.5 Hz, 1H; H-3), 4.07 ppm (brt, 1H; H-2); ^{13}C NMR (101 MHz, D_2O): δ = 165.1 (C-6), 161.1 (Ar), 142.7 (Ar), 126.1 (Ar), 116.9 (Ar), 112.5 (C-4), 97.3 (C-1), 69.3 (C-3), 65.7 ppm (C-2) (carbon 5 signal too weak to detect, but seen in protected form); HRMS: *m/z* calcd: 285.0166 $[M-H]^-$; found: 285.0161.

Unsaturated glucuronide 10h: Protected unsaturated glucuronide **9h** (33 mg, 0.081 mmol) was deprotected by the general method for ester saponification. Purification was by C-18 Sep-pak (5 g, washed with 10% acetonitrile in water, 40% acetonitrile in water, 60% acetonitrile in water, 75% acetonitrile in water and 100% acetonitrile). All fractions determined to contain pure product by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1) were pooled and lyophilised, whereas those containing impure product were pooled, lyophilised and purified again, to give a white powder (13.8 mg, 0.044 mmol, 54%). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.34 (d, $J_{2',3'} = J_{5',6'}$ = 8.7 Hz, 2H; H-3′,5′), 7.10 (d, 2H; H-2′,6′), 6.13 (d, $J_{3,4}$ = 3.6 Hz, 1H; H-4), 5.52 (d, $J_{1,2}$ = 5.6 Hz, 1H; H-1), 4.20 (brt, $J_{2,3}$ = 4.9 Hz, 1H; H-3), 3.90 (brt, 1H; H-2), 1.30 ppm (s, 9H; *tert*-butyl); ^{13}C NMR (151 MHz, CD_3OD): δ = 165.38 (C-6), 156.26 (C-5), 146.90 (Ar), 127.28 (Ar), 117.95 (Ar), 113.17 (C-4), 100.99 (C-1), 72.03 (C-3), 68.37 (C-2), 35.04 (s, *tert*-butyl), 31.91 ppm (s, *tert*-butyl) (aryl carbon 4 signal too weak to detect, but seen in protected form); HRMS: *m/z* calcd: 307.1186 $[M-H]^-$; found: 307.1182.

Unsaturated glucuronide 10i: Protected unsaturated glucuronide **9i** (20 mg, 0.055 mmol) was deprotected by the general method for ester saponification. Purification was by C-18 Sep-pak (5 g, washed with water, 40% acetonitrile in water twice and 100% acetonitrile). All fractions determined to contain pure product by TLC (toluene/ethyl acetate/methanol/acetic acid 3:3:1:1) were pooled and lyophilised to give a white powder (14.1 mg, 0.053 mmol, 96%). ^{1}H NMR (400 MHz, D_2O): δ = 7.35–7.28 (m, 5H; H-2′,3′,4′,5′,6′), 6.06 (d, $J_{3,4}$ = 4.0 Hz, 1H; H-4), 5.16 (d, $J_{1,2}$ = 4.5 Hz, 1H; H-1), 4.77 (d, $J_{7'a,7'b}$ = 10.5 Hz, 1H; H-7′a), 4.71 (d, 1H; H-7′b), 4.06 (brt, $J_{2,3}$ = 4.1 Hz, 1H; H-3), 3.80 ppm (brt, 1H; H-2); ^{13}C NMR (101 MHz, D_2O): δ = 136.86 (Ar), 128.82 (Ar), 128.51 (Ar), 128.46 (Ar), 111.63 (C-4), 99.53 (C-1), 71.54 (s, OCH_2Ar), 69.57 (C-3), 65.76 ppm (C-2) (carbon 5 and 6 signals too weak to detect, but seen in protected form); HRMS: *m/z* calcd: 289.0688 $[M+Na]^+$; found: 289.0690.

Protected equatorial glucuronyl fluoride 11: Globally protected glucuronyl bromide (see first half of general method for Koenigs–Knorr glycosylation, 0.142 g, 0.358 mmol) was dissolved in acetonitrile (4 mL). Silver(I) fluoride (0.214 g, 1.687 mmol, 4.7 equiv) was then added, and the reaction mixture was stirred at ambient temperature overnight in the dark under nitrogen. The reaction mixture was then filtered through Celite and purified by flash column chromatography (petroleum ether/ethyl acetate 2:1) to give a white powder (0.103 g, 0.306 mmol, 85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.41 (dd, $J_{1,F}$ = 51.0, $J_{1,2}$ = 5.2 Hz, 1H; H-1), 5.39 (t, $J_{3,4}$ =

$J_{4,5}$ = 8.2 Hz, 1H; H-4), 5.22 (t, $J_{2,3}$ = 8.2 Hz, 1H; H-3), 5.08 (ddd, $J_{2,F}$ = 9.9 Hz, 1H; H-2), 4.27 (d, 1H; H-5), 3.77 (s, 3H; OMe), 2.09 (s, 3H; OAc), 2.03 ppm (s, 6H; 2OAc); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 169.86 (OAc), 169.34 (OAc), 169.14 (OAc), 167.00 (C-6), 105.93 (d, $J_{C\text{-}1,F}$ = 223.9 Hz, C-1), 72.23 (d, $J_{C\text{-}5,F}$ = 3.6 Hz, C-5), 70.72 (d, $J_{C\text{-}2,F}$ = 27.2 Hz, C-2), 70.48 (d, $J_{C\text{-}3,F}$ = 3.0 Hz, C-3), 68.01 (C-4), 53.14 (OMe), 20.61 (2OAc), 20.55 ppm (OAc); ^{19}F NMR (282 MHz, $CDCl_3$): δ = −135.35 ppm (dd, F-1); MS: *m/z* calcd: 359.1 $[M+Na]^+$; found: 359.3.

Protected pseudo-equatorial unsaturated glucuronyl fluoride 12: Protected equatorial glucuronyl fluoride **11** (0.103 g, 0.306 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (petroleum ether/ethyl acetate 4:1) gave a colourless syrup (0.028 g, 0.101 mmol, 33%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.35 (dd, $J_{3,4}$ = 4.8, $J_{2,4}$ = 1.6 Hz, 1H; H-4), 5.93 (dt, $J_{1,F}$ = 49.7, $J_{1,2}$ = $J_{1,3}$ = 1.6 Hz, 1H; H-1), 5.17–5.16 (m, 2H; H-2,3), 3.85 (s, 3H; OMe), 2.09 ppm (s, 3H; 2OAc); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 169.83 (OAc), 169.21 (OAc), 161.51 (C-6), 141.98 (C-5), 107.47 (C-4), 101.38 (d, $J_{C\text{-}1,F}$ = 227.7 Hz, C-1), 66.18 (d, $J_{C\text{-}2,F}$ = 36.7 Hz, C-2), 62.31 (C-3), 52.94 (OMe), 20.81 (OAc), 20.66 ppm (OAc); ^{19}F NMR (282 MHz, $CDCl_3$): δ = −140.59 ppm (d, F-1); MS: *m/z* calcd: 299.1 $[M+Na]^+$; found: 299.3.

Pseudo-equatorial unsaturated glucuronyl fluoride 13: Protected pseudo-equatorial unsaturated glucuronyl fluoride **12** (28 mg, 0.101 mmol) was subjected to the general method for Zemplén deprotection, followed by that for hydrolysis by lithium hydroxide. The product was purified by C-18 Sep-pak (5 g, elution with water, 10% acetonitrile in water, 20% acetonitrile in water, 40% acetonitrile in water, and 100% acetonitrile). All fractions determined by TLC to contain product were pooled and purified further by flash column chromatography (ethyl acetate/methanol/water 7:2:1) and were then lyophilised to afford an off-white powder (12.5 mg, 0.071 mmol, 70%). ^{1}H NMR (300 MHz, D_2O): δ = 6.18 (dd, $J_{3,4}$ = 4.7, $J_{2,4}$ = 1.2 Hz, 1H; H-4), 5.95 (dd, $J_{1,F}$ = 51.4, $J_{1,2}$ = 2.3 Hz, 1H; H-1), 4.18 (dd, $J_{2,3}$ = 1.2 Hz, 1H; H-3), 4.11 ppm (dt, 1H; H-2); ^{13}C NMR (101 MHz, D_2O): δ = 167.37 (C-6), 108.70 (C-4), 105.10 (d, $J_{C\text{-}1,F}$ = 222.7 Hz, C-1), 67.30 (d, $J_{C\text{-}2,F}$ = 30.3 Hz, C-2), 63.58 ppm (C-3) (carbon 5 signal too weak to detect, but seen in protected form); ^{19}F NMR (282 MHz, D_2O): δ = −142.35 ppm (d, F-1); HRMS: *m/z* calcd: 177.0199 $[M-H]^-$; found: 177.0202.

Protected axial glucuronyl fluoride 14: Globally protected glucuronic acid **7** (0.560 g, 1.488 mmol) was dissolved in HF in pyridine (70%, 10 mL) in a plastic container at 0 °C and then stirred overnight at 4 °C. The reaction was quenched with solid $NaHCO_3$ on ice, and the mixture was then diluted with EtOAc. The organic phase was washed with water, sat. $NaHCO_3$, and brine before drying over $MgSO_4$. Purification by flash column chromatography (petroleum ether/ethyl acetate 5:2) followed by crystallisation from toluene/*n*-heptane yielded sticky white prisms (0.102 g, 0.303 mmol, 20%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.78 (dd, $J_{1,F}$ = 52.5, $J_{1,2}$ = 2.5 Hz, 1H; H-1), 5.51 (t, $J_{2,3}$ = $J_{3,4}$ = 9.9 Hz, 1H; H-3), 5.19 (t, $J_{4,5}$ = 9.9 Hz, 1H; H-4), 4.94 (ddd, $J_{2,F}$ = 24.1 Hz, 1H; H-2), 4.43 (d, 1H; H-5), 3.72 (s, 3H; OMe), 2.06 (s, 3H; OAc), 2.00 ppm (s, 6H; 2OAc); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 169.89 (OAc), 169.79 (OAc), 169.48 (OAc), 166.99 (C-6), 103.61 (d, $J_{C\text{-}1,F}$ = 231.2 Hz, C-1), 69.97 (d, $J_{C\text{-}3,F}$ = 4.4 Hz, C-3), 69.94 (d, $J_{C\text{-}2,F}$ = 24.1 Hz, C-2), 68.68 (C-4), 68.54 (C-5), 53.13 (OMe), 20.63 (OAc), 20.53 (OAc), 20.47 ppm (OAc); ^{19}F NMR (282 MHz, $CDCl_3$): δ = −150.37 ppm (dd, F-1); MS: *m/z* calcd: 359.1 $[M+Na]^+$; found: 359.3.

Protected pseudo-axial unsaturated glucuronyl fluoride 15: Protected axial glucuronyl fluoride **14** (0.080 g, 0.238 mmol) was subjected to the general method for DBU-promoted elimination. Purification by flash column chromatography (petroleum ether/ethyl acetate 5:2) gave a colourless syrup (0.022 g, 0.080 mmol, 34%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.15 (d, $J_{3,4}$ = 2.3 Hz, 1H; H-4), 5.88 (dd, $J_{1,F}$ = 53.7, $J_{1,2}$ = 1.9 Hz, 1H; H-1), 5.71 (dd, $J_{2,3}$ = 8.9 Hz, 1H; H-3), 5.21 (ddd, $J_{2,F}$ = 24.1 Hz, 1H; H-2), 3.83 (s, 3H; OMe), 2.14 (s, 3H; OAc), 2.10 ppm (s, 3H; OAc); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.16 (OAc), 170.07 (OAc), 161.05 (C-6), 141.06 (C-5), 109.69 (C-4), 103.43 (d, $J_{C\text{-}1,F}$ = 232.7 Hz, C-1), 68.39 (d, $J_{C\text{-}2,F}$ = 21.4 Hz, C-2), 65.64 (d, $J_{C\text{-}3,F}$ = 4.6 Hz, C-3), 52.99 (OMe), 20.96 (OAc), 20.77 ppm (OAc); ^{19}F NMR (282 MHz, $CDCl_3$): δ = −146.30 ppm (dd, F-1); MS: *m/z* calcd: 299.1 $[M+Na]^+$; found: 299.3.

Pseudo-axial unsaturated glucuronyl fluoride 16: Protected pseudo-axial unsaturated glucuronyl fluoride **15** (22 mg, 0.080 mmol) was subjected to the general method for Zemplén deprotection. The intermediate was purified by flash column chromatography (dichloromethane/methanol 9:1) and was then further deprotected by the general method for hydrolysis by lithium hydroxide. The final product was purified by flash column chromatography (ethyl acetate/methanol/water 7:2:1) and lyophilised to afford a white powder (11.3 mg, 0.063 mmol, 79%). ^{1}H NMR (300 MHz, D_2O): δ = 5.92 (d, $J_{3,4}$ = 2.2 Hz, 1H; H-4), 5.85 (dd, $J_{1,F}$ = 54.8, $J_{1,2}$ = 1.5 Hz, 1H; H-1), 4.45 (dt, $J_{2,3}$ = 8.7, $J_{3,F}$ = 2.8 Hz, 1H; H-3), 3.87 ppm (ddd, $J_{2,F}$ = 26.4 Hz, 1H; H-2); ^{13}C NMR (75 MHz, D_2O): δ = 169.41 (C-6), 109.84 (C-4), 105.39 (d, $J_{C\text{-}1,F}$ = 191.3 Hz, C-1), 69.72 (d, $J_{C\text{-}2,F}$ = 21.7 Hz, C-2), 65.30 ppm (d, $J_{C\text{-}3,F}$ = 5.2 Hz, C-3) (carbon 5 signal too weak to detect, but seen in protected form); ^{19}F NMR (282 MHz, $CDCl_3$): δ = −147.46 ppm (br dd, F-1); HRMS: *m/z* calcd: 177.0199 $[M+Na]^+$; found: 177.0202.

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