Journal of Medicinal Chemistry

Brief Article

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Exploring the Interactions of Unsaturated Glucuronides with Influenza Virus Sialidase†

Beenu Bhatt,‡ Raphael Böhm,‡ Philip S. Kerry,§ Jeffrey C. Dyason,‡ Rupert J. M. Russell,§ Robin J. Thomson,‡ and Mark von Itzstein*,‡

‡Institute for Glycomics, Gold Coast Campus, Griffith University, Queensland 4222, Australia

§Interdisciplinary Centre for Human and Avian Influenza Research, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, U.K.

Supporting Information

ABSTRACT: A series of C3 *O*-functionalized 2-acetamido-2-deoxy-Δ^4-β-D-glucuronides were synthesized to explore noncharge interactions in subsite 2 of the influenza virus sialidase active site. In complex with A/N8 sialidase, the parent compound (C3 OH) inverts its solution conformation to bind with all substituents well positioned in the active site. The parent compound inhibits influenza virus sialidase at a sub-μM level; the introduction of small alkyl substituents or an acetyl group at C3 is also tolerated.

INTRODUCTION

The continual emergence of new strains of influenza virus presents an ongoing threat to humankind.[1] In addition to the burden of seasonal epidemic human influenza, there are serious concerns about the zoonotic potential of nonhuman, in particular swine and avian, influenza viruses.[2] A number of influenza-specific antiviral therapeutics are currently available, or are in development, which target different stages of virus replication.[1] The drugs recommended by the World Health Organization as a first option for treatment during the 2009 influenza A pandemic (A/H1N1/pdm09), and in human infections with highly pathogenic avian influenza (eg "bird flu" H5N1), are inhibitors of the viral enzyme sialidase (neuraminidase, NA).[3] This enzyme plays an important role in the release of new virus particles as they bud from the surface of an infected host cell; its inhibition leaves the virus particles clumped at the cell surface, thereby reducing virus spread.[4]

The first sialidase inhibitor drugs were the potent (K_i sub-nM) inhibitors zanamivir **1** and oseltamivir carboxylate **2** (OC, the active form of oseltamivir) (Figure 1).[4] A third inhibitor, peramivir **3**, was given temporary Emergency Use Authorization during the 2009 influenza pandemic.[5] During that pandemic, the sialidase inhibitors provided a means of therapeutic intervention while an appropriate vaccine was being developed and manufactured. Development of next-generation influenza virus sialidase inhibitors is ongoing, based in particular upon new insights into the sialidase structure.[6,7]

Both during and since the development of inhibitors **1**−**3**, emphasis has been on structures that incorporate functional groups to engage in predominantly hydrogen bonding and electrostatic interactions with conserved acidic amino acids in a pocket of the active site (subsite 2), which is dominated by negatively charged residues (Glu119, Glu277, Asp151).[4] Subsite 2 accommodates the guanidino and amino groups of inhibitors **1**−**3**. The development of potent inhibitor A-315675 **4** (e.g., K_i 0.2 nM against A/N2 NA),[8] however, gave a new dimension to probing this area of the sialidase active site by showing that the amino acids of subsite 2 can also engage in hydrophobic interactions with an appropriately positioned nonpolar group (as in **4** and **5**).[6,8] This finding was further supported with the synthesis of OC analogue **6**, in which the basic primary amine of OC was replaced by a vinyl group. **6** showed significant inhibition of influenza virus sialidase (K_i 45 nM),[9] despite the smaller 2-propyl *O*-alkyl side chain compared to **2**. The introduction of a hydrophobic, rather than a charged, substituent to bind subsite 2 could be advantageous in terms of drug pharmacokinetics: the zwitterionic character of inhibitors **1**−**3** containing a guanidino or amino group limits the drugs' oral bioavailability.[4,5] Importantly, presumably by virtue of the hydrophobic side-chain binding subsite 2, **4** retains potent inhibition against influenza A viruses harboring the E119 V mutation that confers resistance to OC **2**.[10]

We are interested in the development of readily accessible uronic acid-based sialidase inhibitors of type **7** (Figure 2),[11,12]

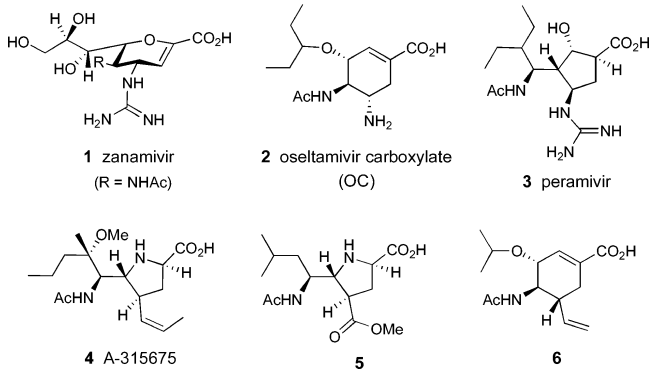


Figure 1. Influenza virus sialidase inhibitors. Drugs **1**−**3** exploit H-bonding and electrostatic interactions in subsite 2 of the active site, whereas inhibitors **4**−**6** exploit hydrophobic interactions.

Received: August 6, 2012
Published: September 27, 2012

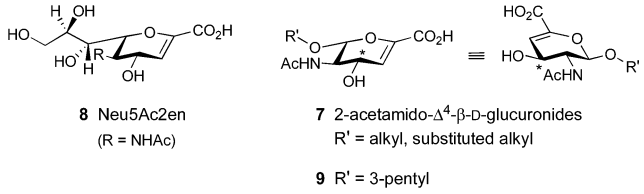


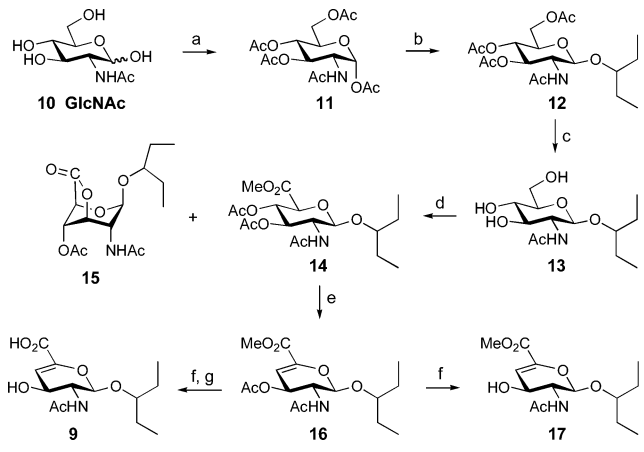
Figure 2. Sialidase inhibitors based on a dihydropyran scaffold: Neu5Ac2en **8** and uronic acid based **7**, a "cross-mimic" of **8** and oseltamivir carboxylate (OC) **2**.

2-acetamido-2-deoxy-Δ^4-β-D-glucuronides which mimic the naturally occurring sialidase inhibitor 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (Neu5Ac2en **8**) but in which the glycerol side-chain of **8** is replaced by an ether (the glycoside of the glucuronide). An advantage of the uronic acid scaffold is its derivation from inexpensive 2-acetamido-2-deoxy-glucose (GlcNAc) compared to *N*-acetylneuraminic acid and quinic acid used for the syntheses of **1** and **2**, respectively.[13] A number of examples of **7** (R = alkyl), but not the OC mimic 3-pentyl analogue **9**, have been evaluated as inhibitors of influenza virus sialidase, where they were found to inhibit in the micromolar range.[11] The objectives of the work described here were two-fold: to evaluate the binding mode of the Δ^4-β-D-glucuronide **9**, which contains the 3-pentyl side-chain of **2** on a dihydropyran scaffold, in complex with influenza virus sialidase, and to explore the hydrophobic paradigm of subsite 2 binding, through C3 *O*-substituted derivatives of **9**.

RESULTS AND DISCUSSION

Synthesis of the Δ^4-β-D-Glucuronide Scaffold. The route to the target 2-acetamido-Δ^4-β-D-glucuronides began with an efficient synthesis of key 3-hydroxy intermediate, methyl (3-pentyl 2-acetamido-2-deoxy-Δ^4-β-D-glucopyranosid)uronate **17**, from GlcNAc (**10**) as outlined in Scheme 1. This approach was used as it allowed selective deprotection at the C3 position,

Scheme 1. Synthetic Route to 9 and 17[a]



[a]Reagents and conditions: (a) Ac_2O, py, DMAP, DCM, rt, 24 h (96%); (b) (i) TMSOTf, DCE, 50 °C, 12 h, (ii) 4 Å mol. sieves, 3-pentanol, rt, 48 h (95%); (c) NaOMe, MeOH, 0 °C–rt, 2 h (99%); (d) (i) TEMPO, KBr, aq NaOCl, aq $NaHCO_3$, 0–4 °C, 12 h, (ii) MeI, DMF, rt, 24 h, (iii) Ac_2O, DMAP, rt, 12 h (61% over 3 steps); (e) DBU, DCM, rt, 24 h (isolated 52%; 74% based on recovered starting material **14**); (f) NaOMe, MeOH, rt, 12 h (97%); (g) NaOH, MeOH/H_2O (1:1), pH 13, 0 °C–rt, 16 h (95%).

which would not have been possible using the previously described[12] 3-*O*-pivaloylated analogue of **16**. High-yielding preparation of the GlcNAc 3-pentyl β-glycoside **12** was achieved through TMSOTf-mediated[14] activation of GlcNAc penta-*O*-acetate **11** to give the oxazolinium ion intermediate and subsequent one-pot reaction with 3-pentanol. This glycosidation method was found to be significantly higher yielding than glycosidation on the preformed GlcNAc 1,2-oxazoline.[12,15] Conversion of trihydroxy derivative **13**, obtained by base-catalyzed de-*O*-acetylation of **12**, to the corresponding 6-carboxy derivative took advantage of the now well-established selective oxidation of primary over secondary hydroxyl groups mediated by the 2,2,6,6-tetramethylpiperidine 1-oxide radical (TEMPO).[16] Accordingly, **13** was reacted with NaOCl in the presence of catalytic TEMPO and KBr in aqueous sodium bicarbonate solution; oxidation was most efficient when the reaction temperature was maintained between 0–4 °C. The resulting carboxylic acid derivative was not isolated but was instead converted[17] directly to the corresponding acetylated methyl ester **14**. The dried crude product was treated[17] with methyl iodide in DMF for esterification, followed by addition of acetic anhydride and catalytic DMAP, affording acetate-protected glucuronide methyl ester **14** in 61% yield over three steps in a one-pot reaction. Also isolated as a byproduct was the 3,6-lactone **15** (19% yield), characterized by the smaller ^3J coupling constants associated with the $^1C_4$ chair conformation of **15** (including a change in $^3J_{1,2}$ from 8.1 Hz in **14** to a broad singlet[18] in **15**). Conversion of glucuronide **14** to the 4,5-unsaturated derivative, Δ^4-β-D-glucuronide **16**, was achieved through DBU-mediated β-elimination.[11,12] To obtain the 3-hydroxy glucuronide **17** for further functionalization at C3, chemoselective cleavage of the C3 *O*-acetate in the presence of the methyl ester was undertaken using sodium methoxide in methanol. The overall yield of key intermediate **17** in 8 steps from GlcNAc **10** was 40%. Further saponification of **17** in aqueous methanolic NaOH (pH 13) gave the "parent" inhibitor on the Δ^4-β-D-glucuronide scaffold, **9**.[12]

Structural Evaluation of the Δ^4-β-D-Glucuronide Scaffold in Complex with Influenza Virus Sialidase. To assess the binding mode of the 3-pentyl 2-acetamido-Δ^4-β-D-glucuronide scaffold with influenza virus sialidase, **9** was soaked into crystals of influenza A virus N8 sialidase [A/duck/Ukraine/1/63 (H3N8)], and the structure of the resultant complex was determined by X-ray crystallography (Figure 3). The N8 sialidase is representative of influenza A virus sialidases as it is equally sensitive to inhibitors **1** and **2** when compared with other influenza A virus sialidases.[19] This study provides the first structural information for a glucuronide-based inhibitor in complex with an influenza virus sialidase. The *apo*-N8 sialidase has an open conformation of the flexible "150-loop".[7] In the N8:**9** complex, this loop has closed over the bound inhibitor, as seen in N8 complexes of **8** and **2**, under similar soaking conditions.[7] Despite **9** having a solution half-chair conformation in which the substituents on the dihydropyran ring are in pseudoaxial positions, as indicated by ^{1}H NMR spectroscopy,[12,15] the compound is clearly bound in the sialidase active site in the alternative half chair conformation which positions all substituents equatorially (Figure 3D). Superimposition of corresponding ring carbons (1, 3, and 5 in glucuronide numbering) of **9** and Neu5Ac2en **8** shows their half-chair conformations to be virtually identical (Figure 3C). By virtue of this conformation, the C1 carboxylate, C4 hydroxyl, and C5 acetamido groups of **9** are oriented, and interacting with key

dx.doi.org/10.1021/jm301145k | J. Med. Chem. 2012, 55, 8963–8968

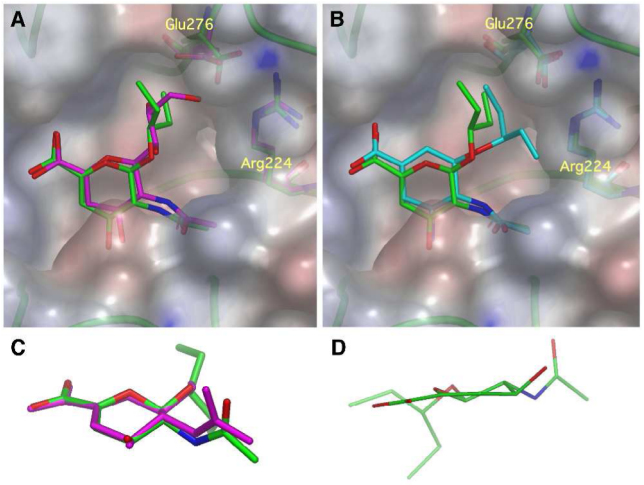


Figure 3. X-ray crystal structure of 3-pentyl 2-acetamido-Δ^4-β-D-glucuronide **9** in complex with influenza A virus N8 sialidase. (A) N8:**9** complex superimposed with N8:Neu5Ac2en **8** complex (**8** magenta, PDB 2HTR). (B) N8:**9** complex superimposed with N8:OC **2** complex (**2** cyan, PDB 2HT8). (C) Superimposition of ligands **9** and **8** (**8** magenta, PDB 2HTR) (the glycerol side-chain of **8** has been removed in this image) showing the very similar dihydropyran ring conformations of **9** and **8**. (D) Half-chair conformation of **9** showing equatorial conformation of substituents.

highly conserved residues in the active site, as seen[7] for Neu5Ac2en (Figure 3A). Specifically, the C1 carboxylate of **9** interacts with the three strictly conserved arginine residues (Arg118, Arg292, and Arg371) in the active site; the C4 hydroxyl group extends toward the pocket (subsite 2) bordered by Glu119 and Asp151 and the C5 acetamido group is directed toward Trp178. Furthermore, in the N8:**9** complex, the conserved residue Glu276 is oriented to form a salt bridge with Arg224, providing hydrophobic character to the active site pocket that accommodates the 3-pentyl side-chain of **9** and of OC **2** (Figure 3B). Comparing **9** and **2** (Figure 3B): while the conformation of the central ring and orientation of the pendant substituents are very similar (not shown), the position of **9** in the active site is slightly shifted from that of **2**, and the arms of the 3-pentyl side chain occupy slightly different regions of the binding pocket.

The free C3 hydroxyl group of **9** is positioned appropriately for substitution to explore hydrophobic interactions in subsite 2 of the active site. To evaluate the potential for a substituent at C3 on **9** to reach the area of subsite 2 accessed by the pyrrolidine-based inhibitors **4** and **5**, the model compound 3-*O*-ethyl substituted Δ^4-β-D-glucuronide **18** was docked into the active site of influenza A sialidase (PDB 2HU4) and superimposed with pyrrolidine-based inhibitor **5**[20] (from PDB 1XOE), the only example of this series available in the PDB. The overlay of the structures of **5** and **18** (Figure 4) shows similar extension of the methyl ester of **5** and the 3-*O*-ethyl group of **18** into subsite 2. To examine if Δ^4-β-D-glucuronides with small hydrophobic substituents at C3 could enhance binding affinity, synthesis and biological evaluation of a small series of compounds with a methyl, ethyl, or allyl ether, or an acetate ester at the C3 position of **9** was undertaken.

Synthesis of 3-*O*-Substituted Δ^4-β-D-Glucuronides. For synthesis of the target 3-*O*-alkylated derivatives, the 3-hydroxy methyl ester derivative **17** was reacted with NaH in DMF in the presence of the alkyl halide (EtI, MeI, allyl bromide) (Scheme 2). Fully or partially trans-esterified product was observed in reactions with ethyl iodide and allyl bromide, respectively. Base-catalyzed saponification of the 3-*O*-alkylated compounds yielded the corresponding free acids **18**, **23**, and **24**. The final target 3-*O*-acetylated derivative was obtained through reaction of fully deprotected glucuronic acid **9** with acetic anhydride in pyridine at room temperature, affording 3-*O*-acetyl derivative **25** in 38% yield (unoptimized).

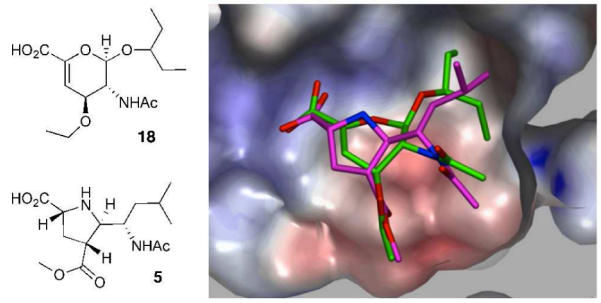
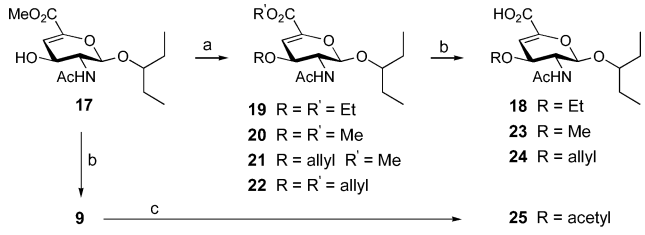


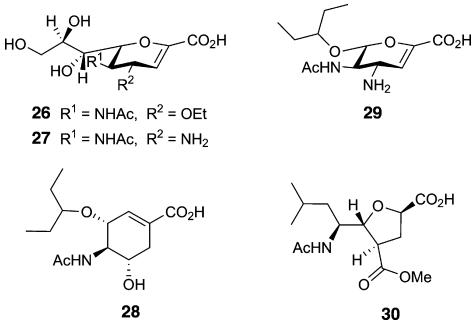
Figure 4. 3-Pentyl 3-*O*-ethyl-2-acetamido-2-deoxy-Δ^4-β-D-glucuronide **18** docked into the active site of influenza A virus sialidase (PDB 2HU4), superimposed with pyrrolidine-based inhibitor **5** (magenta; from PDB 1XOE). The surface is colored by electrostatic potential (blue, positive; red, negative).

Scheme 2. Synthetic Route to 18 and 23–25[a]



[a]Reagents and conditions: (a) RX, NaH, TBAI, DMF, 0 °C–rt, 24–48 h (EtI, **19** 56%; MeI, **20** 66%; AllylBr, **21**, 34%; **22**, 42%); (b) NaOH, MeOH/H_2O (1:1), pH 13, 0 °C–rt, 16 h; (c) Ac_2O, py, rt, 16 h (38%).

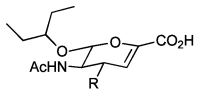
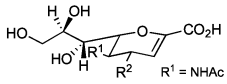
Biological Evaluation. The 3-*O*-substituted 2-acetamido-Δ^4-β-D-glucuronides synthesized were evaluated for their ability to inhibit the enzyme activity of influenza A virus sialidase, using an in vitro fluorometric assay.[21,22] The inhibitory activities of **18** and **23–25**, with comparison to **9**, the general[21] sialidase inhibitor Neu5Ac2en **8**, and previously synthesized[23] 4-*O*-ethyl-Neu5Ac2en **26**, are presented in Table 1.



The structure of 3-pentyl Δ^4-β-D-glucuronide **9** in complex with N8 sialidase (Figure 3) shows **9** positioned in the active site with the central ring and the carboxylate, hydroxyl, and

dx.doi.org/10.1021/jm301145k | J. Med. Chem. 2012, 55, 8963–8968

Table 1. Inhibitory Activity (IC_{50}) of 9, 18, and 23−26 against Influenza A Virus (N2) Sialidase

Template	Δ^4-β-D-Glucuronide			Neu5Ac2en		
						
R or R^2	Cpd	IC_{50} (μM) [a,b]		Cpd	IC_{50} (μM) [a,b]	
O–H	**9**	0.32		**8**	10.5	
$O–CH_2CH_3$	**18**	28.0	[88]	**26**	479	[47]
$O–CH_3$	**23**	1.45	[5]			
$O–CH_2CH=CH_3$	**24**	212	[690]			
$O–C(O)CH_3$	**25**	5.39	[17]			

R or R^2	compd	IC_{50} (μM)[a,b]	compd	C_{50} (μM)[a,b]
O−H	**9**	0.32	**8**	10.5
$O–CH_2CH_3$	**18**	28.0 (88)	**26**	479
$O–CH_3$	**23**	1.45 (5)		
$O–CH_2CH=CH_3$	**24**	212 (690)		
$O–C(O)CH_3$	**25**	5.39 (17)		

[a]Results are given as means for three experimentally determined values; IC_{50} 95% confidence intervals are within ±15% of the IC_{50} value. [b]Number in square parentheses is the fold-difference of IC_{50} from the "parent" inhibitor (R or R^2 = OH). In this assay, benchmark inhibitors were: 4-amino-4-deoxy-Neu5Ac2en **27**, IC_{50} 0.68 μM; 4-deoxy-4-guanidino-Neu5Ac2en **1**; IC_{50} 0.012 μM.

acetamido substituents in similar orientations within the active site to those seen for Neu5Ac2en **8** and OC **2**. In terms of inhibitory activity against A/N2 sialidase, **9** exhibits ∼30-fold greater inhibitory potency than Neu5Ac2en **8**, making the 3-pentyl derivative the most potent inhibitor on the glucuronic acid scaffold, with *O*-substitution at C3, to date.[11] The inhibitory potency of the 3-pentyl Δ^4-β-D-glucuronide (dihydropyran-based) scaffold appears to lie midway between that of the natural dihydropyran scaffold with the glycerol side chain (**8**) and the cyclohexene-based scaffold with the 3-pentyl ether side chain, hydroxy-OC analogue **28**[24] (inhibition of A/N2 NA:[24] **28** IC_{50} 0.015 μM, compared to Neu5Ac2en **8** IC_{50} 1.7 μM). It is interesting that on the 3-pentyl Δ^4-β-D-glucuronide scaffold, the C3-hydroxy derivative (**9**) appears to have a similar inhibitory potency to the corresponding C3-amino analogue **29**[15] reported by Smith et al. (inhibition of A/N2 NA:[15] **29** IC_{50} 0.770 μM; compared to zanamivir **1** IC_{50} 0.005 μM), although comparison across assays should be made with caution. This is in contrast to the Neu5Ac2en scaffold where change in C4 substituent from a hydroxyl (**8**) to an amino (**27**) group gives significant improvement in inhibitory activity[21] (Table 1: see data for **8** and **27** in footnote).

With regard to the effect of functionality introduced to explore hydrophobic interaction in subsite 2, the 3-*O*-alkylated (**18**, **23**, **24**) or acetylated (**25**) glucuronide derivatives showed weaker inhibition than the parent C3-hydroxy glucuronide **9**, with activity decreasing as the size of the alkyl chain increased (Table 1). The weaker activity of the ethyl ether derivative **18** compared to **9** is mirrored in that of 4-*O*-ethyl-Neu5Ac2en **26** compared to Neu5Ac2en **8**. Modeling had indicated adequate space in subsite 2 for, in particular, the 3-*O*-ethyl and acetyl substituents on the Δ^4-β-D-glucuronide scaffold, placing them potentially in a similar region to the methyl ester of the pyrrolidine and furan based inhibitors **5** and **30**[20] (inhibition of A/N2 NA:[20] **5** IC_{50} 0.041 μM; **30** 0.41 μM). The particular hydrophobic substituents introduced onto **9**, however, apparently fail to engage in interactions in subsite 2 that improve overall binding affinity.

The 3-atom extension from the pyrrolidine ring into subsite 2 seen, for example, in **4**, **5**, and **30**, equates to only a 2-atom extension from the C3 hydroxyl group of **9**. As such, the fine-tuning of the nature and "directionality" of this substituent (e.g., 10-fold greater activity on introduction of the *cis*- versus *trans*-propenyl substituent on scaffold **5**[6]), which was required during optimization on the pyrrolidine template,[6] is not as readily accessible on the current Δ^4-β-D-glucuronide template. An exception to this is variation of the acetyl group of **25** that enables further examination of the hydrophobic paradigm.

In conclusion, the 3-pentyl Δ^4-β-D-glucuronide inhibitor scaffold can be efficiently synthesized from an inexpensive and readily accessible starting material. The first structural study of this scaffold in complex with influenza virus sialidase shows that the parent compound **9** binds well into the sialidase active site: this binding results in good inhibitory activity for the "unfunctionalized" template. The single available hydroxyl group can be efficiently functionalized to explore possibilities for binding interactions in subsite 2 such as those previously identified on the pyrrolidine and cyclohexene scaffolds.

EXPERIMENTAL SECTION

General. A full description of materials and methods is provided in the Supporting Information (SI). Final compounds were purified by reversed phase (RP) HPLC. Purities of synthetic intermediates after chromatographic purification were judged to be >90% by analysis of the ^1H and ^{13}C NMR spectra (SI). Purity of tested compounds was ≥95% (HPLC analysis). **1**,[25] **8**,[26] **26**,[23] and **27**[25] used in the sialidase assay were synthesized according to literature procedures and purified by RP-HPLC (≥95% purity). Methods for the N8:**9** complex formation and X-ray structure determination,[27] computational chemistry,[27] and sialidase activity assay,[22] were as previously described.

General Procedure for De-esterification of 2-Acetamido-2-deoxy-Δ^4-β-D-glucuronides. To a solution of ester in MeOH:H_2O (1:1) at 0 °C, was added aq NaOH to achieve pH 13. The reaction mixture was stirred at rt for 16 h and then neutralized with Amberlite IR-120 (H^+) resin. The crude product was purified by chromatography on silica (EtOAc/MeOH/H_2O 8:1.5:0.5) followed by RP-HPLC.

General Procedure for Synthesis of 3-*O*-Alkylated 2-Acetamido-2-deoxy-Δ^4-β-D-glucuronides 18, 23, and 24. To a solution of **17** in anhyd DMF (0.12 M) under Ar was added alkyl halide (7 mol equiv) and nBu_4NI (0.2 mol equiv). The reaction was cooled to 0 °C, NaH (1.3 mol equiv) was added, and the reaction was stirred at 0 °C for 30 min and at rt for 24−48 h. The reaction was quenched at 0 °C by addition of a drop of AcOH, followed by addition of water and extractive workup with EtOAc. The crude product was purified by chromatography on silica to give the 3-*O*-alkylated derivative (**19**−**22**; see Scheme 2). **19**−**22** were de-esterified and the products (**18**, **23**, and **24**) purified according to the general procedure (yields and spectral characterization are given in the SI).

3-Pentyl 2-Acetamido-3-*O*-acetyl-2,4-dideoxy-α-L-*threo*-hex-4-enopyranosiduronic Acid (25). To a solution of **9**[12] (0.03 g, 0.10 mmol) in anhyd pyridine (1 mL) under N_2 was added acetic anhydride (0.5 mL). The reaction was stirred at rt for 16 h, concentrated, and the crude product was purified by chromatography on silica to give **25** (0.013 g, 38%) (spectral characterization is given in the SI).

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and spectral data for synthesized compounds. Protocols for: molecular modeling; N8−**9**

dx.doi.org/10.1021/jm301145k | J. Med. Chem. 2012, 55, 8963−8968

structure determination (including crystallographic statistics); sialidase activity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

PDB ID: 4D8S.

AUTHOR INFORMATION

Corresponding Author

*Phone: +61-(0)7-5552-7025. Fax: +61-(0)7-5552-9040. E-mail: m.vonitzstein@griffith.edu.au.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

M.v.I. thanks the Honda Foundation (Australia). R.J.M.R. thanks the Medical Research Council and the Scottish Funding Council for financial support. B.B. and R.B. thank Griffith University for the award of scholarships.

DEDICATION

†This paper is dedicated to the memory of Professor Stephen J. Angyal.

ABBREVIATIONS USED

NA, neuraminidase/sialidase; A/N8 or A/N2, influenza A virus N8 or N2 sialidase; OC, oseltamivir carboxylate; GlcNAc, 2-acetamido-2-deoxy-glucose; Neu5Ac2en, 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enoic acid

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