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Unsaturated *N*-acetyl-D-glucosaminuronic acid glycosides as inhibitors of influenza virus sialidase

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Abstract The threat of pandemic influenza is a significant concern of governments worldwide. There is a very limited and relatively expensive armament to tackle such a pandemic should it occur. This fact provides much impetus to the scientific community for the discovery of new and less expensive anti-influenza drugs. Our longstanding interest in the inhibition of influenza virus sialidase, coupled with the development of simple carbohydrates that mimic an unsaturated derivative of the enzyme's naturally-occurring ligand, *N*-acetylneuraminic acid, has led us to investigate the development of influenza virus sialidase inhibitors based on these mimetics. We have successfully prepared a range of these compounds, in good yield, from the relatively inexpensive carbohydrate *N*-acetylglucosamine utilising a short synthetic procedure. We have employed a sialidase inhibition assay for biological evaluation of the target compounds and to our delight these mimetics have displayed significant inhibition of influenza virus sialidase.

Keywords Influenza virus · Sialidase · Sialylmimetics · Sialic acids · Inhibitors

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Introduction

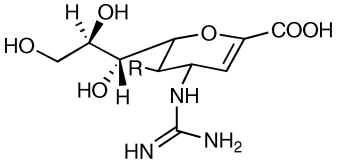
As a result of the emergence of avian influenza it is commonly believed that the next influenza pandemic could arise at any time [1]. As a response to this threat, apart from vaccine development, a number of groups have been engaged in heightened activities towards the discovery and development of small molecule anti-influenza drugs that target the viral surface glycoproteins haemagglutinin and sialidase [2–4].

To date the most successful small molecule influenza virus inhibitors developed have targeted the enzymic function sialidase and these developments have been reviewed elsewhere [2–5]. Both Relenza™(1), a carbohydrate-based drug, and Tamiflu™ (**2a**), a carbocyclic mimetic (a *pseudo*-carbohydrate) are potent and clinically effective anti-influenza drugs [6]. Despite the efficacy of these drugs major concerns remain with the prospect of the development of drug resistance.

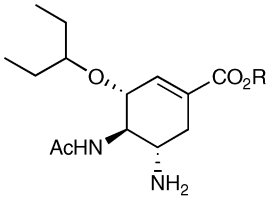
The naturally-occurring sialidase inhibitor 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enoic acid (Neu5Ac2en **3**) inhibits influenza virus sialidase at micromolar levels [7]. We have previously reported some preliminary studies concerning the synthesis of β-*O*-glycosides of 4,5-unsaturated 2-acetamido-D-glucuronic acid (**4**) as Neu5Ac2en mimetics and inhibitors of bacterial sialidases [8,9]. We believe that these mimetics provide an excellent opportunity for the complete replacement of the glycerol sidechain of Neu5Ac2en (**3**) with either functionalised or non-functionalised hydrophobic groups and to investigate the influence of such substitutions on sialidase activity. In general, we have found that while bacterial sialidases can tolerate the introduction of hydrophobic moieties, all of the synthesised mimetics, to date, are less

potent inhibitors of the investigated bacterial sialidases when compared with Neu5Ac2en (**3**).

In complete contrast, our preliminary study [8] and a simultaneous independent report [10] led us to conclude that these aforementioned simple uronic acid mimetics may provide potent inhibitors of influenza virus sialidase. For example the uronic acid mimetic (**4a**) was found to inhibit influenza virus sialidase at similar levels to Neu5Ac2en (**3**) itself [8]. Comparing mimetic (**4a**) with Neu5Ac2en (**3**) it is obvious that the key functional difference is the complete replacement of the glycerol sidechain in **3** with a hydrophobic isopropyl ether moiety. This particular substitution provides a similar hydrophobic environment to that observed in the de-esterified active form (**2b**) of the *pseudo*-carbohydrate (**2a**) and as a consequence we predicted that it should be accommodated within the influenza virus sialidase active site in a similar binding mode to **2b**. To expand the potential of this mimetic framework we have now synthesised and biologically evaluated further examples of these mimetics as inhibitors of influenza virus sialidase.

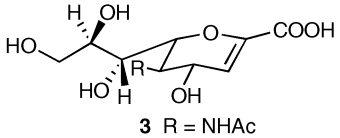


1 R = NHAc

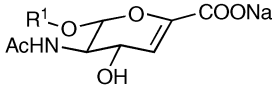


2a R = Et

2b R = H



3 R = NHAc



4 R^1 = aglycone

4a $R^1 = CH(CH_3)_2$

Materials and methods

Synthesis of β-*O*-glycosides of 4,5-unsaturated 2-acetamido-D-glucuronic acid

General methods

Reactions were monitored by TLC using Merck silica gel plates GF_{245}. Detection was typically effected under UV light where applicable, followed by treatment with H_2SO_4 in EtOH (5% v/v) and charring at ~180°C. Purification by flash chromatography was achieved with Merck silica gel 60 (0.040–0.063 mm). ^1H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded using a Bruker Avance 300 spectrometer. Data acquisition and processing were performed with XWINNMR software (version 3.1) running on a Silicon Graphics O2 workstation. Chemical shifts are expressed as parts per million (ppm, δ) and are relative to the solvent as an internal reference ($CDCl_3$: δ7.27 for ^1H; δ77.0 for ^{13}C; CD_3OD: δ4.78 for ^1H; δ49.0 for ^{13}C; D_2O: δ4.67 for ^1H). Two-dimensional COSY and HMQC experiments were recorded in order to assist with spectral assignment. ESI Mass spectra (+ve ion mode) were recorded on a Bruker Esquire 3000 spectrometer for LRMS, or on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III electrospray source for HRMS. All compounds gave satisfactory analytical data. All solvents were distilled prior to use or were of analytical grade. Methyl (2-acetamido-2-deoxy-3,4-di-*O*-pivaloyl-D-glucopyran)uronate (**5**) was synthesised according to the published procedure [8], via 2-acetamido-2-deoxy-3,4-di-*O*-pivaloyl-6-*O*-trityl-D-glucopyranose (Scheme 1). Methyl (2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-pivaloyl-D-glucopyran)uronate (**6**) was synthesized using the same procedure from 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-pivaloyl-6-*O*-trityl-D-glucopyranose. Sodium (isopropyl 2-acetamido-2,4-dideoxy-α-L-*threo*-hex-4-enopyranosid)uronate **4a** was prepared according to the literature [7].

*General procedure for the synthesis of **7c,d** and **8b***

TMSOTf (99 μL, 0.55 mmol) was added to a stirred solution of 5 and 6 (α/β ~2:3) (0.50 mmol) in anhyd 1,2-dichloroethane (DCE, 2.5 mL) under Ar. The clear yellow solution was warmed to 50°C. After 3 d, TLC analysis (EtOAc/hexane 1:3) indicated that the starting material was nearly all consumed. The resulting brown reaction mixture was cooled to rt, and 3 molecular sieves were added. After 30 min, anhyd alcohol (1.50 mmol) was added and the reaction was stirred at rt under Ar for 24 h. NEt_3 was added to adjust to pH 9, the reaction was filtered through Celite®, the residue was washed with $CHCl_3$/MeOH 10:1 (75 mL), and the filtrate was concentrated to give a brown gum. Purification

of the crude product by flash chromatography afforded **7c,d** and **8b** (56–76%, isolated yield).

*Methyl (cyclopentyl 2-acetamido-2-deoxy-3,4-di-*O*-pivaloyl-β-D-glucopyranosid)uronate (**7c**)*

Prepared from reaction between **5** and cyclopentanol in 56% yield after chromatography (EtOAc/hexane 1:3→1:1) as a white amorphous mass. $R_f = 0.24$ (EtOAc/hexane 2:3); ^{1}H NMR ($CDCl_3$) δ 1.10, 1.11 (2 × 9H, 2 × s, 2 × OPiv), 1.38-1.78 (8H, m, H-2′, H-2″, H-3′, H-3″), 1.87 (3H, s, NAc), 3.69 (3H, s, OMe), 3.87 (1H, ddd, $J_{2,3}$ 10.4, $J_{2,NH}$ 9.0, $J_{2,1}$ 8.2 Hz, H-2), 4.09 (1H, d, $J_{5,4}$ 9.6 Hz, H-5), 4.19-4.33 (1H, m, H-1′), 4.76 (1H, d, $J_{1,2}$ 8.2 Hz, H-1), 5.18 (1H, dd, $J_{4,3} = J_{4,5}$ 9.6 Hz, H-4), 5.41 (1H, dd, $J_{3,2}$ 10.4, $J_{3,4}$ 9.6 Hz, H-3), 6.29 (1H, br d, $J_{NH,2}$ 9.0 Hz, NH); ^{13}C NMR ($CDCl_3$) δ 22.9 (NC(O)*Me*), 23.0, 23.2 (C-3′, C-3″, 31.9, 32.9 (C-2′, C-2″), 26.9, 27.0 (2 × OC(O)C*Me*$_3$), 38.6, 38.8 (2 × OC(O)*C*Me$_3$), 52.5 (CO_2*Me*), 54.4 (C-2), 69.4 (C-4), 71.1 (C-3), 72.7 (C-5), 81.0 (C-1′), 99.3 (C-1), 167.7, 170.0, 176.5, 178.2 (*C*O_2Me, N*C*(O)Me, 2 × O*C*(O)CMe$_3$). LRMS m/z 508 ([M+Na]$^+$, 100%). HRMS calcd for $C_{24}H_{39}NNaO_9$ [M+Na] 508.2523, found 508.2525.

*Methyl (benzyl 2-acetamido-2-deoxy-3,4-di-*O*-pivaloyl-β-D-glucopyranosid)uronate (**7d**)*

Prepared from reaction between 5 and BnOH in 76% yield after chromatography (EtOAc/hexane 1:3→1:1) as a clear colourless gum. $R_f = 0.26$ (EtOAc/hexane 1:3); ^{1}H NMR ($CDCl_3$) δ 1.11, 1.12 (2 × 9H, 2 × s, 2 × OPiv), 1.86 (3H, s, NAc), 3.73 (3H, s, OMe), 4.06 (1H, d, $J_{5,4}$ 9.3 Hz, H-5), 4.14 (1H, ddd, $J_{2,3}$ 9.5, $J_{2,NH}$ 9.2, $J_{2,1}$ 8.2 Hz, H-2), 4.60 (1H, d, $J_{1'a,1'b}$ 12.2 Hz, H-1′a), 4.67 (1H, d, $J_{1,2}$ 8.2 Hz, H-1), 4.91 (1H, d, $J_{1'b,1'a}$ 12.2 Hz, H-1′b), 5.21-5.33 (2H, m, H-3, H-4), 5.23 (1H, br d, $J_{NH,2}$ 9.2 Hz, NH), 7.23-7.37 (5H, m, OCH_2*Ph*); ^{13}C NMR ($CDCl_3$) δ23.0 (NC(O)*Me*), 26.9, 27.0 (2 × OC(O)C*Me*$_3$), 38.6, 38.8 (2 × OC(O)*C*Me$_3$), 52.6 (CO_2*Me*), 53.8 (C-2), 69.3 (C-4), 70.6 (C-1′), 71.2 (C-3), 72.9 (C-5), 99.7 (C-1), 127.9, 128.4 (5 × OCH_2*Ph*), 136.8 (C*ipso*), 167.5, 169.8, 176.4, 178.2 (*C*O_2Me, N*C*(O)Me, 2 × O*C*(O)CMe$_3$). LRMS m/z 530 ([M+Na]$^+$, 100%). HRMS calcd for $C_{26}H_{37}NNaO_9$ [M + Na] 530.2366, found 530.2370.

*Methyl (2-ethylbutyl 2-acetamido-4-*O*-acetyl-2-deoxy-3-*O*-pivaloyl-β-D-glucopyranosid)uronate (**8b**)*

Prepared from reaction between **6** and 2-ethyl-1-butanol in 58% yield after chromatography ((EtOAc/hexane 2:1→1:1) as a colourless gum. $R_f = 0.48$ (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ0.81−0.91(6H,m, H-4′, H-4″), 1.14 (9H, s, OPiv), 1.17-1.46 (5H, m, H-2′, H-3′, H-3″), 1.90 (3H, s, OAc), 1.99 (3H, s, NAc), 3.30 (1H, dd, $J_{1'a,1'b}$ 9.5, $J_{1'a,2'}$ 6.4 Hz, H-1′a), 3.74 (3H, s, OMe), 3.83 (1H, dd, $J_{1'b,1'a}$ 9.5, $J_{1'b,2'}$ 5.3 Hz, H-1′b), 4.01 (1H, m, H-2), 4.05 (1H, d, $J_{4,5}$ 9.3 Hz, H-5), 4.63 (1H, d, $J_{1,2}$ 8.1 Hz, H-1), 5.26 (1H, dd, $J_{4,3} = J_{4,5} = 9.6$ Hz, H-4), 5.31 (1H, dd, $J_{3,2}$ 10.2, $J_{3,4}$ 9.6 Hz, H-3), 5.71 (1H, br d, $J_{NH,2}$ 9.3 Hz, NH); ^{13}C NMR ($CDCl_3$) δ 10.6, 11.0 (C-4′, C-4″), 20.4 (OC(O)*Me*), 22.8, 23.0 (C-3′, C-3″), 23.1 (NC(O)*Me*), 26.9 (OC(O)C*Me*$_3$), 38.9 (OC(O)*C*Me$_3$), 40.7 (C-2′), 52.8 (CO_2*Me*), 54.1 (C-2), 69.4 (C-4), 71.3 (C-3), 72.3 (C-5), 72.7 (C-1′), 101.4 (C-1), 167.5 (O*C*(O)Me), 169.1 (*C*O_2Me), 169.7 (N*C*(O)Me), 178.5 (O*C*(O)CMe$_3$). LRMS m/z 482 ([M+Na]$^+$, 100%). HRMS calcd for $C_{22}H_{37}NNaO_9$ [M+Na] 482.2366, found 482.2363.

*General procedure for the synthesis of **9b–d***

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 0.76 mmol) was added to a solution of compound **7c,d** and **8b** (0.38 mmol) in anhyd CH_2Cl_2 (2 mL) under N_2. The pale yellow solution was stirred at rt and monitored by TLC analysis. After 18 h, the reaction mixture was concentrated under reduced pressure to give a clear yellow syrup. Purification of the crude product by flash chromatography afforded **9b–d** (80–89%).

*Methyl (2-ethylbutyl 2-acetamido-2,4-dideoxy-3-*O*-pivaloyl-α-L-*threo*-hex-4-enopyranosid)uronate **(9b)***

Prepared from **8b** in 80% yield after chromatography ((EtOAc/hexane 2:1) as a colourless syrup. $R_f = 0.42$ (EtOAc/hexane 1:1); 1H NMR ($CDCl_3$) δ0.81(6H,t,$J_{4',3'} = J_{4'',3''} = 7.5$ Hz, H-4′, H-4″), 1.18 (9H, s, OPiv), 1.21–1.41 (5H, m, H-2′, H-3′, H-3″), 1.97 (3H, s, NAc), 3.42 (1H, dd, $J_{1'a,1'b}$ 9.2, $J_{1'a,2'}$ 5.0 Hz, H-1′a), 3.67 (1H, dd, $J_{1'b,1'a}$ 9.2, $J_{1'b,2'}$ 5.3 Hz, H-1′b), 3.83 (3H, s, OMe), 4.42 (1H, dd, $J_{2,NH}$ 9.2, $J_{2,3}$ 1.4 Hz, H-2), 4.97 (1H, d, $J_{3,4}$ 4.2 Hz, H-3), 5.15 (1H, d, $J_{1,2}$ 1.8 Hz, H-1), 5.66 (1H, br d, $J_{NH,2}$ 9.0 Hz, NH), 6.23 (1H, dd, $J_{4,3}$ 4.5, $J_{4,2}$ 1.1 Hz, H-4); ^{13}C NMR ($CDCl_3$) δ 10.7, 11.0 (C-4′, C-4″), 22.8, 23.0 (C-3′, C-3″), 23.1 (NC(O)*Me*), 26.9 (OC(O)C*Me*$_3$), 31.9, 33.2 (C-2′, C-2″), 38.7 (OC(O)*C*Me$_3$), 40.9 (C-2′), 48.6 (C-2), 52.6 (CO_2*Me*), 64.3 (C-3), 71.3 (C-1′), 98.2 (C-1), 108.0 (C-4), 142.0 (C-5), 162.6 (*C*O_2Me), 169.5 (N*C*(O)Me), 177.6 (O*C*(O)CMe$_3$). LRMS m/z 422 ([M+Na]$^+$, 100%). HRMS calcd for $C_{20}H_{33}NNaO_7$ [M+Na] 422.2155, found 422.2156.

*Methyl (cyclopentyl 2-acetamido-2,4-dideoxy-3-*O*-pivaloyl-α-L-*threo*-hex-4-enopyranosid)uronate (**9c**)*

Prepared from **7c** in 89% yield after chromatography ((EtOAc/hexane 2:1) as a colourless syrup. $R_f = 0.43$ (EtOAc/hexane 1:1); 1H NMR ($CDCl_3$) δ1.18 (9H, s, OPiv), 1.39-1.79 (8H, m, H-2′, H-2″, H-3′, H-3″), 1.97 (3H, s,

NAc), 3.84 (3H, s, OMe), 4.21-4.30 (1H, m, H-1′), 4.36 (1H, ddd, $J_{2,NH}$ 9.0, $J_{2,4}$ 1.3 Hz, H-2), 4.95 (1H, dd, $J_{3,4}$ 4.8, $J_{3,1}$ 0.9 Hz, H-3), 5.24 (1H, d, $J_{1,2}$ 1.8 Hz, H-1), 5.58 (1H, br d, $J_{NH,2}$ 9.0 Hz, NH), 6.23 (1H, dd, $J_{4,3}$ 4.9, $J_{4,2}$ 1.1 Hz, H-4); ^{13}C NMR ($CDCl_3$) δ 23.1 (NC(O)*Me*), 23.2, 23.4 (C-3′, C-3″), 26.9 (OC(O)C*Me*$_3$), 31.9, 33.2 (C-2′, C-2″), 38.7 (OC(O)*C*Me$_3$), 48.6 (C-2), 52.7 (CO_2*Me*), 64.2 (C-3), 81.1 (C-1′), 97.0 (C-1), 107.5 (C-4), 142.3 (C-5), 162.6 (*C*O$_2$Me), 169.5 (N*C*(O)Me), 177.5 (O*C*(O)CMe$_3$). LRMS m/z 406 ([M+Na]$^+$, 100%). HRMS calcd for $C_{19}H_{29}NNaO_7$ [M+Na] 406.1842, found 406.1847.

*Methyl (benzyl 2-acetamido-2,4-dideoxy-3-*O*-pivaloyl-α-L-*threo*-hex-4-pyranosid)uronate (**9d**)*

Prepared from 7d in 83% yield after chromatography ((EtOAc/hexane 1:1) as an amorphous mass. R_f = 0.12 (EtOAc/hexane 1:1); ^{1}H NMR ($CDCl_3$) δ 1.12 (9H, s, OPiv), 1.98 (3H, s, NAc), 3.81 (3H, s, OMe), 4.89 (1H, dddd, $J_{2,NH}$ 9.0, $J_{2,1}$ = $J_{2,3}$ 2.1, $J_{2,4}$ 1.2 Hz, H-2), 4.64 (1H, d, J_{gem} 11.7 Hz, OCH_2), 4.79 (1H, d, J_{gem} 11.7 Hz, OCH_2), 5.01 (1H, ddd, $J_{3,4}$ 4.8, $J_{3,2}$ 2.1, $J_{3,1}$ 0.9 Hz, H-3), 5.30 (1H, br d, $J_{1,2}$ 2.1 Hz, H-1), 5.75 (1H, br d, $J_{NH,2}$ 9.0 Hz, NH), 6.25 (1H, dd, $J_{4,3}$ 4.8, $J_{4,2}$ 1.2 Hz, H-4), 7.27-7.32 (5H, m, Ph); ^{13}C NMR ($CDCl_3$) δ 22.9 (NC(O)*Me*), 26.8 (OC(O)C*Me*$_3$), 38.6 (OC(O)*C*Me$_3$), 48.5 (C-2), 52.5 (OMe), 64.2 (C-3), 70.7 (O*C*H$_2$Ph), 97.4 (C-1), 107.9 (C-4), 127.5, 127.7, 128.1 (OCH$_2$*Ph*), 136.6 (*ipso*-Ph), 142.0 (C-5), 162.3, 169.6, 177.5 (3 × carbonyl). LRMS m/z 428 ([M+Na]$^+$, 100%). HRMS calcd for $C_{24}H_{39}NNaO_9$ [M+Na] 428.1685, found 428.1692.

*General procedure for deprotection of **9b–d***

A solution of compound **9b–d** (∼0.4 mmol) in aq MeOH (50%, 5 mL) was adjusted to pH 13 using aq NaOH (0.5 M). The solution was stirred at rt and monitored by TLC analysis (EtOAc/MeOH/H_2O 7:2:1). After 18 h, Amberlite® IR-120 (H^+) resin was added to adjust to pH 3, the reaction was filtered, the resin was washed with MeOH/H_2O 1:1 (30 mL), and the filtrate was concentrated to dryness. PivOH was then removed by evaporation under reduced pressure (∼1 mmHg) at 40°C for 3 h. The residue was dissolved in water (5 mL), aq NaOH was added to adjust to pH 7.3, and the solution was lyophilised to afford an amorphous solid. The crude product was purified by HPLC, and then lyophilised to give **4b–d** (70–87%).

*Sodium (2-ethylbutyl 2-acetamido-2,4-dideoxy-α-L-*threo*-hex-4-enopyranosid)uronate (**4b**)*

Prepared from **9b** in 74% as a creamy coloured amorphous solid. R_f = 0.26 (EtOAc/MeOH/H_2O 7:2:1); ^{1}H NMR (D_2O) δ0.67(6H,t, $J_{4',3'}$ = $J_{4'',3''}$ = 7.5 Hz, H-4′, H-4″), 1.08-1.17 (4H, m, H-3′, H-3″), 1.24–1.34 (1H, m, H-2′), 1.84 (3H, s, NAc),), 3.36 (1H, dd, $J_{1'a,1'b}$ 9.9, $J_{1'a,2'}$ 5.7 Hz, H-1′a), 3.67 (1H, dd, $J_{1'b,1'a}$ 9.8, $J_{1'b,2'}$ 5.6 Hz, H-1′b), 3.88 (1H, t, $J_{2,1}$ = $J_{2,3}$ = 5.7 Hz, H-2), 4.06 (1H, dd, $J_{3,2}$ 5.4, $J_{3,4}$ 3.6 Hz, H-3), 4.90 (1H, br d, $J_{1,2}$ 5.7 Hz, H-1), 5.72 (1H, d, $J_{4,3}$ 3.6 Hz, H-4); ^{13}C NMR (D_2O) δ 10.1, 10.2 (C-4′, C-4″), 21.9 (NC(O)*Me*), 22.4, 22.5 (C-3′, C-3″), 40.2 (C-2′), 52.3 (C-2), 65.1 (C-3), 72.1 (C-1′), 99.5 (C-1), 107.8 (C-4), 144.6 (C-5), 160.9 (*C*O$_2$H), 174.3 (N*C*(O)Me). LRMS m/z 300 ([M-H]$^-$, 100%). HRMS calcd for $C_{14}H_{23}NNaO_6$ [M+Na] 324.1423, found 324.1425.

*Sodium (cyclopentyl 2-acetamido-2,4-dideoxy-α-L-*threo*-hex-4-enopyranosid)uronate (**4c**)*

Prepared from **9c** in 87% as a creamy coloured amorphous solid. R_f = 0.39 (EtOAc/MeOH/H_2O 7:2:1); ^{1}H NMR (D_2O) δ 1.35–1.78 (8H, m, H-2′, H-2″, H-3′, H-3″), 1.85 (3H, s, NAc), 3.80 (1H, t, $J_{2,1}$ = $J_{2,3}$ = 6.3 Hz, H-2), 4.08 (1H, br dd, $J_{3,2}$ 6.0, $J_{3,4}$ 3.6 Hz, H-3), 4.21-4.28 (1H, m, H-1′), 5.26 (1H, br d, $J_{1,2}$ 6.3 Hz, H-1), 5.65 (1H, d, $J_{4,3}$ 3.0 Hz, H-4); ^{13}C NMR (D_2O) δ 21.9 (NC(O)*Me*), 22.4, 22.7 (C-3′, C-3″), 31.4, 32.4 (C-2′, C-2″), 52.7 (C-2), 65.4 (C-3), 82.1 (C-1′), 98.1 (C-1), 107.3 (C-4), 145.3 (C-5), 170.4 (*C*O$_2$H), 174.3 (N*C*(O)Me). LRMS m/z 284 ([M-H]$^-$, 100%). HRMS calcd for $C_{13}H_{19}NNaO_6$ [M+Na] 308.1110, found 308.1118.

*Sodium (benzyl 2-acetamido-2,4-dideoxy-α-L-*threo*-hex-4-enopyranosid)uronate (**4d**)*

Prepared from **9d** in 95% as a creamy coloured amorphous solid. R_f = 0.33 (EtOAc/MeOH/H_2O 7:2:1); ^{1}H NMR (D_2O) δ1.78 (3H, s, NAc), 3.91 (1H, t, $J_{2,1}$ = $J_{2,3}$ = 5.0 Hz, H-2), 3.98 (1H, t, $J_{3,2}$ = $J_{3,4}$ = 4.4 Hz, H-3), 4.55 (1H, d, J_{gem} 12.0 Hz, OCH_2), 4.69 (1H, d, J_{gem} 12.0 Hz, OCH_2), 5.00 (1H, d, $J_{1,2}$ 5.1 Hz, H-1), 5.71 (1H, d, $J_{4,3}$ 3.6 Hz, H-4), 7.18–7.29 (5H, m, *Ph*); ^{13}C NMR (D_2O) δ 21.8 (NC(O)*Me*), 52.1 (C-2), 64.7 (C-3), 70.9 (O*C*H$_2$Ph), 97.8 (C-1), 107.3 (C-4), 128.3, 128.4, 128.6 (OCH$_2$*Ph*), 136.6 (*ipso*-Ph), 144.9 (C-5), 169.1 (*C*O$_2$H), 174.2 (N*C*(O)Me). LRMS m/z 308 ([M + H]$^+$, 100%). HRMS calcd for $C_{13}H_{18}NNa_2O_6$ [M+Na] 330.0954, found 330.0933.

Sialidase activity assay

Sialidase activity was assayed by our modification [10] of the fluorometric method of Potier *et al*. [12] using the fluorogenic substrate 4-methylumbelliferyl *N*-acetyl-α-D-neuraminide (MUN). The K_i estimates were calculated based on inhibition observed at an inhibitor concentration of

0.1 mM (i.e., [I] = 0.1 mM), using the following equation [13]:

$$\%\,\text{Inhibition} = [\text{I}]/\{[\text{I}] + K_\text{i}(1 + [\text{S}]/K_\text{m}\}$$

where [I] is the inhibitor concentration, K_i is the inhibition constant, [S] is the substrate concentration, and K_m is the Michaelis-Menten constant.

In a previous study, inhibition for Neu5Ac2en mimetics of this type was determined to be of a linear competitive type [8].

Molecular modelling

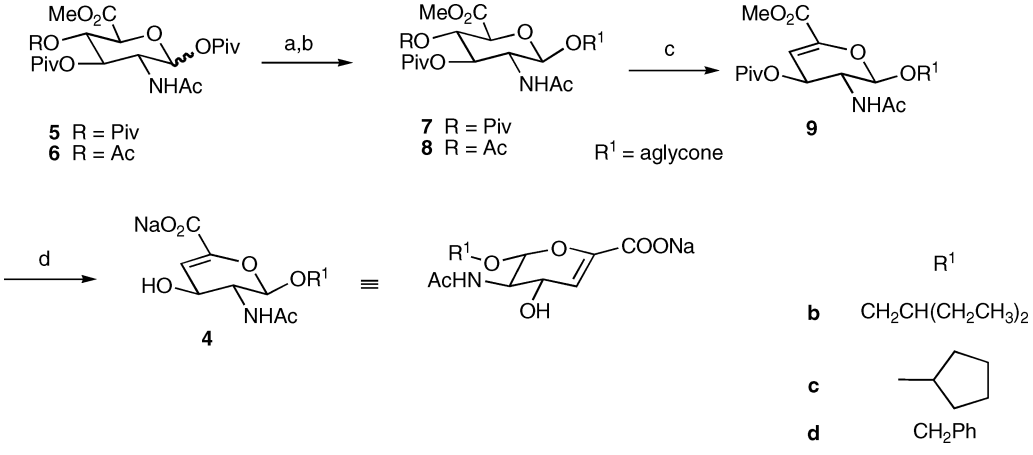
Compounds **4a** and **4d** were built using InsightII [14] with the ring conformation based on that of Neu5Ac2en (**3**) observed in complex with influenza virus sialidase (1F8B) [15]. The influenza virus sialidase (N9) crystal structure used for the docking experiment was 2QWK [16] as it was felt that the conformation of Glu276 (N2 numbering) was more relevant to the binding of these compounds than that observed in the native structure. AutoDock Tools (ADT) [17] was used to prepare the compounds and the protein structure for docking. For the two compounds ADT was used to add Gasteiger charges and define rotatable bonds, while for the protein, polar hydrogen atoms, Kollman atomic charges and solvation parameters were added.

The docking grid was prepared using Autogrid3 and was centred on the active site with the dimensions of 23.25 × 23.25 × 23.25 with a grid spacing of 0.375 . Autodock (version 3.05) [18] was used to perform a flexible docking of the compounds to the protein crystal structure. The AutoDock parameters used were the default, except for the number of energy evaluations which was increased to 1.25×10^{-6}. The top 10 ranking conformations were clustered into families using AutoDock and the results viewed using InsightII.

Results and discussion

We have recently reported [9] a simple method for the preparation of C-6 ether Neu5Ac2en mimetics **4** and we have now extended this method to the preparation of novel influenza virus sialidase inhibitors. Thus, treatment of the pivaloylated glucosaminuronates **5** and **6**, readily synthesised from *N*-acetylglucosamine using the previously described method [9], with TMSOTf in 1,2-dichloroethane (DCE) provided a series of novel β-*O*-glycosides **7c,d** and **8b** in moderate to good yields (56 to 76%) as shown in Scheme 1. The *O*-glycosides **7c, d** and **8b** were then subjected to β-elimination through treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in CH_2Cl_2. Subsequent deprotection employing base-catalysed deacylation and de-esterification at pH 13 using aqueous NaOH afforded the corresponding 4,5-unsaturated derivatives **4b–d** in good yields (Scheme 1). ^{1}H NMR spectroscopy of **4b–d** showed olefinic H-4 resonances at δ ~5.7 and the absence of resonances for H-5.

Sialidase activity was measured using a fluorometric assay, which was based upon a modification [11] of a method developed by Potier *et al*. [12] and measures the hydrolysis of 4-methylumbelliferyl 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-non-2-ulopyranosidonic acid (4-methylumbelliferyl *N*-acetyl-α-D-neuraminide, MUN). Neu5Ac2en mimetics **4a–d** were evaluated for inhibition against influenza virus sialidase (see Materials and Methods section for details), and the K_i estimates are shown in Table 1. To our delight all of the mimetics displayed significant inhibitory activity against both N9 and



Scheme 1 Synthesis of influenza virus sialidase inhibitors **4b–d**. *Reagents and Conditions:* (a) 1.1 eq. TMSOTf, $ClCH_2CH_2Cl$ (DCE), 50°C, 3 d; (b) (i) 3 Å MS, rt, 30 min, (ii) 3 eq. ROH, 24 h, rt; (c) 2 eq. 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), CH_2Cl_2, rt, 18 h; (d) 50% aq MeOH, aq NaOH, pH 13, 18 h

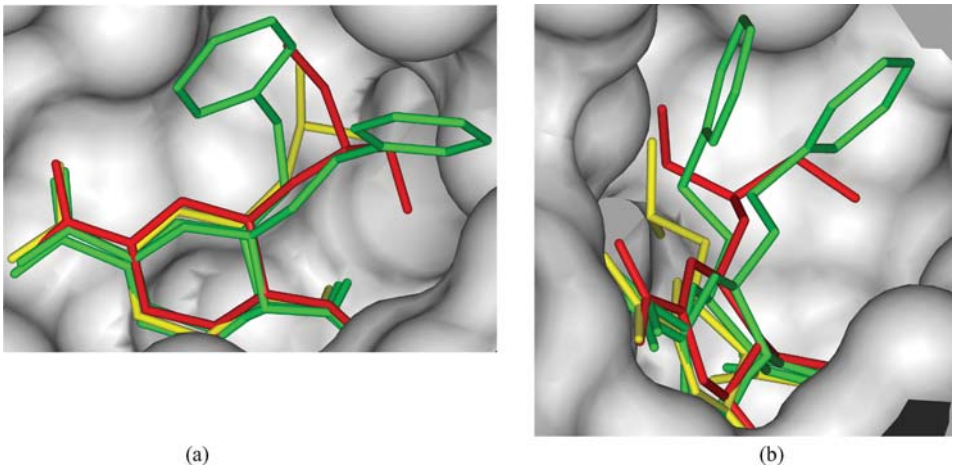


Fig. 1 (a) and (b): Two views of a Connolly surface over the active site of influenza virus sialidase (N9) with **2b** (1-ethylpropyl aglycone of Tamiflu active) from the crystal structure (2QWK) shown in dark grey. From the Autodock calculations **4a** (isopropyl aglycone) is shown in light grey, while the two clusters of **4d** (benzyl aglycone) are shown in steel grey

Table 1 K_i estimates (M) of Neu5Ac2en mimetics **4a-d** determined against sialidase from influenza virus N9 and N2

Inhibitor R^1 = Enzyme	**4a** $CH(CH_3)_2$	**4b** $CH_2CH(CH_2CH_3)_2$	**4c** cyclopentyl	**4d** CH_2Ph	**3** Neu5Ac2en
N9	1×10^{-6}	1×10^{-6}	1×10^{-6}	2.5×10^{-5}	5×10^{-6}
N2	1×10^{-6}	1×10^{-6}	1×10^{-6}	2.5×10^{-4}	1×10^{-6}

N2 influenza virus sialidase. In fact all of the prepared β-*O*-glycosides of 4,5-unsaturated 2-acetamido-D-glucuronic acid, except **4d**, were found to be μM inhibitors and therefore as potent as Neu5Ac2en (**3**). As a consequence it would appear that the enzyme's active site can readily accommodate a range of hydrophobic groups.

To investigate why **4d** may be a poorer inhibitor than any of the other mimetics, we thought it of value to undertake a molecular modelling study utilising an Autodock [18] approach and available crystal structures [15,16]. An induced-fit phenomenon has been observed for other hydrophobic C-6 glycerol sidechain replacements such as those reported for C-6 carboxamide-Neu5Ac2en derivatives [19] and **2b** [16]. Consequently we have used in our study the crystal structure of N9 influenza virus sialidase in complex with **2b**. A preliminary x-ray crystallographic study of an N9 influenza virus sialidase—**4a** complex also supports the notion that induced fit is observed upon binding of β-*O*-glycosides of 4,5-unsaturated 2-acetamido-D-glucuronic acid [20]. The Autodock calculations of **4a** resulted in the top ten structures belonging to a single conformationally similar family. Similar calculations for **4d** resulted in the top ten structures belonging to four families that can be further grouped into two spatially-related clusters. Figure 1 shows *O*-benzyl glycoside (**4d**) and **4a** docked into the influenza virus sialidase active site referenced to the crystallographically-determined binding mode of **2b** [16]. Inspection of Figure 1 enables a comparison of the binding modes of each of the ligands **4a**, **4d**, and **2b** and provides a reasonable explanation of why **4d** does not inhibit influenza virus sialidases with the same level of potency as **4a–c**. Unlike the hydrophobic groups in either **4a** or **2b** the benzyl group in **4d** does not make significant interaction with the active site and is directed out into the solvent space. The result of the loss of such hydrophobic interactions would be a reduction in binding affinity which is what is experimentally observed.

Conclusions

We have successfully prepared a number of β-*O*-glycosides of 4,5-unsaturated 2-acetamido-D-glucuronic acid and evaluated them as inhibitors of influenza virus sialidase. The majority of the synthesised inhibitors are equipotent to Neu5Ac2en (**3**). We believe that the 4,5-unsaturated 2-acetamido-D-glucuronic acid framework provides an exciting opportunity for the further development of novel influenza virus sialidase inhibitors that may deliver new potent anti-influenza drugs.

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