Galactose-conjugates of the oseltamivir pharmacophore—new tools for the characterization of influenza virus neuraminidases†

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We describe the synthesis of mimetics of the α2–3 and α2–6 sialogalactoside substrates of influenza neuraminidase which include the oseltamivir pharmacophore, and report the sub-nanomolar affinities for these novel neuraminidase inhibitors. The challenge of synthesizing a Phospha-Oseltamivir/Tamiphosphor monoester involving the secondary 3-hydroxy group of galactose required to mimic the α2–3 sialogalactoside has been overcome by palladium-promoted coupling of the oseltamivir-derived vinyl iodide with a protected galactose-3-phosphonate. The difference in binding of these two inhibitors to a given influenza neuraminidase should be a function of its α2–3/α2–6-selectivity, an important, but not yet fully understood factor in the adaptation of highly pathogenic avian influenza viruses to human hosts.

Introduction

Influenza continues to be a major health concern for the world population with epidemics occurring worldwide on a yearly basis.[1] Influenza is caused by negative-strand RNA viruses, classified into influenza types A and B, which are important targets of current medicinal chemistry. In particular, type A influenza viruses, most of which are avian viruses, are at the centre of attention as a possible source of a severe flu pandemic.[2,3] They are further classified as subtypes based on the antigenic behaviour of their two surface glycoproteins, a haemagglutinin (subtypes H1–H16) and a neuraminidase (subtypes N1–N9).[4] The interplay between the neuraminidase (NA) and haemagglutinin (HA) crucially affects both the host range and pathogenicity of influenza virus subtypes. Consequently, one important aim of medicinal chemists, virologists, and structural biologists alike is to understand the molecular basis of how an avian influenza virus, such as the notorious H5N1 virus, could adapt to the environment of the human respiratory tract and thus becomes a major threat to human health.

The natural receptors (for HA) and substrates (for NA) for influenza viruses are the *N*-acetylneuraminic acids on the host cell surface, which are α-ketosidically linked to galactose (Fig. 1). A relatively clear picture has emerged for the trimeric haemagglutinins. Those of human viruses bind more strongly to α2–6-linked sialylgalactosides (predominant in the human lung) and those of avian viruses bind more strongly to α2–3-linked sialylgalactosides (predominant in the intestines of birds) (Fig. 1).[5]

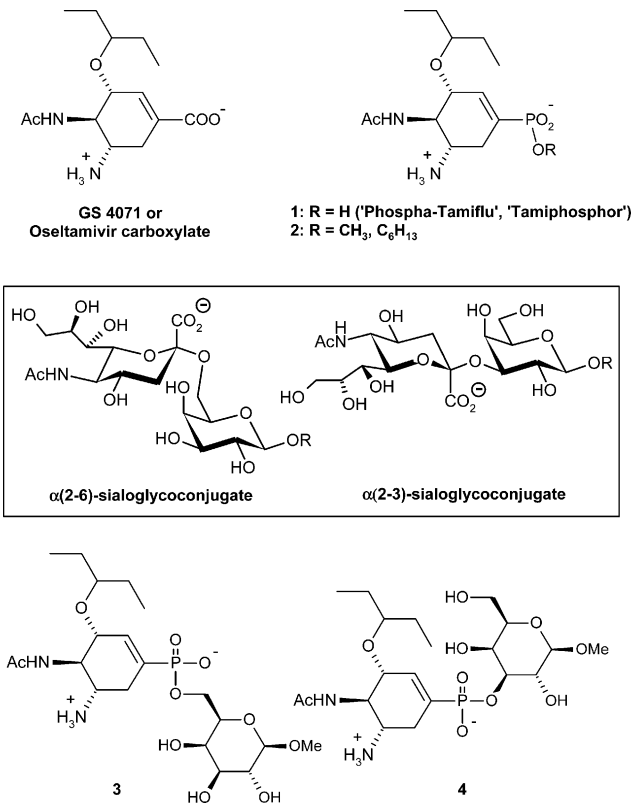

Fig. 1 Sialoglycoconjugates, influenza neuraminidase inhibitors and targeted galactose-conjugates.

For the tetrameric neuraminidases, which hydrolyze the ketosidic linkage in sialosides and thus cleave the neuraminic acid from the host cell sialoglycoconjugates, the situation is less clear. Avian influenza neuraminidases have a pronounced preference for α2–3-linked sialylgalactosides while human influenza neuraminidases act on both linkages but, in the majority of studies

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reported, still retain a higher activity with the α2–3-linked sialylgalactosides.[6] These data are generally obtained by analyzing the reaction kinetics for the hydrolysis of the two substrates by a given neuraminidase or whole virus.

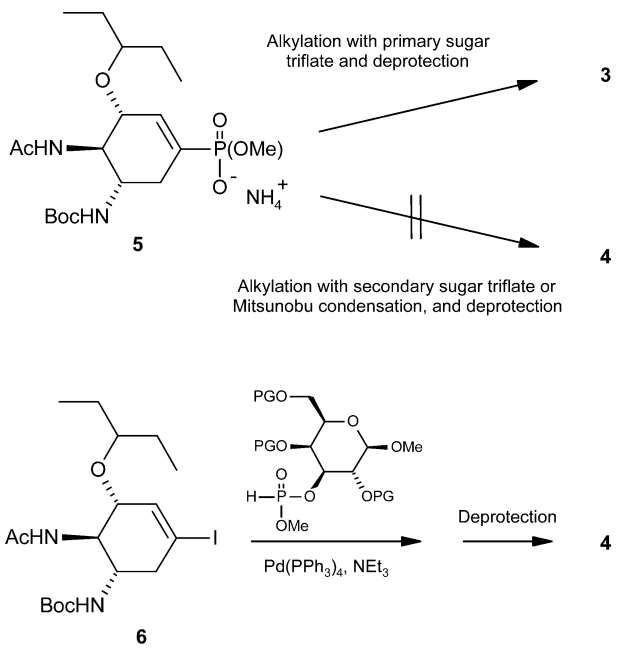
In this contribution, we suggest inhibitor-based tools for the characterization of influenza neuraminidases. Oseltamivir carboxylate, marketed as its ester prodrug Tamiflu™, is a sub-nanomolar inhibitor of all influenza virus A neuraminidases (Fig. 1).[7] We recently published an effective synthesis of highly active phospha-isosteres **1–3** of oseltamivir, where a phosphonate or phosphonate monoester replaces the carboxylate without impairing the potency of the oseltamivir pharmacophore.[8] **3** represents a chemically stable mimetic of an α2–6-linked sialylgalactoside which has been shown to inhibit the neuraminidase activity of influenza virus A/Norway/1758/07 with an inhibitory constant in the sub-nanomolar range (Fig. 1).[8]

Any differential inhibition of an influenza neuraminidase by **3** and the corresponding α2–3 sialylgalactoside mimetic **4** would necessarily depend on interactions beyond the primary binding site and hence be determined by linkage recognition. For example, such data could help to shed light on the adaptation process undergone by the notorious bird flu virus (H5N1) when it changes preference from an α2–3 sialylgalactoside receptor environment in birds to the α2–6 sialylgalactoside receptor environment of the human lung.

Results and discussion

Synthesis

Cyclohexenephosphonate monoesters of type **2** and **3** have so far been synthesized by us through alkylation of monomethyl phosphonates such as **5** with the protected sugar triflate or condensation under Mitsunobu conditions (Scheme 1).[8,9]



Scheme 1 Synthetic routes to galactose conjugates of the oseltamivir motif (PG = protecting group).

Unfortunately, compound **4**, the mimetic of an α2–3-linked sialylgalactoside (Fig. 1), proved not to be accessible *via* these routes, which so far remain limited to primary hydroxy groups.

In order to alleviate the steric congestion probably responsible for the problem, we decided to attempt a direct coupling of the appropriate vinyl iodide **6**[8,10] (Fig. 2) with the mixed methyl, 3-*O*-galactosyl phosphonate **10**. To obtain **10**, the methoxymethyl protecting group was introduced into selectively protected methyl β-D-galactopyranoside **7**[11] in 55% yield to give **8**, followed by cleavage of the 3-*O*-allyl group with $PdCl_2$ in 69% yield to give **9** (Scheme 2).

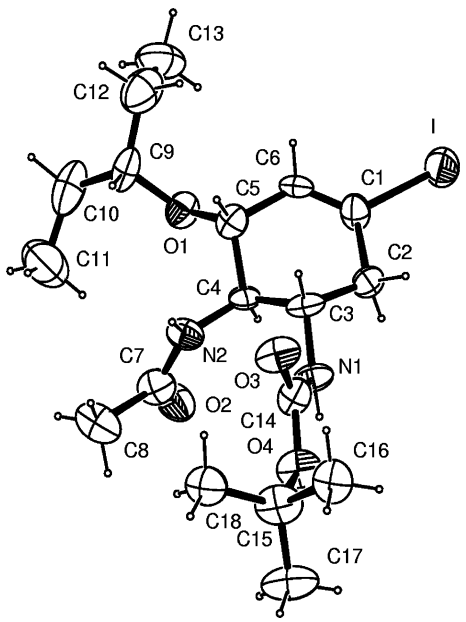


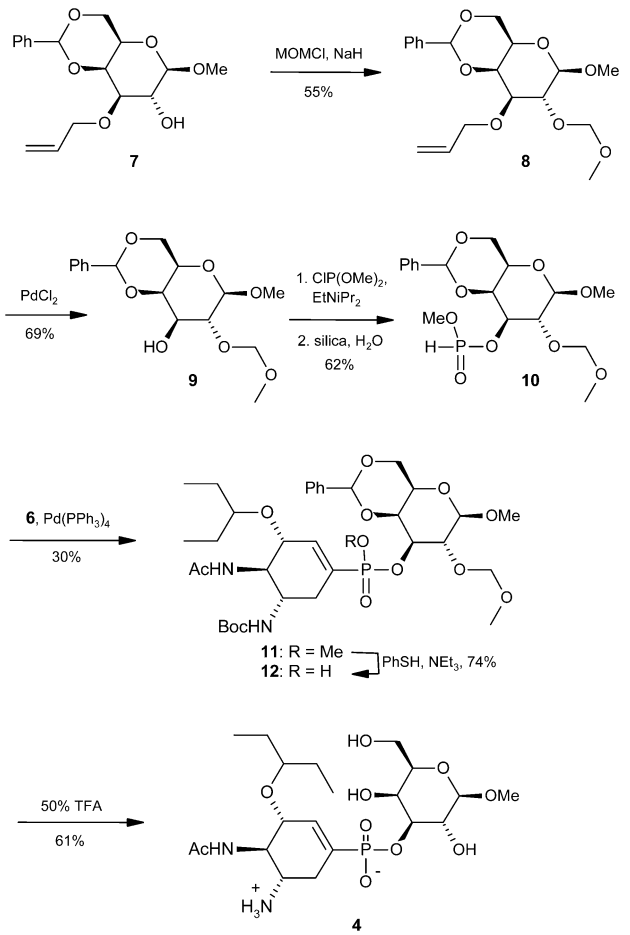
Fig. 2 Crystal structure of vinyl iodide **6**.

The phosphonate was introduced by esterification with dimethyl chlorophosphonate,[12] yielding initially the triester which was immediately hydrolyzed on silica to give diester **10** in 62% yield.

Coupling of phosphonate **10** with vinyl iodide **6** using $Pd(PPh_3)_4$ proceeded with only moderate 30% yield which was, however, sufficient for our purposes. Interestingly, a vinyl diphenylphosphine oxide by-product arising from coupling with displaced palladium ligand was also isolated. Selective phosphonate demethylation converted the mixture of diastereomers **11** into the monoester **12** in 74% yield. Final deprotection was achieved in aqueous trifluoroacetic acid, furnishing sialoside mimetic **4** in 61% yield after purification by gel-permeation chromatography. A spin-echo difference (SED) spectrum[13] of **4** unequivocally confirmed the linkage of the 'Phospha-Tamiflu' moiety to the 3-position of the galactose (see also the ESI†).

Inhibition of neuraminidase activity

To compare the inhibitory potency of **3**, **4** and oseltamivir, a whole-virus assay using allantoic fluid from infected eggs was employed. In this standard assay, which is widely used in the influenza field,



Scheme 2 Synthesis of an α2–3 sialylgalactoside mimetic.

Table 1 Inhibition constants (K_i [nM]) for target phosphonates[a]

3	4	Oseltamivir
0.74 ± 0.08	0.29 ± 0.04	0.14 ± 0.02

[a] Inhibition of MUNANA hydrolysis catalysed by neuraminidase from influenza virus A/Norway/1758/07; inhibition constants were determined as described in the ESI†; K_M for MUNANA = 6.4 ± 0.6 μM.

the inhibition constant K_i is obtained by measuring the effect of the inhibitor on the rate of MUNANA hydrolysis.[14]

When tested for inhibition of the neuraminidase activity of an H1N1 virus (A/Norway/1758/07), compounds **3** and **4** indeed displayed a significant difference, with the α2–3 sialylgalactoside mimetic **4** inhibiting somewhat more strongly (Table 1). In the absence of detailed structural data, this finding cannot be directly correlated with the substrate specificity of the virus but it does demonstrate that the set of the two compounds is useful to establish a 'fingerprint' for a neuraminidase which is, at least partly, governed by its substrate specificity. In other words, the ratio of the inhibition constants of **3** and **4** should be to some extent proportional, or inversely proportional, to the ratio of the binding constants of the respective α2–3 and α2–6 sialylgalactoside substrates.

Experimental

General

Reaction solvents were purchased anhydrous and used as received. Solvents for chromatography were distilled before use. Reactions were monitored by TLC using precoated silica gel 60 F_{254} plates. Compounds were detected by UV absorption and/or by staining with a molybdenum phosphate reagent (20 g ammonium molybdate and 0.4 g cerium(IV) sulfate in 400 mL of 10% aq. sulfuric acid) or a basic $KMnO_4$-solution and subsequent heating at 120 °C for 5 min. Silica gel 60 A 'Davisil' (particle size 35–70 μm) from Fisher Scientific, UK was used for flash chromatography. ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and all multidimensional NMR spectra were recorded on Varian VNMRS spectrometers (600 MHz, 500 MHz or 400 MHz, see compound characterisation for individual experiments). Chemical shifts in ^{1}H NMR and ^{13}C NMR spectra were referenced to the residual proton resonance of the respective deuterated solvent, $CDCl_3$ (7.24 ppm), D_2O (4.80 ppm), D_2O in CD_3OD (4.88 ppm). For ^{31}P NMR spectra H_3PO_4 was used as external standard (0 ppm). In some cases, ^{13}C chemical shifts were deduced from heteronuclear multiple spin correlation (HSQC) spectra. The H-6_{ax} and H-6_{eq} assignments refer to the pseudoaxial and pseudoequatorial protons in the cyclohexene systems, respectively, obtained by ROESY spectroscopy. In pseudo-disaccharidic systems, the cyclohexene ring is indicated by the suffix 'a', the sugar by the suffix 'b'. Diastereomeric mixtures of mixed diesters are indicated by the suffix h (higher moving) and l (lower moving) but no attempts of separation were made. HR-ESI MS spectra were recorded on a Bruker Daltonics Apex III in positive mode with MeOH/H_2O as solvent.

Fluorescence was measured in a JASCO FP-6300 fluorimeter. Silica-based MPLC chromatography was carried out on the Büchi Sepacore system equipped with glass columns packed with LiChroprep Si 60 (15–25 μm) from Merck, Darmstadt, Germany. Gel permeation chromatography was carried out in the 1–10 mg scale on a XK 16/70 column (bed volume 130 mL), from Amersham packed with Sephadex G-10 (particle size 40–120 μm) and 0.1 M NH_4HCO_3 as buffer. Detection was achieved with a differential refractometer from Knauer, Berlin, Germany. Fine chemicals were purchased from Aldrich-, Sigma- or Acros-Chemicals and were of the highest purity available.

(Methyl-β-D-galactopyranos-3-yl) [(3*R*,4*R*,5*S*)-4-acetamido-3-amino-5-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate] (4). Compound **12** (6 mg, 8.2 μmol) was dissolved in a solution of 1:1 TFA/H_2O (2 mL) and the mixture was stirred at room temperature for 3 hours. The mixture was lyophilised and the residue was purified by gel permeation chromatography to afford compound **4** (2.5 mg, 5 μmol, 61%) as a white powder after lyophilisation. δ_H (600 MHz, D_2O) 0.87 (3 H, t, $J_{2,P}$ 7.4 Hz, pentyl-CH_3), 0.92 (3 H, t, J 7.4 Hz, pentyl-CH_3), 1.44–1.52 (1 H, m), 1.54–1.63 (3 H, m), 2.10 (3 H, s, $COCH_3$), 2.37–2.45 (1 H, m, 6a′-H), 2.73–2.81 (1 H, m, 6a-H), 3.27 (1 H, s, 5a-H), 3.54 (1 H, tt, J 5.6 Hz, pentyl-CH), 3.60 (3 H, s, OCH_3), 3.64 (1 H, dd, J 9.6, 8.2 Hz, 2b-H), 3.71–3.84 (3 H, m, H-6b, H-6b′, 5b-H), 3.89 (1 H, dd, $J_{4a,5a}$ 11.0, $J_{4a,3a}$ 9.4 Hz, 4a-H), 4.02 (1 H, ddd, $J_{3b,2b}$ 8.8, $J_{3b,4b}$ 3.4 Hz, 3b-H), 4.08 (1 H, d, $J_{4b,3b}$ 3.3 Hz, 4b-H), 4.22 (1 H, d, $J_{3a,4a}$ 8.5 Hz, 3a-H), 4.39 (1 H, d, $J_{1b,2b}$ 8.3 Hz, 1b-H), 6.42 (1 H, d, $J_{2a,P}$ 20.4 Hz, 2a-H); δ_C (150.9 MHz, D_2O) 8.48, 8.51, 25.10, 22.26

($NHCOCH_3$), 25.45, 31.15 (6a-C), 49.49 (5a-C), 57.12 (OCH_3), 60.80 (6b-C), 68.35 (4b-C), 69.66 (d, $J_{2b,P}$ 5.6 Hz, 2b-C), 74.68 (5b-C), 76.32 (d, $J_{3a,P}$ 20.4 Hz, 3a-C), 76.61 (d, $J_{3b,P}$ 5.8 Hz, 3b-C), 81.14, 84.08 (pentyl-CH), 103.52 (1b-C), 136.77 (d, $J_{2a,P}$ 7.3 Hz, 2a-C), 174.92 ($NHCOCH_3$); δ_P (242.9 MHz, D_2O) 13.06; HR-ESI-MS calculated for $C_{19}H_{35}N_2O_{10}P$ $(M + Na)^+$ 519.2078, found 519.2062.

Ammonium [methyl (3*R*,4*R*,5*S*)-4-acetamido-3-(1,1-dimethylethyloxycarbonylamino)-5-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate] (5). The synthesis of compound **5** has been described by us before.[8]

(3*R*,4*R*,5*S*)-4-Acetamido-3-(1,1-dimethylethyloxycarbonylamino)-5-(1-ethylpropoxy)-1-iodocyclohexene (6). Compound **6** was synthesised from the corresponding azide as described before.[8] Crystals were obtained from a solution in CH_2Cl_2.

Methyl 3-*O*-allyl-4,6-*O*-benzylidene-β-D-galactopyranoside (7). Compound **7** was synthesised as described in the literature.[15]

Methyl 2-*O*-methoxymethyl-3-*O*-allyl-4,6-*O*-benzylidene-β-D-galactopyranoside (8). To a solution of compound **7** (0.514 g, 1.59 mmol) in dry THF (4 mL), cooled to 0 °C in an atmosphere of nitrogen, was added NaH (57.4 mg, 2.39 mmol) as a suspension in dry THF (3mL). The mixture was stirred for 15 min and then cooled to −20 °C. MOMCl (157 μL, 2.07 mmol) was added dropwise, the mixture was allowed to warm up to room temperature and stirred overnight. The reaction was quenched by addition of saturated aqueous NH_4HCO_3 (5 mL). CH_2Cl_2 (40 mL) was added and the mixture was extracted with NH_4HCO_3 (2 × 10 mL), washed with brine (2 × 10 mL), the organic phase was dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (Tol:EA 5:1 → 1:5) gave compound **8** (0.323 g, 0.88 mmol, 55%) as a white solid. R_f = 0.53 (Tol:EA 1:1). δ_H (500 MHz, $CDCl_3$) 3.34 (1 H, s, 5-H), 3.42 (3 H, s, methoxymethyl-CH_3), 3.44 (1 H, dd, $J_{3,2}$ 9.8, $J_{3,4}$ 3.6 Hz, 3-H), 3.53 (3 H, s, OCH_3), 3.84 (1 H, dd, $J_{2,3}$ 9.7, $J_{2,1}$ 7.9 Hz, 2-H), 4.03 (1 H, dd, $J_{6',6}$ 12.3, $J_{6',5}$ 1.6 Hz, 6′-H), 4.11–4.21 (3 H, m, 4-H, allyl-OCH_2), 4.25 (1 H, d, $J_{1,2}$ 7.8 Hz, 1-H), 4.29 (1 H, dd, $J_{6,6'}$ 12.3, $J_{6,5}$ 1.4 Hz, 6-H), 4.80, 4.82 (2 H, 2d, J 6.2 Hz, methoxymethyl-CH_2), 5.16 (1 H, dd, J 10.4, 1.5 Hz, allyl-$CHCH_2$), 5.29 (1 H, dd, J 17.2, 1.6 Hz, allyl-$CHCH_2$), 5.50 (1 H, s, CHPh), 5.91 (1 H, ddd, J 17.3, 10.4, 5.7 Hz, allyl-$CHCH_2$), 7.31–7.36 (3 H, m), 7.50 (2 H, m); δ_C (125.8 MHz, $CDCl_3$) 56.07 (methoxymethyl-CH_3), 56.79 (OCH_3), 66.61 (5-C), 69.40 (6-C), 71.02, 73.72 (4-C), 74.46 (2-C), 79.21 (3-C), 97.65 (methoxymethyl-CH_2), 101.52 (Ph-CH), 104.22 (1-C), 117.34, 126.72, 128.24, 129.08, 135.17, 138.01; HR-ESI-MS calculated for $C_{19}H_{26}O_7$ $(M + Na)^+$ 389.1575350, found 389.1570743.

Methyl 2-*O*-methoxymethyl-4,6-*O*-benzylidene-β-D-galactopyranoside (9). A mixture of compound **8** (0.316 g, 0.86 mmol), NaOAc (0.212 g, 2.59 mmol) and $PdCl_2$ (0.168 g, 0.95 mmol) in AcOH (4 mL) and H_2O (0.4 mL) was stirred for 5 hours at room temperature. The reaction mixture was evaporated in *vacuo.* The residue was dissolved in CH_2Cl_2 (20 mL) and washed successively with saturated aqueous $NaHCO_3$ (10 mL) and H_2O (10 mL), the organic phase was dried over $MgSO_4$ and evaporated to dryness. Purification of the residue by flash chromatography (Tol:EA; 3:1 → 1:3) gave compound **9** (0.194 g, 0.595 mmol, 69%). R_f = 0.17 (Tol:EA 1:1). δ_H (500 MHz, $CDCl_3$) 3.01 (1 H, bd, J 5.4 Hz, OH), 3.41–3.44 (4 H, m, 5-H, methoxymethyl-CH_3), 3.52–3.55 (3 H, m, OCH_3), 3.62–3.70 (2 H, m, 3-H, 2-H), 4.05 (1 H, d, $J_{6',6}$ 12.5 Hz, 6′-H), 4.20 (1 H, s, 4-H), 4.25 (1 H, d, $J_{1,2}$ 6.5 Hz, 1-H), 4.32 (1 H, d, $J_{6,6'}$ 12.5 Hz, 6-H), 4.76, 4.84 (2 H, 2d, J 6.5 Hz, methoxymethyl-CH_2), 5.53 (1 H, s, CHPh), 7.30–7.37 (3 H, m), 7.46–7.52 (2 H, m); δ_C (125.8 MHz, $CDCl_3$) 56.03, 57.15 (OCH_3), 66.71 (5-C), 69.30 (6-C), 72.48, 75.78 (2-C, 4-C), 77.92 (3-C), 97.74, 101.76 (pentyl-CH), 103.77 (1-C), 126.74, 128.38, 129.36, 137.81; HR-ESI-MS calculated for $C_{16}H_{22}O_7$ $(M + Na)^+$ 349.1252960, found 349.1257742.

***O*-Methyl *O*-(methyl 2-*O*-methoxymethyl-4,6-*O*-benzylidene-β-D-galactopyranos-3-yl) phosphonic acid (10).** Under an atmosphere of nitrogen, compound **9** (31 mg, 0.095 mmol) was dissolved in dry CH_2Cl_2 (1 mL). *N*,*N*-diisopropyl ethyl amine (49 μL, 0.285 mmol) was added to the solution which was then cooled to 0 °C. Chloro(dimethyl)phosphine[12] (40 μL) was added dropwise, the reaction mixture was allowed to come to room temperature and stirred overnight. Solid $NaHCO_3$ (50 mg) was added to the reaction mixture, followed by MeOH (0.5 mL) and silica (50 mg). The mixture was evaporated *in vacuo* to dryness. Purification by flash chromatography (Tol:EA:MeOH 1:1:0 → 0:10:1) gave compound **10** (24 mg, 0.059 mmol, 62%). Upper spot on tlc: R_f = 0.18, lower spot on tlc: R_f= 0.16 (EA:MeOH 20:1). NMR data of the isomer corresponding to the upper spot: δ_H (600 MHz, $CDCl_3$) 3.38 (3 H, s, methoxymethyl-CH_3), 3.44 (1 H, s, 5-H), 3.54 (3 H, s, OCH_3), 3.70 (3 H, d, J 12.1 Hz, $POCH_3$), 3.90 (1 H, dd, $J_{2,3}$ 9.8, $J_{2,1}$ 7.7 Hz, 2-H), 4.05 (1 H, dd, $J_{6',6}$ 12.5, $J_{6',5}$ 1.6 Hz, 6′-H), 4.28 (1 H, d, $J_{1,2}$ 7.7 Hz, 1-H), 4.32 (1 H, dd, $J_{6,6'}$ 12.4, $J_{6,5}$ 1.2 Hz, 6-H), 4.38 (1 H, d, J 3.7 Hz, 4-H), 4.50–4.45 (1 H, m, 3-H), 4.72, 4.87 (2 H, 2d, J 6.3 Hz, methoxymethyl-CH_2), 5.55 (1 H, s, CHPh), 6.93 (1 H, d, $J_{H,P}$ 717.2 Hz, P-H), 7.32–7.36 (3 H, m), 7.48–7.51 (2 H, m); δ_C (150.9 MHz, $CDCl_3$) 51.90 (d, J 5.8 Hz, $POCH_3$), 56.42 (methoxymethyl-CH_3), 57.23 (OCH_3), 66.13 (5-C), 69.14 (6-C), 73.98 (d, $J_{2,P}$ 5.7 Hz, 2-C), 75.85 (4-C), 76.25 (d, $J_{3,P}$ 5.9 Hz, 3-C), 97.64 (methoxymethyl-CH_2), 101.43 (benzylidene-CH), 104.17 (1-C), 126.57, 128.40, 129.33, 137.73; δ_P (161.9 MHz, $CDCl_3$) (8.19), 9.92; HR-ESI-MS calculated for $C_{17}H_{25}O_9P$ $(M + Na)^+$ 427.1143900, found 427.1128400.

Methyl (methyl 2-*O*-methoxymethyl-4,6-*O*-benzylidene-β-D-galactopyranos-3-yl) [(3*R*,4*R*,5*S*)-4-acetamido-3-(1,1-dimethylethyloxycarbonylamino)-5-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate] (11). The phosphinic acid **10**, tetrakistriphenylphosphine palladium (7 mg, 6.3 μmol) and vinyl iodide **6** (20 mg, 0.042 mmol) were mixed under an atmosphere of dry nitrogen. The mixture was dissolved in anhydrous toluene (3 mL) and triethylamine (8.8 μL, 0.063 mmol) was added. The mixture was stirred at 85 °C for 2.5 hours. After cooling to room temperature, the reaction was quenched by addition of saturated aqueous NH_4Cl (10 mL). CH_2Cl_2 (15 mL) was added to the solution which was then extracted with NH_4Cl (5 mL), washed with brine (2 × 5 mL), the organic phase was dried over $MgSO_4$ and the solvent was evaporated. Purification by flash chromatography (Tol:EA:MeOH 1:2:0 → 0:10:1) gave a mixture of phosphonate diastereoisomers **11** (9 mg, 0.012 mmol, 30%). R_f = 0.34 (EA:MeOH 10:1). The product contains a cyclohexenenyl diphenylphosphine oxide impurity which could be separated and characterised only after

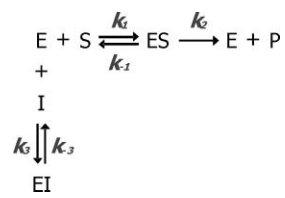
the following synthetic step (compound **12**). δ_H (500 MHz, $CDCl_3$) 6.53/6.66 (1 H, 2d, $J_{2a,P}$ 22 Hz, 2a-H of the 2 diastereoisomers); δ_P (161.9 MHz, $CDCl_3$) 18.07/18.93 (2 diastereoisomers); HR-ESI-MS calculated for $C_{35}H_{55}N_2O_{13}P$ $(M + Na)^+$ 765.3321640, found 765.3333975.

(Methyl 2-*O*-methoxymethyl-4,6-*O*-benzylidene-β-D-galactopyranos-3-yl) [(3*R*,4*R*,5*S*)-4-acetamido-3-(1,1-dimethylethyloxycarbonylamino)-5-(1-ethylpropoxy)-1-cyclohexene-1-phosphonate] (12). Under an atmosphere of dry nitrogen, compound **11** (8 mg, 0.011 mmol) was dissolved in anhydrous THF (1 mL). Anhydrous NEt_3 (22 μL, 0.156 mmol) and thiophenol (8 μL, 0.078 mmol) were added to the solution. After 48 h of stirring at room temperature, more anhydrous NEt_3 (22 μL, 0.156 mmol) and thiophenol (8 μL, 0.078 mmol) were added to the mixture which was stirred for another 24 h at room temperature. After evaporation of the solvent, purification by flash chromatography (EA:MeOH 10:1→1:5) afforded compound **12** (6 mg, 8.2 μmol, 74%). δ_H (600 MHz, CD_3OD) 0.88–0.95 (6 H, m), 1.41 (9 H, s), 1.43–1.60 (4 H, m), 1.97 (3 H, s), 2.35–2.43 (1 H, m, 6a′-H), 2.70–2.77 (1 H, m, 6a-H), 3.42–3.45 (1 H, m, pentyl-CH), 3.46 (3 H, s, methoxymethyl-CH_3), 3.57 (4 H, s, 5b-H, OCH_3), 3.71–3.77 (1 H, m, 5a-H), 3.81 (1 H, dd, *J* 8.7 Hz, 2b-H/3b-H), 3.89 (1 H, dd, *J* 10.0 Hz, 4a-H), 4.08 (1 H, d, *J* 6.7 Hz, 3a-H), 4.14–4.21 (2 H, m, 6b′-H-, 2b-H/3b-H), 4.23 (1 H, d, $J_{6b,6b'}$ 12.4 Hz, 6b-H), 4.36–4.41 (2 H, m, 1b-H, 4b-H), 4.82–4.89 (1 H, 2d, *J* 5.9 Hz, methoxymethyl-CH_2), 5.65 (1 H, s, CHPh), 6.48 (1 H, d, $J_{2a,P}$ 19.8 Hz, 2a-H), 7.33–7.38 (3 H, bs), 7.52–7.56 (2 H, bs); δ_C (150.9 MHz, CD_3OD) 9.84, 10.44, 23.19, 26.91, 27.55, 28.91, 32.96 (d, $J_{6a,P}$ 9.6 Hz, 6a-C), 51.20 (d, $J_{5a,P}$ 13.6 Hz, 5a-C), 56.64 (methoxymethyl-CH_3), 56.94 (d, $J_{4a,P}$ 2.6 Hz, 4a-C), 57.31 (OCH_3), 67.86 (5b-C), 70.19 (6b-C), 74.88 (d, $J_{3b\text{-}C/2b\text{-}C,P}$ 5.95 Hz, 3b-C/2b-C), 76.05 (d, $J_{3b\text{-}C/2b\text{-}C,P}$ 5.1 Hz, 3b-C/2b-C), 77.04 (4b-C), 78.10 (d, $J_{3a,P}$ 19.85 Hz, 3a-C), 80.12, 83.39 (pentyl-CH), 98.20 (methoxymethyl-CH_2), 102.27 (benzylidene-CH), 105.40 (1b-C), 127.76, 129.04, 129.85, 137.66 (m, 2a-C), 139.95, 158.09 ($NHCOOC(CH_3)_3$), 173.71 ($NHCOCH_3$); δ_P (161.9 MHz, CD_3OD) 10.87; HR-ESI-MS calculated for $C_{34}H_{53}N_2O_{13}P$ $(M + Na)^+$ 775.3140430, found 775.3153421.

Inhibition of neuraminidase activity

Neuraminidase (NA) enzymatic activity was studied using the fluorescent substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA). Measurements were made at 37 °C in 32.5 mM MES (pH 6.5) + 4 mM $CaCl_2$ using a JASCO FP-6300 fluorimeter with excitation at 365 nm and emission at 450 nm. Michaelis–Menten constants for the enzyme, $K_m = (k_2 + k_{-1})/k_1$, were determined using standard initial rate measurements with estimated neuraminidase concentrations in the range 0.1 to 0.5 nM and MUNANA concentrations in the range 2 to 200 μM.

Inhibition constants (K_I) were determined by measuring the extent to which different concentrations of inhibitor reduced the steady-state rate of MUNANA hydrolysis. The data were interpreted using the following simple model for competitive inhibition in which E, S, and I represent neuraminidase, MUNANA, and inhibitor, respectively:



The reduced rate of hydrolysis of MUNANA observed in the presence of inhibitor is predicted by the following equation (Rameix-Welti *et al.*)[14a]:

$$V_I = \frac{V_0([S] + K_m)}{[S] + K_m\left(1 + \frac{[I]}{K_I}\right)}$$

where V_I is the steady-state rate for MUNANA hydrolysis in the presence of inhibitor at concentration [I], V_0 is the steady-state rate for MUNANA hydrolysis in the absence of inhibitor, [S] is the MUNANA concentration, K_m is the Michaelis–Menten constant for hydrolysis of MUNANA, and K_i is the dissociation constant for the enzyme–inhibitor complex. Because these inhibitors show a slow approach to the new steady state rate (see Collins *et al.*[14b]) it was necessary to confirm that the new steady-state rate had been reached; this was done by demonstrating that the first derivative of the fluorescence change (proportional to the NA activity) became constant at long times.

Conclusion

Our work provides access to galactose-conjugates of the oseltamivir motif mimicking the natural α2–3 and α2–6 sialylgalactoside substrates of influenza virus neuraminidases. We found a difference in binding depending on the position of the oseltamivir pharmacophore on a β-galactoside, the natural sialic acid aglycone. We conclude that this difference must be a function of the preference of a given neuraminidase, and the respective virus, for a α2–3 or a α2–6 sialylgalactoside substrate. Consequently, the set of **3** and **4** can be used to characterize a neuraminidase beyond the primary sialic acid or oseltamivir binding site, based on inhibition.

Abbreviations

THF, tetrahydrofuran; TBDPS, *tert*-butyldiphenylsilyl; EA, ethyl acetate; Tol, toluene; TFA, trifluoroacetic acid; TBAF, tetrabutylammonium fluoride; TBAI, tetrabutylammonium iodide, DMAP, *p*-dimethylaminopyridine; BnBr, benzyl bromide; DMF, dimethyl formamide; MES, 4-morpholinoethanesulfonic acid; MOMCl, methoxymethyl chloride; MUNANA, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid.

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References

1 R. B. Belshe, *New Eng. J. Med.*, 2005, **353**, 2209–2211.
2 M. D. de Jong and T. T. Hien, *J. Clin Virol.*, 2006, **35**, 2–13.

3 E. de Clercq and J. Neyts, *Trends Pharm. Sci*, 2007, **28**, 280–285.
4 World Health Organisation, *Bull. World Health Organ.*, 1980, 58, 585–591.
5 (*a*) I. A. Wilson, J. J. Skehel and D. C. Wiley, *Nature*, 1981, **289**, 366–373; (*b*) Y. Suzuki, T. Ito, T. Suzuki, R. E. Holland, Jr., T. M. Chambers, M. Kiso, H. Ishida and Y. Kawaoka, *J. Virol.*, 2000, **74**, 11825–11831.
6 D. Katinger, L. Mochalova, A. Chinarev, N. Bovin and J. Romanova, *Arch. Virol.*, 2004, **149**, 2131–2140; S. J. Baigent, R. C. Bethell and J. W. McCauley, *Virology*, 1999, **263**, 323–328.
7 (*a*) C. U. Kim, W. Lew, M. A. Williams, L. Zhang, X. Chen, P. A. Escarpe, D. B. Mendel, W. G. Laver and R. C. Stevens, *J. Med. Chem.*, 1998, **41**, 2451–2460; (*b*) P. J. Collins, L. F. Haire, Y. P. Lin, R. J. Russell, P. A. Walker, J. J. Skehel, S. R. Martin, A. J. Hay and S. J. Gamblin, *Nature*, 2008, **453**, 1258–1261.
8 B. Carbain, P. J. Collins, L. Callum, S. R. Martin, A. J. Hay, J. McCauley and H. Streicher, *ChemMedChem*, 2009, **4**, 335–337.
9 H. Streicher and H. Busse, *Bioorg. Med. Chem.*, 2006, **14**, 1047–1057.
10 J.-J. Shie, J.-M. Fang and C.-H. Wong, *Angew. Chem.*, 2008, **120**, 5872–5875.
11 A. Larsson, J. Ohlsson, K. W. Dodson, S. J. Hultgren, U. Nilsson and J. Kihlberg, *Bioorg. Med. Chem.*, 2003, **11**, 2255–2262.
12 Dimethyl chlorophosphonate was synthesised according to the method of S.-B. Chen, Y.-M. Li, S.-Z. Luo, G. Zhao, B. Tan and Y.-F. Zhao, *Phosphorous Sulfur Silicon*, 2000, **164**, 277–291.
13 J. S. Cohen, C.-W. Chen and A. Bax, *J. Magn. Res.*, 1984, **59**, 181–184.
14 (*a*) M. A. Rameix-Welti, F. Agou, P. Buchy, S. Mardy, J. T. Aubin, M. Veron, S. van der Werf and N. Naffakh, *Antimicrobial Agents Chemother.*, 2006, **50**, 3809–3815; (*b*) P. J. Collins, L. F. Haire, J. Liu, R. J. Russel, P. A. Walker, J. J. Skehel, S. R. Martin, A. J. Hay and S. J. Gamblin, *Nature*, 2008, **453**, 1258–1262; (*c*) R. G. Duggleby, P. V. Attwood, J. C. Wallace and B. D. Keech, *Biochemistry*, 1982, **21**, 3364–3370; (*d*) W. M. Kati, A. S. Saldivar, F. Mohamadi, H. L. Sham, W. G. Laver and W. E. Kohlbrenner, *Biochem. Biophys. Res. Comm.*, 1998, **244**, 408–413; (*e*) M. Z. Wang, C. Y. Tai and D. B. Mendel, *Antimicrobial Agents Chemother.*, 2002, **46**, 3809–3816.
15 A. Larsson, J. Ohlsson, K. W. Dodson, S. J. Hultgren, U. Nilsson and J. Kihlberg, *Bioorg. Med. Chem.*, 2003, **11**, 2255–2262.