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Synthesis and chemical characterization of several perfluorinated sialic acid glycals and evaluation of their *in vitro* antiviral activity against Newcastle disease virus†

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Newcastle Disease Virus (NDV), belonging to the Paramyxoviridae family, causes a serious infectious disease in birds, resulting in severe losses in the poultry industry every year. Haemagglutinin neuraminidase glycoprotein (HN) has been recognized as a key protein in the viral infection mechanism, and its inhibition represents an attractive target for the development of new drugs based on sialic acid glycals, with the 2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid (Neu5Ac2en) as their backbone. Herein we report the synthesis of several Neu5Ac2en glycals and of their perfluorinated C-5 modified derivatives, including their respective stereoisomers at C-4, together with evaluation of their *in vitro* antiviral activity. While all synthesized compounds were found to be active HN inhibitors in the micromolar range, we found that their potency was influenced by the chain-length of the C-5 perfluorinated acetamido functionality. Thus, the binding modes of the inhibitors were also investigated by performing a docking study. Moreover, the perfluorinated glycals were found to be more active than the corresponding normal C-5 acylic derivatives. Finally, cell–cell fusion assays on NDV infected cells revealed that the addition of a newly synthesized C-4α heptafluorobutyryl derivative almost completely inhibited NDV-induced syncytium formation.

Introduction

Newcastle disease is an avian viral infection caused by Newcastle Disease Virus (NDV), a single-stranded RNA virus belonging to the Paramyxoviridae family, which represents a serious problem for the poultry industry resulting in a significant reduction of productivity with a consistent economic loss.[1] Moreover, despite the availability of various vaccines,[2] NDV outbreaks have increased, calling for novel preventive and control measures. For this reason, several recent studies have revealed novel antiviral compounds from different sources,[3] yet the development of new and more active compounds remains a constant goal. Clearly, a key step is to block NDV infection. Actually, the virus enters the host cells through two main pathways: direct fusion between the viral envelope and the plasma membrane, and receptor-mediated endocytosis.[4] The NDV infection involves two transmembrane glycoproteins, a fusion protein (F) and haemagglutinin-neuraminidase (HN). The NDV–HN complex is a multifunctional glycoprotein that mediates (i) the binding of the virus to the host cell by its interaction with sialic acid **1** (Sia, Neu5Ac) residues of cell-surface glycoconjugates, (ii) the promotion of fusion activity allowing the virus to enter the cells and (iii) the virion release acting as sialidase of nascent virion particles, preventing their self-agglutination and permitting the efficient spreading of the virus.[1c,5]

The involvement of the HN protein in various crucial stages of the viral life-cycle makes it an attractive target for the development of antiviral drugs for NDV.

Actually, the availability of the crystal structure of NDV–HN, also in complex with its inhibitors such as 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid **2a** (DANA),[6] allowed the rational design and synthesis of HN DANA-analogue inhibitors, that were found to be active on viruses belonging to the Paramyxoviridae family. In recent years, major attention has been devoted to inhibitors against HN of human parainfluenza viruses (hPIVs) that led to the identification of

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† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR of glycals **2a–f** and **7a–d**; MTT assay; docking poses of **2a–2d**. See DOI: 10.1039/c7md00072c

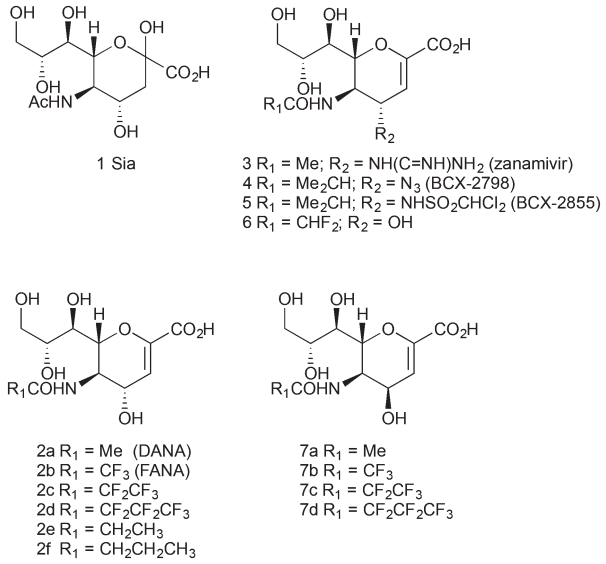


Fig. 1 Structures of sialidase inhibitors and their C-5 normal or perfluorinated modified analogues.

several active compounds,[7] such as zanamivir **3**,[7c] BCX-2798 **4** and BCX-2855 **5**.[7f] On the other hand, little has been published on NDV HN inhibitors, and only a few active analogues of DANA **2a** have been reported in early studies, which include difluoroacetyl **6** and trifluoroacetyl (FANA) **2b** as the most active compounds (Fig. 1).[8]

Based on these initial results and on our involvement in sialidase and sialoconjugate research,[9] the main goal of this work is to study the effects of a structural modification of the DANA framework on NDV–HN inhibition.

Starting from the structure of FANA **2b**, the best inhibitor to date,[8a] and considering that the C-5 binding domain within the NDV–HN active site can accommodate bulky hydrophobic acyl moieties,[6c,7b,e] we planned to study the effects of replacing the C-5 acyl group with longer perfluorinated chains. Indeed, incorporating fluorine atoms in bioactive compounds has been extensively used in medicinal chemistry to enhance the binding affinity of a compound to a target protein.[10] In fact, the presence of fluorine atoms adds polar-hydrophobicity to the residue together with the capability of weak hydrogen-bonding. In addition, this modification generally causes an enhancement in the metabolic stability and molecular bioavailability.[10b]

Thus, we prepared the C-5 perfluorinated homologues **2c–d** together with their corresponding normal acylic analogues **2e–f** and subjected all compounds to HN–NDV inhibition assay to compare the effects of fluorine incorporation.

Furthermore, to better understand the role played by the C-4 and C-5 substituents in the interaction with the HN active site, we also synthesized the epimers **7a–d**, with an inverted stereochemistry of the C-4 hydroxyl group. We then determined the IC_{50} of all compounds and subjected the best new inhibitor, the C-4α heptafluorobutyryl derivative **2d**, to an *in vitro* cytotoxicity test, as well as cell–cell fusion assay on COS7 cells infected with NDV.

Results and discussion

Chemistry

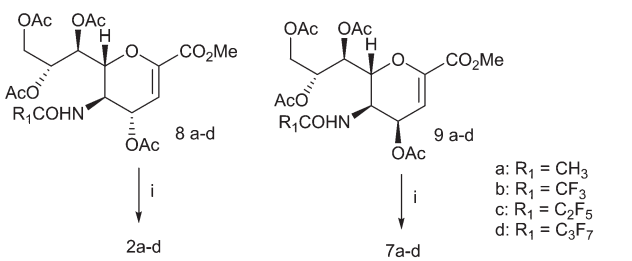
The crystal structures (PDB codes: 1E8T, 1E8U, 1E8V) of the complex of NDV with DANA **2a**, as well as the computational chemistry studies previously reported in the literature,[6,7b] prompted us to further investigate the notion that both the C-4 and C-5 positions[6,7b,e] are crucial for the inhibitory activity of DANA derivatives. In particular, analysis of the NDV–HN-DANA complex suggests that a bulkier hydrophobic group could be accommodated in place of the methyl group of the acetamido moiety at C-5, while the effects of a configuration inversion of the C-4 group is yet to be reported. Based on these premises, considering that the presence of a perfluorinated acyl chain should have improved the hydrophobicity of the C-5 moiety, we prepared the previously reported,[8d] yet chemically uncharacterized perfluorinated C-5 analogues of DANA, **2c–d**, together with the corresponding 4β epimers, **7b–d**, previously unreported.

As depicted in Scheme 1, the synthesis of both glycals **2a–d** and **7a–d** was achieved starting from the common precursor methyl ester of peracetylated Neu5Ac.

The protected intermediates **8a–d** (Scheme 1) of the 4α series were obtained through glycal formation promoted by Na_2HPO_4[11] followed, in the case of compounds **8b–d**, by a rapid basic *N*-transacylation reaction.[12] On the other hand, peracetylated glycals **9a–d**, having a 4β stereochemistry, were achieved *via* oxazoline formation, ring opening under acetylating conditions,[13] and, for **9b–d**, a final basic *N*-transacylation.[12b]

All the protected derivatives **8a–d** and **9a–d** were subjected to deacetylation with $NaOCH_3$ in methanol followed by selective hydrolysis to remove the ester function, while keeping the labile fluorinated amide groups. The best selective hydrolytic conditions, for pentafluoropropionic and heptafluorobutyric amido derivatives **8c**, **d** and **9c**, **d**, were K_2CO_3 in moist methanol, according to the procedure previously optimized by us for other perfluorinated sialic acid derivatives.[9a,e,14] Under the described conditions, glycals **2c**, **d** and **7c**, **d** were formed in satisfactory yields (71–86%) and in very short reaction times.

In contrast, the hydrolysis of FANA **2b** and of its C-4 epimer **7b**, both bearing a base-labile trifluoroacetyl amido group, could be achieved in good yields (82–87%) only in the



Scheme 1 Reagents and conditions: i, NaOMe, MeOH, 23 °C, 1 h, then for **8a**, **c–d** and **9a**, **c–d**: K_2CO_3, MeOH/H_2O (10 : 1, v/v), 23 °C, 12 h, 71–86% and for **8b** and **9b**: Et_3N, aq. MeOH–H_2O, 23 °C, 12 h, 82–87%.

presence of triethylamine in aqueous methanol.[13b] After purification by reverse phase preparative HPLC and lyophilization, all final glycals showed physicochemical properties consistent with their structures. In particular, the ^{13}C-NMR spectra proved the survival of the perfluorinated acyl groups in the molecules, as confirmed by the coupling constants (37 Hz for **2b** and **7b**; 26 Hz for **2c**, **d** and **7c**, **d**) between the carbonyl carbon (~159 ppm) and the fluorine atoms at the α-carbon.

Finally, in order to further investigate the role played by fluorine atoms in the inhibition activity, we synthesized the known compounds **2e–f**,[15] bearing an unfluorinated analogue chain (propionic and butyric, respectively) at the C-5 amidic functionality. For this purpose, we synthesized the target compounds **2e–f** starting from compound **8b** by a two-step hydrolytic procedure, affording the intermediate zwitterion **10**, which was then reacted with the appropriate acyl chloride, in a dioxane–water mixture containing triethylamine (Scheme 2).

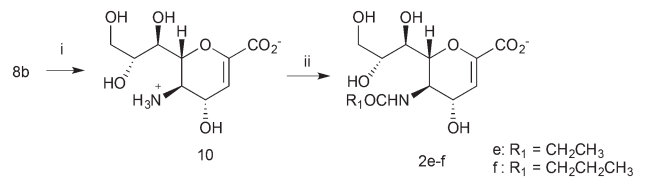
Biological evaluation

All synthesized compounds **2a–d** and **7a–d** were tested for NDV neuraminidase inhibition with an *in vitro* assay based on the hydrolysis of 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid, performed using *in toto* purified NDV.[16] To evaluate the activity of the synthesized inhibitors, the concentration causing 50% inhibition of the sialidase activity (IC_{50}) was determined by plotting the percentage inhibition against the logarithm of concentration of each compound.

The IC_{50} values found for the known compounds DANA **2a** and FANA **2b** are in agreement with those reported in the literature (13 μM and 1.9 μM respectively, Table 1).[8a] On the other hand, inhibitory activities measured for compounds **2b–2d** differ from those described in a dated symposium communication,[8d] where these compounds were reported to possess little inhibitory activity on NDV. However, we may speculate that these apparent discrepancies with our findings are due to the thiobarbituric assay used in the previous report, which is now considered obsolete and quite unreliable.

All the perfluorinated inhibitors were found to be more active than the corresponding unfluorinated analogues **2a**, **2e** and **2f** (Table 1).

In addition, while FANA **2b** was confirmed as the most active inhibitor (IC_{50} = 2.4 μM), an interesting trend was observed in both perfluorinated 4α and 4β series (**2b–d** and **7b–d** respectively) as we increased the perfluorinated chain-length at the C-5 position. In fact, while we observed increased IC_{50} values, *i.e.* less activity, moving from the trifluoroacetamido (**2b**, **7b**) to the corresponding *N*-pentafluoroacetamido derivatives (**2c**, **7c**), the activity was restored when we further elongated the chain to the *N*-heptafluorobutyramides (**2d**, **7d**).



Scheme 2 Reagents and conditions (**8b** to **2e–f**): i, NaOMe, MeOH, 23 °C, 1 h, then NaOH 1 M, MeOH/H_2O (1 : 1, v/v), 40 °C, o/n; ii, acyl chloride, Et_3N, dioxane–H_2O (5 : 1, v/v), 0 °C to 40 °C, 1 h, overall yield, 55–57%.

Table 1 IC_{50} values of DANA **2a**, FANA **2b**, perfluorinated analogues **2c**, **d** and **7a–d** and unfluorinated glycals **2e**, **f** established by NDV neuraminidase inhibition assay

Compound	IC_{50}[a] (μM)
DANA (**2a**)	14.6 ± 1.2
FANA (**2b**)	2.42 ± 0.29
2c	11.6 ± 1.4
2d	3.73 ± 0.11
2e	25.7 ± 4.8
2f	46.2 ± 3.0
7a	12.2 ± 1.6
7b	4.16 ± 0.48
7c	20.8 ± 2.9
7d	6.38 ± 0.87

[a] Each value represents the mean of three independent experiments carried out in triplicate.

In contrast, this trend was not observed in the unfluorinated derivatives (**2a**, **2e**, **2f**), as the activity kept decreasing while we elongated the C-5 chain. While an in-depth SAR study would be required to fully understand these findings, our results are consistent with the notion that C-5 side chains, even longer than the methyl group of DANA **2a**, could be accommodated in the known hydrophobic pocket in the enzyme active site, generating new interactions depending on the different chain-lengths and the tendency of fluorine atoms to form hydrophobic and/or weak hydrogen bonds.[6c,7b]

In order to further investigate this hypothesis, we performed a docking study using compounds **2a–2d**. To assess the quality of the method, ligand **2a** was docked and the experimental binding mode could be reproduced (RMSD of 0.5 Å). Ligand interactions of compound **2a** are shown in the ESI† (Fig. S2) and the overall binding mode is the same as the experimental one previously reported in the literature.[6a] Compounds **2a** and **2b** have almost identical binding modes (RMSD 1.0 Å), with an additional interaction between the CF_3 group of FANA **2b** and the hydroxyl group of Tyr 299. This additional interaction could explain the observed decrease of the IC_{50} of **2b**. In addition, in both compounds **2a** and **2b**, we observed an intramolecular hydrogen bond between the hydroxyl function at the C-4 position and the carbonyl group of the amide at C-5, which could influence the position of the C-5 chain in the hydrophobic pocket. Another interesting difference between **2a** and **2b** is that **2b** loses the interaction between the second hydroxyl group of the C-6 glyceric chain (at C-8) and Glu 401 in favor of a new intramolecular bond with the pyranosidic oxygen.

As shown in the ESI† (Fig. S2 and S3), the extension of the C-5 perfluorinated chain present in compounds **2c** and **2d**

has different effects on the binding mode of the ligand compared to **2b**. In particular, the intramolecular hydrogen bond between the hydroxyl group at C-4 and the carbonyl oxygen of the amide, observed in **2b** (and **2a**), is broken in the superior homologues, due to a change in the conformation to accommodate the extended C-5 chain. Furthermore, the glyceric chain at C-6 of **2c** and **2d** shows a different conformational arrangement compared with that of FANA **2b**: (i) the first hydroxyl group (at C-7) interacts with Glu 401 (for **2c**) instead of Glu 258; (ii) the second hydroxyl group (at C-8) interacts with Glu 258, losing the intramolecular hydrogen bond with the pyranosidic oxygen. In addition, of particular relevance is the loss of the vdW interaction of perfluorinated derivatives **2c** and **2d** with the Tyr-299 residue, a characteristic binding observed in the most active FANA **2b**. Overall, these results could explain the decrease of the inhibitory activity observed for the perfluorinated derivatives **2c** and **2d**, as compared to that of the trifluoracetamido one.

Finally, an explanation for the improvement in the inhibitory activity of **2d**, as compared to ligand **2c**, might be due to a new interaction between the terminal trifluoromethyl residue of the heptafluorobutyric chain and Lys-236.

Moreover, we found that all C-4β epimers (**7a–d**) are active but generally less effective than the corresponding C-4 α-glycals (**2a–d**), with the exception of β-epimer **7a**, which was slightly more active than its counterpart α-epimer DANA **2a**. Altogether these results confirm that the HN cavity near the 4-hydroxy group is relatively large, as previously reported,[6a] as it can accommodate both α and β-epimers. On the other hand, as the activity trend for the C-5 chain is confirmed also for the β-epimers, the C-4 stereochemistry seems to have little influence on the binding of the C-5 chain.

Then, we selected compound **2d**, showing the lowest IC_{50} value among the newly synthesized inhibitors, and subjected it to further biological evaluation. In particular, the cytotoxicity of **2d** was determined by MTT assay and compared with that of the endogenous glycal DANA **2a**. No significant cytotoxicity was detected for both glycal **2d** and DANA **2a**, with a minimum cell viability of 96% at 3 and 10 μM concentrations (values are in the same range as the IC_{50} of compound **2d**). Then, we studied the inhibitory effect of **2d** on virus propagation by looking at its ability to reduce the formation of syncytia (cell–cell fusion) in NDV-infected COS7 cells, as previously reported (Fig. 2).[9a] Since COS7 cells themselves have some tendency to form syncytia, uninfected cells were used to normalize the results (Fig. 2B, condition a). The results showed that NDV infection causes a significant increase (+63%) of syncytia formation as compared to uninfected cells, as expected (Fig. 2B, condition b). Then, we assessed whether **2d** was able to reduce the NDV ability to infect cells. For this purpose, COS7 cells were infected with NDV (1 MOI) for 1 h at 37 °C in the presence of 3.7 μM **2d**. Cells were then washed with a fresh medium (DMEM + 10% v/v FBS) and then cultured for an additional 5 h post-infection without the inhibitor **2d** before trypsin digestion, to activate the fusion protein (Fig. 2B, condition c). Syncytia formation was then assayed 16 h after trypsin digestion. The results showed that **2d** treatment during NDV infection caused complete inhibition of syncytia formation (Fig. 2B, condition c), as their number was comparable to that of uninfected COS7 cells.

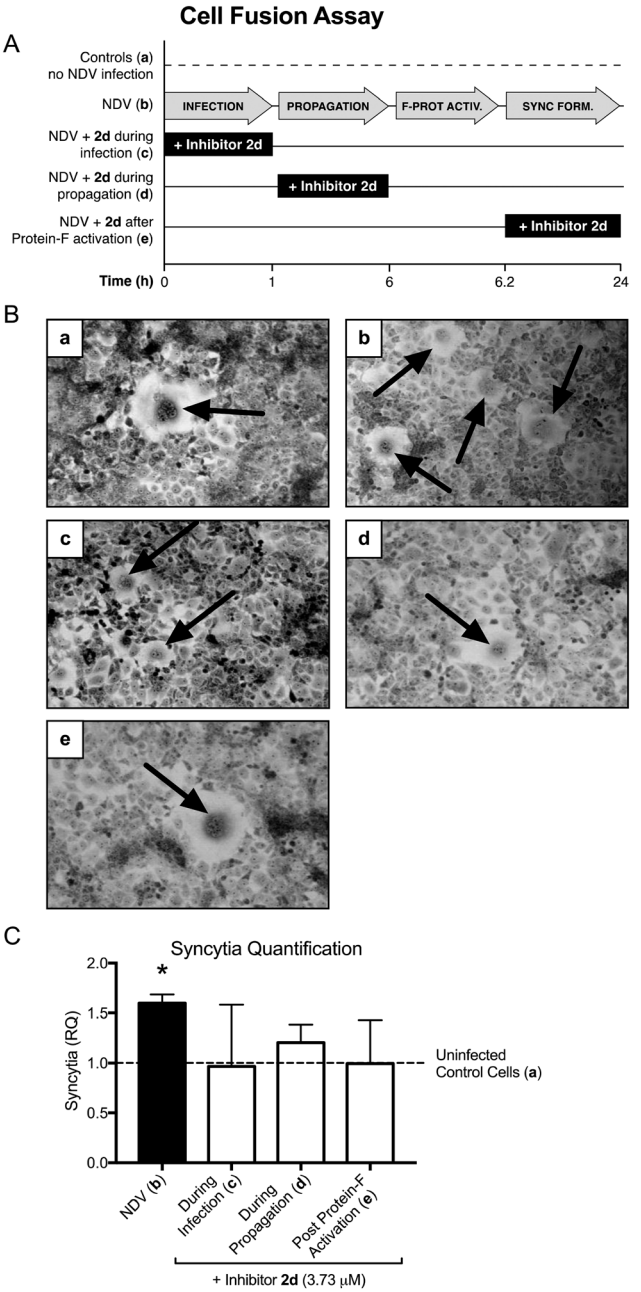


Fig. 2 Inhibition of avirulent NDV (Clone 30) strain-mediated cell fusion. A) Schematic representation of the cell–cell fusion assay; B) microscopy images of COS7 cells under all the different experimental conditions (100× magnification, arrows indicate syncytia) of: uninfected cells (a); NDV infected cells (b); NDV infected cells + **2d** during infection (c); NDV infected cells + **2d** during virus propagation (d); NDV infected cells + **2d** after F-protein activation (e); C) quantification of cell fusion. Data are expressed as relative quantification (RQ) of cell syncytia as compared to uninfected controls (dotted line). Shown are the mean ± SD of independent experiments performed in triplicate, counting 20 fields per well. $*p < 0.05$.

These data support the notion that **2d** binds to NDV–HN, thus preventing it from entering the cell. Actually, we could also speculate that the binding between compound **2d** and the HN protein could also modify the interaction between HN and the F-protein, destabilizing the F protein structure, thus indirectly affecting the fusion process.[6e,17]

To test this hypothesis, we first infected cells with NDV without the inhibitor **2d**. Then, we washed cells, and treated them with **2d** before (Fig. 2B, condition d) or after (Fig. 2B, condition e) activating the F-protein with trypsin, and checked for syncytia formation after 16 h. In both cases, the results confirmed that **2d** treatment was able to inhibit syncytia formation also in NDV-infected cells, as no significant increase in the syncytia number could be observed, as compared to uninfected controls. These results support the notion that inhibitor **2d** also plays a role in preventing NDV propagation, conceivably through the destabilization of the interaction between HN and the F-protein (Fig. 2).

Conclusions

Altogether these results show that **2d** could be a promising candidate for the development of new drugs against NDV infections. In particular, we unveiled (also by docking studies) the key role played by the C-5 chain of the DANA framework and its interactions with the amino acids present in the hydrophobic pocket of NDV–HN.

On the other hand, while at this stage the C-4 stereochemistry seems to have little effect on the binding, it would be interesting to test new substituents other than the hydroxyl group. Studies in this direction are ongoing in our laboratories.

Experimental

Chemistry

General information. All chemicals and solvents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared by filtering water on a Milli-Q Simplicity 185 filtration system from Millipore (Bedford, MA, USA). Solvents were dried using standard methods and distilled before use. The progress of all reactions was monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60 F254) using UV light, anisaldehyde/H_2SO_4/EtOH solution or 0.2% ninhydrin in ethanol and heat as the developing agent. Flash chromatography was performed with normal phase silica gel (E. Merck 230–400 mesh silica gel), following the general protocol of Still.[18]

Nuclear magnetic resonance spectra were recorded at 303 K on a Bruker AM-500 spectrometer equipped with a 5 mm inverse-geometry broadband probe and operating at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C. Chemical shifts are reported in parts per million (ppm, δ units) and are referenced for ^{1}H spectra, to a solvent residue proton signal (δ 3.31 ppm CD_3OD solution), and for ^{13}C spectra, to a solvent carbon signal (central line at δ 49.05 ppm CD_3OD solution). The ^{1}H and ^{13}C resonances were assigned by ^{1}H–^{1}H (COSY) and ^{1}H–^{13}C (HSQC and HMBC) correlation 2D experiments. ^{1}H NMR data are tabulated in the following order: number of protons, multiplicity (s, singlet; d, doublet; br s, broad singlet; m, multiplet), coupling constant(s) in hertz and assignment of proton(s). Optical rotations were taken on a Perkin-Elmer 241 polarimeter equipped with a 1 dm tube; $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1} and the concentrations are given in g per 100 mL.

High-resolution mass spectrometry (HRMS) analyses were performed using a QTof 5600 ABSciex mass spectrometer equipped with an ESI ion source. The spectra were collected in continuous flow mode by connecting the integrated Harvard syringe pump directly to the ESI source. Compound solutions were infused at a flow rate of 0.01 mL min^{-1} and the spray voltage was set at 4.5 kV in the negative ion mode with a capillary temperature of 400 °C. Full-scan mass spectra were recorded by scanning a m/z range of 50–700.

The preparative HPLC purifications were performed on a Dionex Ultimate 3000 instrument equipped with a Dionex RS variable wavelength detector, using an Atlantis C-18-Preper T3 ODB (5 μm, 19 × 10 mm) column and starting from 100% aqueous 0.1% (v/v) formic acid to 100% CH_3CN as the eluent. The crude product was dissolved in water and the solution was filtered (polypropylene, 0.45 μm, 13 mm ø, PK/100) and injected into the HPLC, affording purified products.

Preparation of *N*-perfluoracyl glycals 2c, d and 7a–d

General procedure. All the peracetylated glycals **8c**, **d** and **9b–d** (0.2 mmol) were treated with a methanolic solution of NaOMe, freshly prepared by dissolving sodium metal (0.22 mmol, 5 mg) in anhydrous MeOH (2 mL). Each reaction mixture was stirred at 25 °C for 2 h, and then quenched with acidic resin (Dowex 50WX8, H^+). The resin was filtered off and washed with MeOH (2 mL × 3) and the combined filtrate and washes were evaporated under vacuum. Each crude compound was purified with flash chromatography and directly subjected to opportune selective hydrolysis (Method A or B).

Method A) Selective hydrolysis using moist K_2CO_3 methanol solution. The free glycals **2c**, **d** and **7c**, **d** were prepared by selective hydrolysis of appropriate methyl ester derivatives (0.14 mmol) performed in a methanol–water solution (0.75 mL, 10 : 1 v/v) containing K_2CO_3 (13 mg), kept at 25 °C for 6–24 h. At this time, the reaction mixture was treated with acidic resin (Dowex 50WX8, H^+) until acidic pH, and then, the resin was filtered and washed with MeOH (2 mL × 3). Finally, the solvent was removed under reduced pressure and the residue was recovered with aqueous methanol and lyophilized to afford, after preparative HPLC, the desired free glycals.

Method B) Selective hydrolysis using moist Et_3N methanol solution. The free glycal **7b** was prepared by selective hydrolysis of its methyl ester derivative (0.14 mmol) performed in a methanol–water solution (1.5 mL, 2 : 1 v/v)

containing Et_3N (0.90 mL), kept at 23 °C for 12 h. Then the mixture was treated with acidic resin (Dowex 50WX8, H^+) until acidic pH and the resin was filtered and washed with MeOH (2 mL × 3). Finally, the solvent was removed under reduced pressure and the residue was recovered with aqueous methanol and lyophilized, to afford, after preparative HPLC, the desired free glycal **7b**.

*Preparation of 2,6-anhydro-5-(2,2,3,3,3-pentafluoropropionamido)-3,5-dideoxy-D-glycero-D-galacto-non-2-enoic acid (**2c**).* Starting from protected glycal **8c**[12c] (80 mg, 0.14 mmol), according to the general two step procedure Zemplén reaction followed by selective hydrolytic method A, glycal **2c** was obtained (48 mg, 86%), as a white solid, showing: $[\alpha]_D^{23}$, +26.7 (*c* 1 in MeOH); *δH* (CD_3OD) 5.96 (1H, d, $J_{3,4}$ 2.2 Hz, 3-H), 4.50 (1H, dd, $J_{4,3}$ 2.2, $J_{4,5}$ 9.0 Hz, 4-H), 4.41 (1H, d app, $J_{6,5}$ 11.0 Hz, 6-H), 4.24 (1H, dd, $J_{5,4}$ 9.0, $J_{5,6}$ 11.0 Hz, 5-H), 3.86 (1H, ddd, $J_{8,9a}$ 2.9, $J_{8,9b}$ 5.6, $J_{8,7}$ 9.2 Hz, 8-H), 3.80 (1H, dd, $J_{9a,8}$ 2.9, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.62 (1H, dd, $J_{9b,8}$ 5.6, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.51 (1H, d app, $J_{7,8}$ 9.2 Hz, 7-H); *δC* (CD_3OD) 165.6 (C-1), 160.0 (t, $J_{C,F}$ 26 Hz, $COCF_2$), 145.3 (C-2), 125.0–110.0 (2C, CF_2CF_3), 113.6 (C-3), 77.2 (C-6), 71.5 (C-8), 70.1 (C-7), 68.0 (C-4), 65.0 (C-9), 52.2 (C-5); HRMS (ESI-TOF, *m*/*z*): calcd for $C_{12}H_{13}F_5NO_8$ $[M - H]^-$ 394.0567, found 394.0559.

*Preparation of 2,6-anhydro-5-(2,2,3,3,4,4,4-heptafluorobutanamido)-3,5-dideoxy-D-glycero-D-galacto-non-2-enoic acid (**2d**).* Starting from protected glycal **8d**[12c] (88 mg, 0.14 mmol), according to the general two step procedure Zemplén reaction followed by selective hydrolytic method A, glycal **2d** was obtained (52 mg, 84%), as a white solid, showing: $[\alpha]_D^{23}$, +19.9 (*c* 1 in MeOH); *δH* (CD_3OD) 5.96 (1H, d, $J_{3,4}$ 2.3 Hz, 3-H), 4.50 (1H, dd, $J_{4,3}$ 2.3, $J_{4,5}$ 8.9 Hz, 4-H), 4.41 (1H, dd, $J_{6,7} < 1.0$, $J_{6,5}$ 11.0 Hz, 6-H), 4.24 (1H, dd, $J_{5,4}$ 8.9, $J_{5,6}$ 11.0 Hz, 5-H), 3.89 (1H, ddd, $J_{8,9a}$ 2.8, $J_{8,9b}$ 5.6, $J_{8,7}$ 9.2 Hz, 8-H), 3.82 (1H, dd, $J_{9a,8}$ 2.8, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.56 (1H, dd, $J_{9b,8}$ 5.6, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.52 (1H, dd, $J_{6,7} < 1.0$, $J_{7,8}$ 9.2 Hz, 7-H); *δC* (CD_3OD) 165.6 (C-1), 159.8 (t, J_{C-F} 26 Hz, $COCF_2$), 145.3 (C-2), 122.0–110.0 (3C, $CF_2CF_2CF_3$), 113.6 (C-3), 77.2 (C-6), 71.5 (C-8), 70.2 (C-7), 67.9 (C-4), 65.0 (C-9), 52.3 (C-5); HRMS (ESI-TOF, *m*/*z*): calcd for $C_{13}H_{13}F_7NO_8$ $[M - H]^-$ 444.0535, found 444.0551.

*Preparation of 2,6-anhydro-5-acetamido-3,5-dideoxy-D-glycero-D-talo-non-2-enoic acid (**7a**).* Starting from protected glycal **9a**[13,14] (66 mg, 0.14 mmol), according to the general two step procedure Zemplén reaction followed by selective hydrolytic method A, glycal **7a** was obtained (31 mg, 75%), as a white solid, showing: $[\alpha]_D^{23}$, −98.9 (*c* 1 in MeOH); *δH* (CD_3OD) 6.02 (1H, d, $J_{3,4}$ 5.0 Hz, 3-H), 4.27–4.14 (3H, overlapping, 4-H, 6-H and 5-H), 3.92 (1H, ddd, $J_{8,9a}$ 2.3, $J_{8,9b}$ 5.3, $J_{8,7}$ 9.2 Hz, 8-H), 3.83 (1H, dd, $J_{9a,8}$ 2.3, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.67 (1H, dd, $J_{9b,8}$ 5.3, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.58 (1H, d app, $J_{7,8}$ 9.2 Hz, 7-H), 2.05 (1H, s, $NHCOCH_3$); *δC* (CD_3OD) 174.2 (C-1), 167.5 ($NHCOCH_3$), 148.2 (C-2), 108.6 (C-3), 73.3 (C-6), 71.5 (C-8), 70.1 (C-7), 65.0 (C-9), 62.1 (C-5), 49.4 (C-4), 22.8 ($NHCOCH_3$); HRMS (ESI-TOF, *m*/*z*): calcd for $C_{11}H_{16}NO_8$ $[M - H]^-$ 290.0881, found 290.0855.

*Preparation of 2,6-anhydro-5-(2,2,2-trifluoroacetamido)-3,5-dideoxy-D-glycero-D-talo-non-2-enoic acid (**7b**).* Starting from protected glycal **9b**[13,14] (74 mg, 0.14 mmol), according to the general two step procedure Zemplén reaction followed by selective hydrolytic method B, glycal **7b** was obtained (42 mg, 87%), as a white solid, showing: m.p. 112–114 °C (from MeOH–diisopropylether); $[\alpha]_D^{23}$, −98.8 (*c* 1 in MeOH); *δH* (CD_3OD) 6.14 (1H, d, $J_{3,4}$ 5.6 Hz, 3-H), 4.45 (1H, d app, $J_{6,5}$ 11.3 Hz, 6-H), 4.32 (1H, dd, $J_{5,4}$ 3.4, $J_{5,6}$ 11.3 Hz, 5-H), 4.28–4.23 (1H, m, 4-H), 4.00 (1H, ddd, $J_{8,9a}$ 2.1, $J_{8,9b}$ 5.4, $J_{8,7}$ 9.2 Hz, 8-H), 3.84 (1H, dd, $J_{9a,8}$ 2.1, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.67 (1H, dd, $J_{9b,8}$ 5.4, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.67 (1H, d app, $J_{7,8}$ 9.2 Hz, 7-H); *δC* (CD_3OD) 165.8 (C-1), 158.9 (1C, q, $J_{C,F}$ 37 Hz, $COCF_3$), 146.4 (C-2), 121.0–114.0 (1C, q, $J_{C,F}$ 287 Hz, CF_3), 110.4 (C-3), 72.9 (C-6), 71.8 (C-8), 69.9 (C-7), 64.9 (C-9), 61.3 (C-5), 50.1 (C-4); HRMS (ESI-TOF, *m*/*z*): calcd for $C_{11}H_{13}F_3NO_8$ $[M - H]^-$ 344.0599, found 344.0583.

*Preparation of 2,6-anhydro-5-(2,2,3,3,3-pentafluoropropionamido)-3,5-dideoxy-D-glycero-D-talo-non-2-enoic acid (**7c**).* Starting from protected glycal **9c**[13,14] (81 mg, 0.14 mmol), according to the general two step procedure Zemplén reaction followed by selective hydrolytic method A, glycal **7c** was obtained (40 mg, 72%), as a white solid, showing: m.p. 107–109 °C (from MeOH–diisopropylether); $[\alpha]_D^{23}$, −119.2 (*c* 1 in MeOH); *δH* (CD_3OD) 6.14 (1H, d, $J_{3,4}$ 5.7 Hz, 3-H), 4.46 (1H, d app, $J_{6,5}$ 11.4 Hz, 6-H), 4.36 (1H, dd, $J_{5,4}$ 3.6, $J_{5,6}$ 11.4 Hz, 5-H), 4.27–4.23 (1H, m, 4-H), 3.91 (1H, ddd, $J_{8,9a}$ 2.7, $J_{8,9b}$ 5.6, $J_{8,7}$ 9.2 Hz, 8-H), 3.84 (1H, dd, $J_{9a,8}$ 2.7, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.65 (1H, dd, $J_{9b,8}$ 5.6, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.57 (1H, d app, $J_{7,8}$ 9.2 Hz, 7-H); *δC* (CD_3OD) 165.8 (C-1), 159.4 (1C, t, $J_{C,F}$ 26 Hz, $COCF_2$), 146.4 (C-2), 123.0–106.0 (2C, CF_2CF_3), 110.5 (C-3), 72.9 (C-6), 71.8 (C-8), 70.0 (C-7), 65.0 (C-9), 61.3 (C-5), 50.1 (C-4); HRMS (ESI-TOF, *m*/*z*): calcd for $C_{12}H_{13}F_5NO_8$ $[M - H]^-$ 394.0567, found 394.0560.

*Preparation of 2,6-anhydro-5-(2,2,3,3,4,4,4-heptafluorobutanamido)-3,5-dideoxy-D-glycero-D-talo-non-2-enoic acid (**7d**).* Starting from protected glycal **9d** (88 mg, 0.14 mmol), according to the general two step procedure Zemplén reaction followed by selective hydrolytic method A, glycal **7d** was obtained (44 mg, 71%), as a white solid, showing: $[\alpha]_D^{23}$, −103.6 (*c* 1 in MeOH); *δH* (CD_3OD) 6.13 (1H, d, $J_{3,4}$ 5.6 Hz, 3-H), 4.47 (1H, d app, $J_{6,5}$ 11.3 Hz, 6-H), 4.36 (1H, dd, $J_{5,4}$ 3.2, $J_{5,6}$ 11.3 Hz, 5-H), 4.24 (1H, dd, $J_{4,5}$ 3.2, $J_{4,3}$ 5.6 Hz, 4-H), 3.91 (1H, ddd, $J_{8,9a}$ 2.1, $J_{8,9b}$ 5.6, $J_{8,7}$ 9.1 Hz, 8-H), 3.85 (1H, dd, $J_{9a,8}$ 2.1, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.65 (1H, dd, $J_{9b,8}$ 5.6, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.58 (1H, d app, $J_{7,8}$ 9.1 Hz, 7-H); *δC* (CD_3OD) 166.0 (C-1), 159.2 (1C, t, $J_{C,F}$ 26 Hz, $COCF_2$), 146.5 (C-2), 121.0–106.0 (3C, $CF_2CF_2CF_3$), 110.3 (C-3), 72.9 (C-6), 71.9 (C-8), 70.1 (C-7), 65.0 (C-9), 61.3 (C-5), 50.2 (C-4); HRMS (ESI-TOF, *m*/*z*): calcd for $C_{13}H_{13}F_7NO_8$ $[M - H]^-$ 444.0535, found 444.0535.

Synthesis of *N*-unfluoracyl glycals 2e–f

General procedure. The peracetylated glycal **8b**[12c] (260 mg, 0.5 mmol) were treated with a methanolic solution of NaOMe, freshly prepared by dissolving sodium metal (0.22 mmol, 5 mg) in anhydrous MeOH (2 mL). The reaction mixture was stirred at 23 °C for 1 h, and then quenched with acidic resin (Dowex 50WX8, H^+). The resin was filtered off and washed with MeOH (2 mL) and the combined filtrates

were evaporated under vacuum. The crude compound was purified with flash chromatography and directly submitted to hydrolysis. The obtained compound was dissolved in a MeOH/H_2O mixture (4 mL, 1 : 1, v/v) and treated with 1 M NaOH solution (1.5 mL) at 40 °C and stirred at room temperature overnight. The reaction was neutralized with Amberlite DP-1 (H^+) weakly acidic ion exchange resin, the resin was washed twice with methanolic 0.5 M NH_3 solution and the combined filtrates were concentrated under vacuum to afford compound **10** as a solid (100 mg, 78%). The obtained intermediate zwitterion **10** was dissolved in water (1 mL) and dioxane was added (5 mL) followed by Et_3N (0.5 mL). The reaction mixture was then cooled to 0 °C and the appropriate acyl chloride (0.72 mmol) in dioxane (0.5 mL) was added dropwise to the reaction mixture. The reaction temperature was raised to 40 °C for 1 h. Then, the reaction mixture was cooled and concentrated under reduced pressure to give a residue, which was purified by preparative HPLC to afford the desired free glycal.

*Preparation of 2,6-anhydro-5-propionamido-3,5-dideoxy-D-glycero-D-galacto-non-2-enoic acid (**2e**).* Starting from protected glycal **8b** (0.5 mmol), according to the general procedure, glycal **2e**[15] was obtained (83 mg, 55%), as a white solid, showing: *δH* (CD_3OD) 5.87 (1H, br s, 3-H), 4.42 (1H, d app, $J_{4,5}$ 8.3 Hz, 4-H), 4.15 (1H, d app, $J_{6,5}$ 10.7 Hz, 6-H), 3.99 (1H, dd, $J_{5,4}$ 8.7, $J_{5,6}$ 10.7 Hz, 5-H), 3.93–3.86 (1H, m, 8-H), 3.82 (1H, d app, $J_{9a,9b}$ 11.3 Hz, 9a-H), 3.65 (1H, dd, $J_{9b,8}$ 4.9, $J_{9b,9a}$ 11.3 Hz, 9b-H), 3.55 (1H, d app, $J_{7,8}$ 8.9 Hz, 7-H), 2.32 (2H, q, $J_{H,H}$ 7.5 Hz, $COCH_2$), 1.16 (3H, t, $J_{H,H}$ 7.5 Hz, $COCH_2CH_3$); *δC* (CD_3OD) 178.7 (C-1), 165.6 (CONH), 145.5 (C-2), 111.8 (C-3), 77.9 (C-6), 71.3 (C-8), 70.3 (C-7), 68.2 (C-4), 65.0 (C-9), 51.9 (C-5), 30.2 ($COCH_2$), 10.3 ($COCH_2CH_3$); MS (ESI negative, *m*/*z*): 304. 2 $[M - H]^-$.

*Preparation of 2,6-anhydro-5-butyramido-3,5-dideoxy-D-glycero-D-galacto-non-2-enoic acid (**2f**).* Starting from protected glycal **8b** (0.5 mmol), according to the general procedure, glycal **2f**[15] was obtained (88 mg, 57%), as a white solid, showing: *δH* (CD_3OD) 5.97 (1H, d, $J_{3,4}$ 2.1 Hz, 3-H), 4.46 (1H, dd, $J_{4,3}$ 2.1, $J_{4,5}$ 8.7 Hz, 4-H), 4.19 (1H, d app, $J_{6,5}$ 10.8 Hz, 6-H), 4.02 (1H, dd, $J_{5,4}$ 8.7, $J_{5,6}$ 10.8 Hz, 5-H), 3.92 (1H, ddd, $J_{8,9a}$ 2.6, $J_{8,9b}$ 5.4, $J_{8,7}$ 9.1 Hz, 8-H), 3.85 (1H, dd, $J_{9a,8}$ 2.6, $J_{9a,9b}$ 11.4 Hz, 9a-H), 3.66 (1H, dd, $J_{9b,8}$ 5.4, $J_{9b,9a}$ 11.4 Hz, 9b-H), 3.59 (1H, d app, $J_{7,8}$ 9.1 Hz, 7-H), 2.30 (2H, t, $J_{H,H}$ 7.2 Hz, $COCH_2$), 1.70 (2H, m, $COCH_2CH_2$), 1.00 (3H, t, $J_{H,H}$ 7.2 Hz, $COCH_2CH_2CH_3$); *δC* (CD3OD) 178.1 (C-1), 165.6 (CONH), 145.5 (C-2), 113.5 (C-3), 78.2 (C-6), 71.1 (C-8), 70.4 (C-7), 68.0 (C-4), 65.1 (C-9), 51.9 (C-5), 39.0 ($COCH_2$), 20.3 ($COCH_2CH_2$), 14.1 ($COCH_2CH_2CH_3$); MS (ESI negative, *m*/*z*): 318.1 $[M - H]^-$.

Biological screening

Cell lines and viruses. COS7 cells were obtained from the American Type Culture Collection and maintained at 37 °C (in an atmosphere of 5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (4 mM), penicillin–streptomycin (100 U ml^{-1}–100 μg ml^{-1}), and heat-inactivated fetal bovine serum (FBS) at 10% v/v (all reagents were from Sigma).

NDV "Clone 30" was grown and purified as described previously in the literature.[19] Stock viruses were harvested, titrated, and stored at −80 °C until use.

Cytotoxicity assay. The cytotoxicity of the compounds was analyzed by MTT assay following the manufacturer's protocol (Sigma Aldrich). Briefly, COS7 cells were seeded in 6-well plates at an initial density of 1×10^5 cells per well. The cells were incubated with different concentrations of the compounds at 37 °C in 5% CO_2 in an incubator for 24 h. MTT solution (5 mg mL^{-1}) was added to the cells, which were further incubated for 30 min. Then, the medium was discarded and 1 ml of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added for 10 min to dissolve the resulting formazan product. The absorbance of each well was measured at 570 nm using a spectrophotometer (Jasco V-530 Spectrophotometer).

Each experiment was performed in triplicate, and experiments were repeated at least three times. The cytotoxicity was expressed as the CC_{50}, which was the concentration of the test substances that inhibited the growth of COS7 cells by 50% compared with the growth of the untreated cells.

Neuraminidase inhibition assay. Neuraminidase activity inhibition assay was performed, according to Venerando *et al.*,[16] using 4-MUNeu5Ac as the artificial substrate. Briefly, the incubation mixture (final volume of 100 μL) contained 0.1 μg of NDV "Clone 30", different amounts of the inhibitors (0-500 mM), 0.3 mM 4-MU-Neu5Ac, 600 μg of bovine serum albumin (BSA) and 200 mM sodium citrate/phosphate buffer pH 6.8. After incubation at 37 °C for 15 min, the reactions were stopped by the addition of 1.5 mL of 0.2 M glycine buffered with NaOH at pH 10.8, and the neuraminidase activity is determined by spectrofluorometric measurement of the 4-methylumbelliferone released (λ excitation 365 nm, λ emission 448 nm). One unit of neuraminidase was defined as the amount of enzyme releasing 1 μmol of *N*-acetylneuraminic acid per minute at 37 °C.

Five concentrations of each inhibitor were used to determine the IC_{50} with a fixed concentration (0.3 mM) of 4-MU-Neu5Ac.

IC_{50} values were obtained using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) by nonlinear regression (curve fit), dose–response inhibition and three parameter logistic. Typical concentration–response plots were obtained from the average values of triplicate assay results.

NDV infection assay in COS7 cells. COS7 cells were cultured at 37 °C (in an atmosphere of 5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (4 mM), penicillin–streptomycin (100 U ml^{-1}–100 μg ml^{-1}), and 10% (v/v) heat-inactivated fetal bovine serum (FBS). Briefly, plated COS7 cells were infected with NDV at a multiplicity of infection (MOI) of 1 for 1 h at 37 °C in DMEM supplemented with 2% (v/v) FBS. After infection, the inoculum was removed and the cells were washed 3 times with DMEM + 2% (v/v) FBS and incubated in DMEM + 10% (v/v) FBS for 24 h. Then cells

were harvested by scraping in phosphate-buffered saline (PBS) at pH 7.4, and centrifuged (250 × *g* for 10 minutes at 4 °C). The cellular pellet was resuspended in PBS buffer at pH 7.4 containing 1 mM EDTA, and a protease inhibitor mixture (10 μg per mL Aprotinin, 10 μg per mL Leupeptin, 1 μg per mL Pepstatin A). Crude cell extracts were prepared by sonication and centrifugation at 800 × *g* for 10 minutes at 4 °C to eliminate cell nuclei and unbroken cells.

The protein concentration was determined using the Bradford method,[20] and aliquots of the crude extracts were used for sialidase activity. This assay was performed in triplicate with 30 μg of crude extract proteins, 0.3 mM 4-MU-Neu5Ac, 600 μg of BSA, 200 mM sodium-citrate/phosphate buffer pH 6.8, in a final volume of 100 μL. The procedure was the same as those reported above.

Syncytium inhibition assay. Fusion inhibition assay was performed according to the method reported by Anastasia.[9a] Plated COS7 cells were infected with NDV at a multiplicity of infection (MOI) of 1. The virus was left to be adsorbed for 1 h at 37 °C, after which the inoculum was removed and cells were incubated in DMEM + 10% (v/v) FBS. For syncytium assays, trypsin digestion of the precursor of viral protein F (F0) was accomplished at 6 h post-infection as described previously. Briefly, cells were incubated in the presence of 5 μg ml^{-1} of acetylated trypsin (acetyl-trypsin, Sigma) in DMEM containing 2% (v/v) FBS for 10 min at room temperature. Then, cells were washed twice with an acetyltrypsin inhibitor (Sigma) at 7.5 μg ml^{-1} in DMEM with 2% (v/v) FBS and incubated in complete medium for an additional 16 h. Cells were then fixed with methanol for 10 min, stained with Giemsa, and the number of syncytia (cells containing more than 3 nuclei) was counted in 20 random areas of the well.

The selected inhibitor **2d** was added at its IC_{50} value at different times: during the infection, at 5 h post-infection or immediately after trypsin treatment.

Statistical analysis. The IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). The results were presented as the mean of three independent experiments carried out in triplicate. For cell–cell fusion tests One-Way ANOVA with the multiple comparison Dunnett test was performed using Prism 7 (Graph Pad Software Inc., San Diego, CA). Values of $p < 0.05$ were considered statistically significant.

Computational chemistry. The protein structure (PDB id 1E8V[6a]) was prepared for docking using Schrödinger Protein Preparation Wizard (Schrödinger release 2016-3 (ref. 21)) with standard settings, except that all water molecules were retained. All ligands **2a–2d** were prepared using Schrödinger LigPrep and the protonation state assessed with Epik.[21] The most stable ligand conformations were identified using Monte Carlo Multiple Minimum (MCMM) in Macromodel.[22,23] The OPLS3 force field was used (OPLS3, Schrödinger, Inc., New York, NY, 2013).[24] Docking of the substrates was performed with Glide[25–27] using the extra precision (XP) mode.[25] Unless otherwise stated, default settings were applied. The best pose was selected using the glide E-model score, and the Glide XP score was used to compare the docking poses of the different substrates. The small difference of the IC_{50} values is reflected in the small variation of the Glide XP G score. Poses with a difference in energy within 2 kcal mol^{-1} are difficult to distinguish because of the limitations of the scoring function[25,26] so the values of the G score cannot be used to rank the quality of the interaction.

Confilct of interest

The authors declare no conflicts of interest.

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Notes and references

1 (*a*) D. J. Alexander, E. W. Aldous and C. M. Fuller, *Avian Pathol.*, 2012, **41**, 329; (*b*) J. C. F. M. Dortmans, G. Koch, P. J. M. Rottier and B. P. H. Peeters, *Vet. Res.*, 2011, 42; (*c*) K. Ganar, M. Das, S. Sinha and S. Kumar, *Virus Res.*, 2014, **184**, 71.

2 (*a*) J. C. F. M. Dortmans, B. P. H. Peeters and G. Koch, *Vet. Microbiol.*, 2012, **160**, 17; (*b*) P. J. Miller, C. L. Afonso, J. El Attrache, K. M. Dorsey, S. C. Courtney, Z. Guo and D. R. Kapczynski, *Dev. Comp. Immunol.*, 2013, **41**, 505.

3 (*a*) R. Elizondo-Gonzalez, L. E. Cruz-Suarez, D. Ricque-Marie, E. Mendoza-Gamboa, C. Rodriguez-Padilla and L. M. Trejo-Avila, *Virol. J.*, 2012, **9**, 307; (*b*) T. L. Nguyen, J. Chen, Y. Hu, D. Wang, Y. Fan, J. Wang, S. Abula, J. Zhang, T. Qin, X. Chen, X. Chen, S. K. Khakame and B. K. Dang, *Carbohydr. Polym.*, 2012, **90**, 1254; (*c*) X. Song, Z. Yin, X. Zhao, A. Cheng, R. Jia, G. Yuan, J. Xu, Q. Fan, S. Dai, H. Lu, C. Lv, X. Liang, C. He, G. Su, L. Zhao, G. Ye and F. Shi, *J. Gen. Virol.*, 2013, **94**, 2164; (*d*) J. K. Young, R. P. Hicks, G. E. Wright and T. G. Morrison, *Virology*, 1997, **238**, 291; (*e*) J. Zhu, X. Jiang, Y. Liu, P. Tien and G. F. Gao, *J. Mol. Biol.*, 2005, **354**, 601; (*f*) J. A. Aguilar-Briseno, L. E. Cruz-Suarez, J. F. Sassi, D. Ricque-Marie, P. Zapata-Benavides, E. Mendoza-Gamboa, C. Rodriguez-Padilla and L. M. Trejo-Avila, *Mar. Drugs*, 2015, **13**, 697.

4 (*a*) C. Cantin, J. Holguera, L. Ferreira, E. Villar and I. Munoz-Barroso, *J. Gen. Virol.*, 2007, **88**, 559; (*b*) K. San Roman, E. Villar and I. Munoz-Barroso, *Virology*, 1999, **260**, 329.

5 E. Villar and I. M. Barroso, *Glycoconjugate J.*, 2006, **23**, 5.

6 (*a*) S. Crennell, T. Takimoto, A. Portner and G. Taylor, *Nat. Struct. Biol.*, 2000, **7**, 1068; (*b*) U. Neu, J. Bauer and T. Stehle, *Curr. Opin. Struct. Biol.*, 2011, **21**, 610; (*c*) C. Ryan, V. Zaitsev, D. J. Tindal, J. C. Dyason, R. J. Thomson, I. Alymova, A.

Portner, M. von Itzstein and G. Taylor, *Glycoconjugate J.*, 2006, **23**, 135; (*d*) P. Yuan, T. B. Thompson, B. A. Wurzburg, R. G. Paterson, R. A. Lamb and T. S. Jardetzky, *Structure*, 2005, **13**, 803; (*e*) V. Zaitsev, M. von Itzstein, D. Groves, M. Kiefel, T. Takimoto, A. Portner and G. Taylor, *J. Virol.*, 2004, **78**, 3733.

7 (*a*) M. Von Itzstein, I. El-Deeb, L. Dirr, P. Guillon and M. Winger, *Patent WO*, 2016033660A1, 2016; (*b*) I. M. El-Deeb, P. Guillon, M. Winger, T. Eveno, T. Haselhorst, J. C. Dyason and M. von Itzstein, *J. Med. Chem.*, 2014, **57**, 7613; (*c*) M. Porotto, M. Murrell, O. Greengard, M. C. Lawrence, J. L. McKimm-Breschkin and A. Moscona, *J. Virol.*, 2004, **78**, 13911; (*d*) T. Suzuki, K. Ikeda, N. Koyama, C. Hosokawa, T. Kogure, T. Takahashi, K. I. P. J. Hidari, D. Miyamoto, K. Tanaka and Y. Suzuki, *Glycoconjugate J.*, 2001, **18**, 331; (*e*) D. J. Tindal, J. C. Dyason, R. J. Thomson, T. Suzuki, H. Ueyama, Y. Kuwahara, N. Maki, Y. Suzuki and M. von Itzstein, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 1655; (*f*) I. V. Alymova, G. Taylor, T. Takimoto, T. H. Lin, P. Chand, Y. S. Babu, C. Li, X. Xiong and A. Portner, *Antimicrob. Agents Chemother.*, 2004, **48**, 1495; (*g*) P. Guillon, L. Dirr, I. M. El-Deeb, M. Winger, B. Bailly, T. Haselhorst, J. C. Dyason and M. von Itzstein, *Nat. Commun.*, 2014, **5**, 5268.

8 (*a*) P. Meindl, G. Bodo, P. Palese, J. Schulman and H. Tuppy, *Virology*, 1974, **58**, 457; (*b*) P. Palese, J. L. Schulman, G. Bodo and P. Meindl, *Virology*, 1974, **59**, 490; (*c*) A. Portner, *Virology*, 1981, **115**, 375; (*d*) R. Dernick and J. Heukeshoven, *Antiviral Chemother.: Des. Inhib. Viral Funct., [Proc. Symp. Antiviral Chemother.]*, 1981, 327.

9 (*a*) L. Anastasia, J. Holguera, A. Bianchi, F. D'Avila, N. Papini, C. Tringali, E. Monti, E. Villar, B. Venerando, I. Munoz-Barroso and G. Tettamanti, *Biochim. Biophys. Acta*, 2008, **1780**, 504; (*b*) S. Bergante, E. Torretta, P. Creo, N. Sessarego, N. Papini, M. Piccoli, C. Fania, F. Cirillo, E. Conforti, A. Ghiroldi, C. Tringali, B. Venerando, A. Ibatici, C. Gelfi, G. Tettamanti and L. Anastasia, *J. Lipid Res.*, 2014, **55**, 549; (*c*) F. Cirillo, A. Ghiroldi, C. Fania, M. Piccoli, E. Torretta, G. Tettamanti, C. Gelfi and L. Anastasia, *J. Biolumin. Chemilumin.*, 2006, **291**, 10615; (*d*) M. Piccoli, E. Conforti, A. Varrica, A. Ghiroldi, F. Cirillo, G. Resmini, F. Pluchinotta, G. Tettamanti, A. Giamberti, A. Frigiola and L. Anastasia, *Int. J. Cardiol.*, 2016, **18**, E1; (*e*) P. Rota, F. Cirillo, M. Piccoli, A. Gregorio, G. Tettamanti, P. Allevi and L. Anastasia, *Chem. – Eur. J.*, 2015, **21**, 14614; (*f*) R. Scaringi, M. Piccoli, M. N. Papini, F. Cirillo, E. Conforti, S. Bergante, C. Tringali, A. Garatti, C. Gelfi, B. Venerando, L. Menicanti, G. Tettamanti and L. Anastasia, *J. Biolumin. Chemilumin.*, 2013, **288**, 3153; (*g*) E. Torretta, M. Vasso, C. Fania, D. Capitanio, S. Bergante, M. Piccoli, G. Tettamanti, L. Anastasia and C. Gelfi, *Electrophoresis*, 2014, **35**, 1319; (*h*) P. Rota, P. Allevi and L. Anastasia, *Asian J. Org. Chem.*, 2015, **4**, 1315; (*i*) P. Rota, L. Anastasia and P. Allevi, *Org. Biomol. Chem.*, 2015, **13**, 4931.

10 (*a*) E. P. Gillis, K. J. Eastman, M. D. Hill, D. J. Donnelly and N. A. Meanwell, *J. Med. Chem.*, 2015, **58**, 8315; (*b*) S. Purser, P. R. Moore, S. Swallowb and V. Gouverneur, *Chem. Soc. Rev.*, 2008, **37**, 320.

11 N. Y. Kulikova, A. M. Shpirt and L. O. Kononov, *Synthesis*, 2006, 4113.

12 (*a*) P. Rota, P. Allevi, R. Colombo, M. L. Costa and M. Anastasia, *Angew. Chem., Int. Ed.*, 2010, **49**, 1850; (*b*) P. Rota, P. Allevi, M. L. Costa and M. Anastasia, *Tetrahedron: Asymmetry*, 2010, **21**, 2681; (*c*) P. Rota, P. Allevi, R. Mattina and M. Anastasia, *Org. Biomol. Chem.*, 2010, **8**, 3771.

13 (*a*) I. S. Agnolin, P. Rota, P. Allevi, A. Gregorio and M. Anastasia, *Eur. J. Org. Chem.*, 2012, 6537; (*b*) P. Allevi, P. Rota, I. S. Agnolin, A. Gregorio and M. Anastasia, *Eur. J. Org. Chem.*, 2013, **19**, 4065; (*c*) P. Rota, P. Allevi, I. S. Agnolin and M. Anastasia, *Eur. J. Org. Chem.*, 2012, **13**, 2508.

14 P. Rota, P. Allevi, I. S. Agnolin, R. Mattina, N. Papini and M. Anastasia, *Org. Biomol. Chem.*, 2012, **10**, 2885.

15 (*a*) P. Chand, Y. S. Babu, S. R. Rowland and T.-H. Lin, *Patent WO*, 2002076971A1, 2002; (*b*) P. Meindl and H. Tuppy, *Hoppe-Seyler's Z. Physiol. Chem.*, 1969, **350**, 1088.

16 B. Venerando, B. Cestaro, A. Fiorilli, R. Ghidoni, A. Preti and G. Tettamanti, *Biochem. J.*, 1982, **203**, 735.

17 (*a*) L. Sanchez-Felipe, E. Villar and I. Munoz-Barroso, *Biochim. Biophys. Acta*, 2014, **1838**, 300; (*b*) J. K. Young, D. Li, M. C. Abramowitz and T. G. Morrison, *J. Virol.*, 1999, **73**, 5945; (*c*) S. H. Kim, M. Subbiah, A. S. Samuel, P. L. Collins and S. K. Samal, *J. Virol.*, 2011, **85**, 8582; (*d*) T. Takimoto, G. L. Taylor, H. C. Connaris, S. J. Crennell and A. Portner, *J. Virol.*, 2002, **76**, 13028.

18 W. C. Still, M. Kahn and A. Mitra, *J. Organomet. Chem.*, 1978, **43**, 2923.

19 A. Garcìa-Sastre, C. Cobaleda, J. A. Cabezas and E. Villar, *Biol. Chem. Hoppe-Seyler*, 1991, **372**, 923.

20 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.

21 G. M. Sastry, M. Adzhigirey, T. Day, R. Annabhimoju and W. Sherman, *J. Comput.-Aided Mol. Des.*, 2013, **27**, 221.

22 G. Chang, W. C. Guida and W. C. Still, *J. Am. Chem. Soc.*, 1989, **111**, 4379.

23 M. Saunders, K. N. Houk, Y.-D. Wu, C. W. Still, M. Lipton, G. Chang and W. C. Guida, *J. Am. Chem. Soc.*, 1990, **112**, 1419.

24 D. Shivakumar, E. Harder, W. Damm, R. A. Friesner and W. Sherman, *J. Chem. Theory Comput.*, 2012, **8**, 2553.

25 R. A. Friesner, R. B. Murphy, M. P. Repasky, L. L. Frye and J. R. Greenwood, *J. Med. Chem.*, 2006, **49**, 6177.

26 T. A. Halgren, R. B. Murphy, R. A. Friesner, H. S. Beard, L. L. Frye, W. T. Pollard and J. L. Banks, *J. Med. Chem.*, 2004, **47**, 1750.

27 R. A. Friesner, J. L. Banks, R. B. Murphy, T. A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky, E. H. Knoll, D. E. Shaw, M. Shelley, J. K. Perry, P. Francis and P. S. Shenkin, *J. Med. Chem.*, 2004, **47**, 1739.