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Novel 3,4-disubstituted-Neu5Ac2en derivatives as probes to investigate flexibility of the influenza virus sialidase 150-loop

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ABSTRACT

Novel 3,4-disubstituted-Neu5Ac2en derivatives have been synthesised to probe the open 150-loop conformation of influenza virus sialidases. Both equatorially and axially (*epi*) substituted C4 amino and guanidino 3-(*p*-tolyl)allyl-Neu5Ac2en derivatives were prepared, via the 4-*epi*-hydroxy derivative. The equatorially-substituted 4-amino derivative showed low micromolar inhibition of both group-1 (pdm09 H1N1) and group-2 (pdm57 H2N2) sialidases, and provides the first in vitro evidence that a group-2 sialidase may exhibit 150-loop flexibility.

1. Introduction

Influenza remains a significant and continuous threat to humanity, as evidenced by seasonal epidemics that exact a high toll in morbidity, estimated to be in the range of three to five million cases of severe illness, and mortality, with up to half a million deaths worldwide.[1] The threat, and potentially devastating consequences (an estimated global mortality of greater than 60 million[2]) of adaptation of, for example, a highly pathogenic avian influenza A (H5N1) virus to a human-to-human transmissible virus has fuelled a drive to develop strategies to minimise, and new therapies to prevent or treat, influenza virus infection.

Influenza A viruses, which cause the most significant disease in humans, including seasonal epidemics and global pandemics, are classified into subtypes based on the antigenic properties of the virus' surface glycoproteins, the lectin 'haemagglutinin' (HA: H1-17) and the glycohydrolase 'sialidase' (neuraminidase, NA: N1-9).[3] All of the influenza A subtypes are found in wild birds, but only three HA subtypes (H1, H2, and H3) and two of the NA subtypes (N1 and N2) have circulated widely in the human population.[3] Influenza A virus sialidases have been further sub-classified based upon primary sequence, into two phylogenetically distinct groups: group-1 (N1, N4, N5 and N8) and group-2 (N2, N3, N6, N7 and N9).[4] During the last century, influenza viruses carrying N1 (H1N1) or N2 (H2N2, H3N2) subtypes have circulated in humans, first as pandemic strains and then, after subsequent adaptation to humans, as seasonal epidemic strains.[3]

A major role of the sialidase in the virus' infective cycle is to facilitate release of virus progeny from an infected cell by cleaving terminal sialic acid residues from cell surface and virus particle associated sialoglycoconjugates that would otherwise be bound by HA of the new virus particles and keep them clumped at the cell surface.[5] This important functional role, and the large number of highly conserved active site amino acid residues in influenza virus sialidases, led to the targeting of this enzyme for anti-influenza drug development.[6] Inhibitor design—based on available N2 and N9 (group-2) NA X-ray crystal structures—targeting highly-conserved residues of the influenza virus sialidase active site, led to the development in the 1990s of two drugs that mimic the putative enzyme transition state: zanamivir (Relenza®) **1**,[7] and oseltamivir (Tamiflu®, an orally-bioavailable pro-drug form of the sialidase inhibitor oseltamivir carboxylate, OC **2**) (Fig. 1).[8] Both drugs are effective in the prophylaxis and treatment of influenza virus infection. The worldwide stockpiling of these two anti-viral drugs as a major component of pandemic preparedness planning highlights the current importance of the sialidase inhibitor drug class.

Recently, the first X-ray crystal structures of influenza A virus group-1 sialidases [(H5)N1, N4 and N8 NAs,[4] and subsequently N5 NA[9]] were solved. These structures showed never-before-seen flexibility of an amino acid loop—the so-called '150-loop' (residues 147–152)—that lies along one edge of the active site cavity. The flexibility of the 150-loop leads to generation of a large cavity (the '150-cavity') adjacent to the enzyme active site when the loop is in an 'open' conformation. Computational studies using molecular dynamics (MD) simulations of the avian (H5)N1 subtype suggest that the 150-loop is even more flexible and able to open into a significantly wider conformation than observed in the crystal structures.[10]

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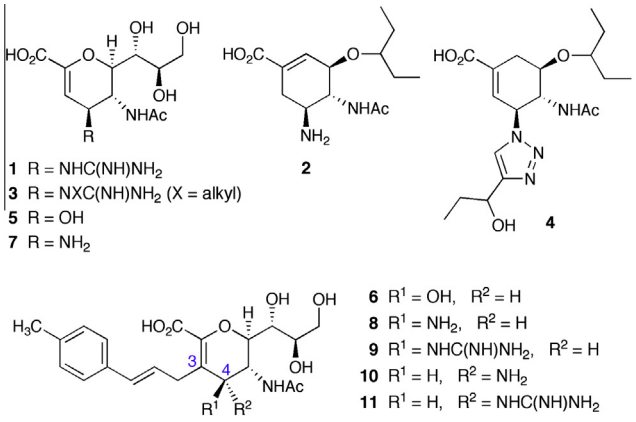


Fig. 1. Anti-influenza drugs (**1** and **2**), and influenza virus sialidase inhibitors.

To date, the 150-loop has always been seen in the 'closed' form in both *apo* and inhibitor-complexed group-2 (N2 and N9) NA crystal structures.[11,12] Interestingly, the X-ray crystal structure of *apo* human pdm09 H1N1 NA, despite being a group-1 sialidase, also showed the 150-loop only in a closed conformation.[13] The factors (e.g., aa sequences) that influence the propensity for, and extent of, 150-loop flexibility in influenza virus sialidases are currently a matter of speculation.[13,14] MD simulation studies have suggested, however, that both human pdm09 N1, and N2 sialidases should also exhibit 150-loop flexibility but with potentially lower population of the open 150-loop conformations.[14]

The extended active site cavity of an open 150-loop conformation sialidase, presents a potential new target for inhibitor design.[4,15] We[16,17] and others[18–20] have begun to explore the 150-loop region of influenza virus sialidases, introducing onto established sialidase-inhibitor scaffolds substituents anticipated to reach towards the 150-cavity; for example from the C4 position of zanamivir[18,20] (e.g., **3**[18]) or the 'equivalent' C3 position of an oseltamivir isostere (e.g., **4**),[19] and from the C3 position of the natural sialidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en **5**) (e.g., **6**).[16,17] Molecular modelling[18] and STD NMR spectroscopy[19] studies, respectively, suggest that an extended alkyl substituent on the internal guanidinyl nitrogen of a zanamivir derivative (**3**),[18] and the triazole 4′-substituent of oseltamivir isostere **4**,[19] could make additional interactions within the 150-cavity in the open form of N1 sialidases. We have recently published the first X-ray crystallographic evidence of sialidase inhibitors binding the 150-cavity.[16,17] C3-functionalised Neu5Ac2en derivative **6**, which inhibits both human (including pdm09) and avian N1 NAs at the micromolar level, was shown to bind the representative group-1 sialidase N8 with the 150-loop in the open conformation and the C3 substituent occupying an area of the 150-cavity.[16] That study provided proof-of-concept that designed compounds could lock-open the 150-loop of an influenza A virus sialidase. In the present study, we report our further work in this area with the synthesis and evaluation of 3,4-difunctionalised Neu5Ac2en-based inhibitor probes of influenza A virus sialidases.

2. Results and discussion

2.1. Structure analysis

A point of focus in the development of potent influenza virus sialidase inhibitors, has been the incorporation of functionality to interact with conserved amino acids lining the pocket of the active site that accommodates the C4 hydroxyl group of Neu5Ac2en **5** (designated for this work as the 'C4 pocket'). This pocket is dominated in the closed 150-loop conformation by negatively-charged acidic residues (including Glu119, Glu227, and Asp151), and has been targeted with the introduction of positively charged guanidino or amino groups as in inhibitors **1**, **2** and **7**.[6,21] The nature (spatial and electrostatic characteristics) of the C4 pocket when the 150-loop is in a more open conformation, is somewhat different to the closed 150-loop conformation.[4] When the 150-loop is open,[4] Glu119 and Asp151 are seen to move away from the positions that they occupy in the closed 150-loop conformation (Fig. 2), giving the C4 pocket a less negative electrostatic potential when the 150-loop is open.

We were interested to examine the effect of introducing functionality at the C4 position on the C3-substituted Neu5Ac2en inhibitor scaffold. While this scaffold holds the 150-loop in the open conformation, the flexibility of the 150-loop, and the 'plasticity/mobility' of adjacent active site residues, as observed crystallographically in complexes of N8 NA with **2**[4] and with different C3-substituted Neu5Ac2en-based inhibitors,[16,17] may allow the protein to 'mold' to the new inhibitor structure. We therefore began a program to functionalise the C4 position of C3-substituted Neu5Ac2en, using 3-(*p*-tolyl)allyl-Neu5Ac2en **6** as the template. The initial modifications chosen were the introduction of amino and guanidino groups at C4 in both the 'natural' equatorial (**8** and **9**) and in the axial (or *epi*) (**10** and **11**) configurations.

2.2. Chemistry

Two main approaches for the synthesis of the C4-N-functionalised C3-substituted Neu5Ac2en-based inhibitor probes **8**–**11** were apparent; functionalisation at C-3 of protected 4-azido-4-deoxy-Neu5Ac2en, using methodology developed for the synthesis of **6** from a 4-acetoxy-Neu5Ac2en derivative;[17,22] or functionalisation at C4 of a C3-substituted Neu5Ac2en derivative. In the first instance, C3 allylation of a protected 4-azido-4-deoxy-Neu5Ac 2,3-bromohydrin was attempted using the method[17,22] employed to install the allyl group at C3 on the corresponding 4-acetoxy derivative (allyltributyltin in the presence of AIBN as free radical initiator). This chemistry was, however, unsuccessful on the 4-azido substrate, resulting in polymerization and decomposition. The alternative route to the target C4-N-functionalised 3-(*p*-tolyl)al-

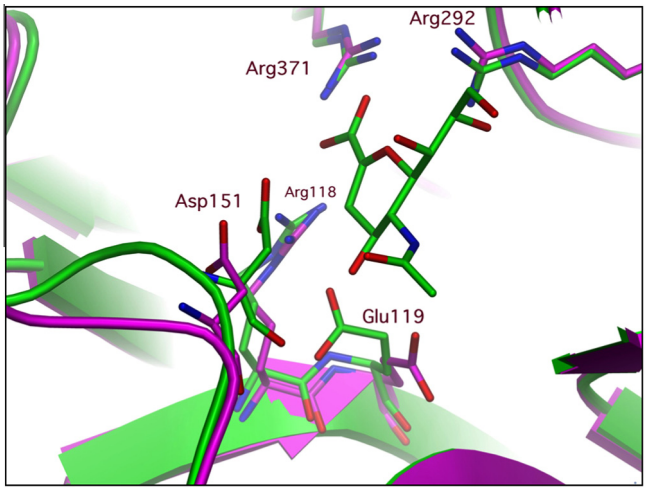


Fig. 2. Positions of important active site residues of group-1 influenza A virus sialidase N8 in the 150-loop open (*apo* form, magenta: PDB 2ht5) and closed (complexed with Neu5Ac2en **5**, green: PDB 2htr) forms. The three arginine residues (Arg118, Arg 371, Arg292) that bind the carboxylate group of **5** hold essentially the same positions in both the open and closed 150-loop forms. In contrast, in the 150-loop open form (magenta) the negatively charged Asp151 and Glu119 move away from the positions adopted in the closed 150-loop form (green), leading to a somewhat less negatively-charged C4 pocket.

lyl-Neu5Ac2en derivatives requires 'activation' of the allylic C4 position of the C3-substituted Neu5Ac2en derivative, for installation of an azido group. In the synthesis of 4-N-substituted Neu5Ac2en derivatives (e.g., zanamivir), introduction of the C4 azide begins with formation of a 4,5-oxazoline, which is either opened directly by nucleophilic azide, or hydrolysed to the 4-*epi*-hydroxy derivative for further manipulation. The efficient synthesis of the 3-(*p*-tolyl)allyl-Neu5Ac2en 4,5-oxazoline **17** and the key 4-*epi*-hydroxy intermediate **19** from peracetylated Neu5Ac2en derivative **12** is outlined in Scheme 1.

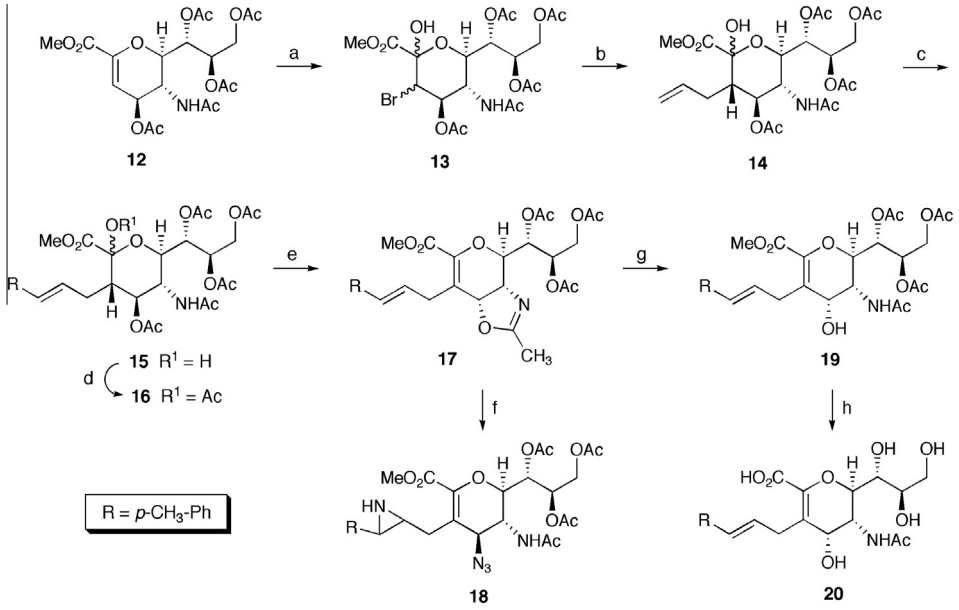
Bromohydroxylation of protected Neu5Ac2en **12** using *N*-bromosuccinimide (NBS) in aqueous acetonitrile gives a mixture of bromohydrins **13** (*trans*-2,3-diaxial bromohydrin and *trans*-2,3-diequatorial bromohydrin ratio = 3:1) in excellent overall yield.[23] The reaction of bromohydrin mixture[22] **13** with allyltributyltin in the presence of AIBN affords solely the required C3 equatorially-allylated product **14**.[22,24] Ruthenium (second generation Grubbs' catalyst) catalysed olefin-cross metathesis[25] of 3-allyl derivate **14** with 4-methylstyrene in anhydrous dichloromethane at 40 °C, affording cross-metathesis coupled product **15** in 85% yield, was followed by acetylation of the C2 hydroxyl group of **15** to give 2-*O*-acetate derivative **16**. The preparation of the 4,5-oxazoline was then achieved by using a modification of the procedure described by Zbiral and co-workers.[26] Reaction of peracetylated derivative **16** with trimethylsilyl trifluoromethanesulfonate (TMSOTf) in ethyl acetate at 52 °C gave the 2,3-eliminated 4,5-oxazoline derivative **17** in high yield (95%).

The next synthetic step involved introduction of the azide functionality at the C4 position. The C4 azide could lead into a range of nitrogen-based substituents—including amine, guanidino, amide, and triazole substituents—to probe interactions in the sialidase C4 pocket. In the case of C3-unsubstitued Neu5Ac2en derivatives, an azide functionality can be introduced at C4 through reaction of the 4,5-oxazoline with trimethylsilyl azide.[27] However, following literature conditions,[27] the reaction of C3-functionalised oxazoline intermediate **17** with trimethylsilyl azide in *tert*-butyl alcohol at 80 °C resulted in an unexpected product, aziridine derivative **18**. Varying the reaction conditions with respect to solvent, temperature and equivalents of trimethylsilyl azide did not avoid aziridination. Owing to the high reactivity of the allylic double bond towards nucleophilic azide addition, we decided to explore an alternative method[28] for introduction of azide at the C4 position. Accordingly, 4,5-oxazoline **17** was hydrolysed with trifluoroacetic acid in aqueous ethyl acetate to yield 4-*epi*-hydroxy derivative **19**. Intermediate **19** was saponified to provide 4-*epi*-3-(*p*-tolyl)allyl-Neu5Ac2en **20**.

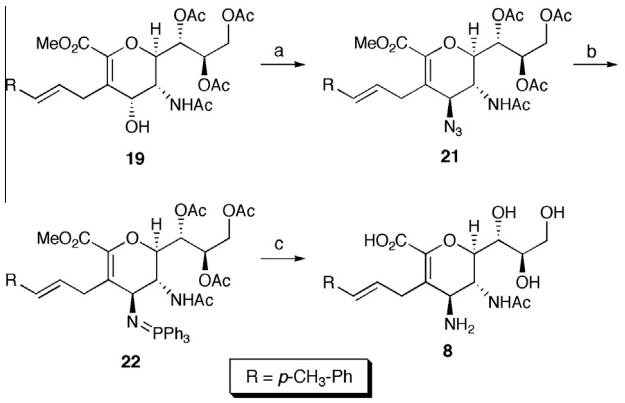
Reaction of 4-*epi*-hydroxy derivative **19** with diphenylphosphoryl azide[28] (DPPA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene gave the desired 4-azido derivative **21** in good yield (Scheme 2). This reaction proceeds with inversion of configuration at C4. Mechanistically, the reaction takes place in two discrete steps—formation of the reactive 4-*O*-phosphate followed by displacement with in situ generated azide.[29] It is important to mention here that our effort to replace benzene as solvent with toluene, THF or S_N2 displacement-favorable DMF resulted mainly in decomposition.

We next turned to the reduction of the C4 azide functionality. Our initial attempts towards selective hydrogenation of the C4 azide of **21** using palladium on carbon (Pd/C 10%)[30] or Lindlar catalyst,[27] conditions used successfully for selective azide reduction in 4-azido-4-deoxy-Neu5Ac2en,[27,30] led to unwanted side reactions, for example acetate migration or even over-reduction of the allylic and/or ring double bonds. Finally, the previously reported[26] method for triphenylphosphine-mediated reduction of 4-azido-4-deoxy-Neu5Ac2en derivatives, successfully gave the desired 4-amino compound **8**. The reaction of the C4 azide group of **21** with triphenylphosphine in THF at 50 °C resulted in the unstable P–N ylide **22** which upon in situ hydrolysis with aqueous sodium hydroxide[31] afforded target 4-amino derivative **8** in good yield.

To install the more basic guanidino group at C4, the amino derivative can be reacted with *N*,*N*′-bis-*t*-butoxycarbonyl-1*H*-pyrazole-1-carboxamidine (bisBocPCH).[31] Attempted guanidinylation of the 4-amino group of fully deprotected acid **8**, however, was unsuccessful. We therefore explored an alternative strategy as shown in Scheme 3. The C4 azide derivative **21** was de-*O*-acety-



Scheme 1. Reagents and conditions: (a) NBS, MeCN/H_2O (2.5:1), 80 °C, 2 h (70% di-axial bromohydrin, 24% di-equatorial bromohydrin, combined yield 94%); (b) Bu_3SnAll, AIBN, toluene, 100 °C, 8 h (68%); (c) Grubbs' catalyst 2nd generation (15 mol %), 4-methylstyrene, DCM, 40 °C, 48 h (85%); (d) Ac_2O, pyridine, DMAP, rt, 16 h (95%); (e) TMSOTf, ethyl acetate, 60 °C, 2 h (95%); (f) azidotrimethylsilane, *tert*-butyl alcohol, 80 °C, 4 h (68%); (g) TFA, H_2O, ethyl acetate, rt, 16 h (96%) and (h) aq NaOH, MeOH/H_2O (1:1), 5 °C-rt, 16 h (90%).



Scheme 2. Reagents and conditions: (a) DPPA, DBU, benzene, 5–10 °C, 15 min, rt, 5 h (85%); (b) PPh_3, THF, 50 °C, 10 min. and (c) aq NaOH, 50 °C, 2 h (72% over 2 steps).

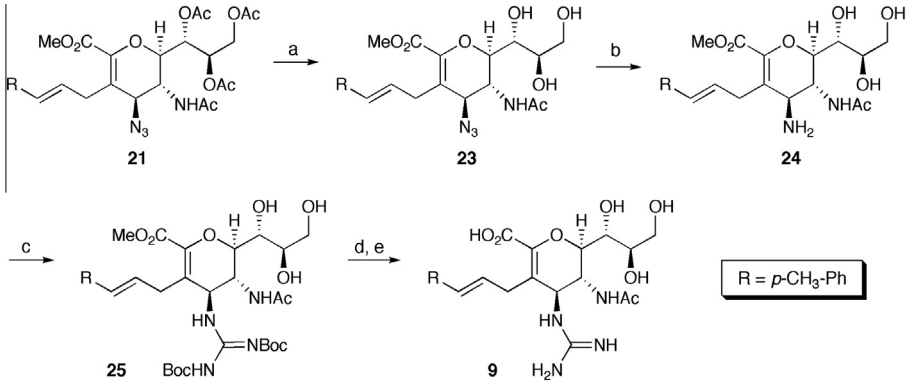
lated using sodium methoxide to give triol methyl ester **23**. Reduction of the azide of **23** with H_2S in pyridine[32] gave the amino derivative **24** in 80% yield. Guanidinylation of the 4-amino group in **24** with bisBocPCH successfully produced the protected guanidine derivative **25**. It is noteworthy that the reaction required several days for complete consumption of starting material; reaction time was not improved upon addition of excess guanidinylating reagent or raising the reaction temperature to 40 °C. The longer reaction time (as compared to 18–20 h reaction time for C3-unsubstituted Neu5Ac2en derivatives[31]) may be due to the steric effect of the C3 *p*-tolylallyl substituent, which could make access to the bulky guanidinylating reagent difficult. Removal of the Boc protecting groups of **25** by treatment with TFA, followed by de-esterification with aqueous sodium hydroxide afforded 4-guanidino derivative **9** in good yield.

The syntheses of the corresponding 4-*epi*-(axially) configured amino and guanidino derivatives also began with 4-*epi*-hydroxy derivative **19**, following the route shown in Scheme 4. Treatment of the peracetylated methyl ester of 4-*epi*-3-(*p*-tolyl)allyl-Neu5Ac2en (**26**), obtained by acetylation of **19**, with sodium azide in aqueous THF in the presence of a catalytic amount of tetrakis(triphenylphosphine)palladium(0) resulted in formation of 4-*epi*-azido derivative **27** in 82% yield. This reaction was highly stereoselective and no detectable amount of the other C4 isomer **21** was observed by ^1H NMR spectroscopy. It is reported[33,34] that palladium(0)-catalysed allylic azidation proceeds with oxidative addition of the palladium catalyst, yielding a (π-allyl)palladium complex with inversion of configuration. Subsequent nucleophilic attack by azide also occurs with inversion of configuration to yield the product with overall retention of configuration at C4.[33,34] In the next sequence, base-catalysed de-*O*-acetylation of **27** gave triol methyl ester **28**. The azide of **28** was then reduced with H_2S in pyridine to give the 4-*epi*-amino derivative **29** in 84% yield. Finally, de-esterification of amine **29** using NaOH in aqueous MeOH gave desired 4-*epi*-amino derivative **10** in 68% yield, along with a side product resulting from acetate migration to give 4-acetamido-4-deoxy-4-*epi*-Neu2en derivative **30**. 4-*epi*-Amino derivative **29** was efficiently converted into the 4-*epi*-guanidino compound **31** by reaction with bisBocPCH. Subsequent processing of **31** as for the C4 equatorially-substituted analogue **25**—deprotection of Boc groups and de-esterification—provided the 4-*epi*-guanidino derivative **11** in good yield. This is, to the best of our knowledge, the first reported example of a 4-*epi*-guanidino substituent on a Neu5Ac2en scaffold.

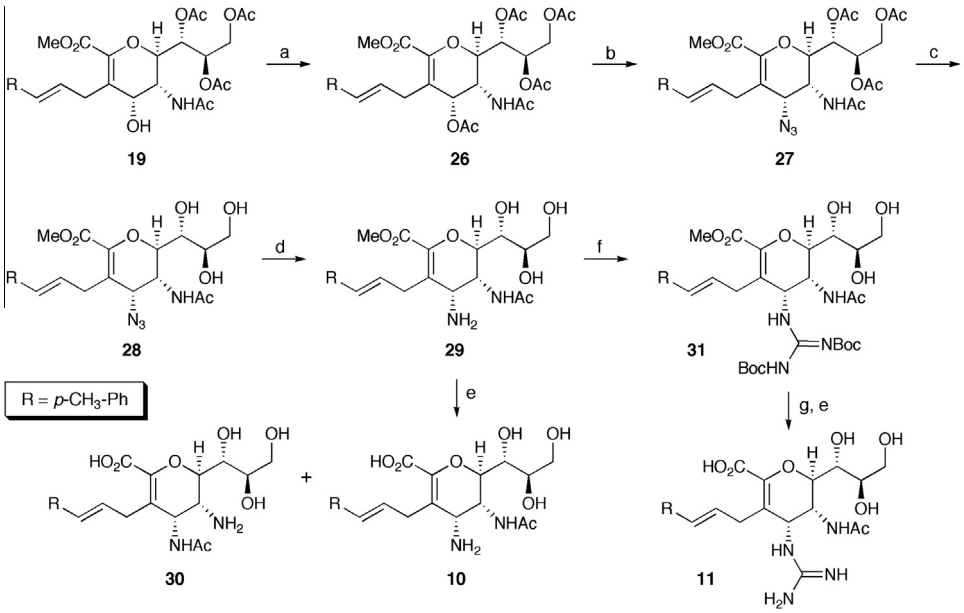
2.3. Biological evaluation

The synthesised 3,4-disubstituted Neu5Ac2en derivatives **8**–**11**, were evaluated[19] for their ability to inhibit the hydrolysis of the fluorogenic substrate 2-α-(4′-methylumbelliferyl)-*N*-acetylneuraminic acid (MUN) by group-1 (pdm09 N1) and group-2 (pdm57 N2) influenza A virus sialidases. The sialidase inhibitory activities of **8**–**11**, the parent template 3-(*p*-tolyl)allyl-Neu5Ac2en **6**, and the natural sialidase inhibitor Neu5Ac2en **5**, are presented in Table 1.

Against the N1 sialidase, the C4-amino derivative **8** shows comparable low micromolar inhibition to the parent (C4-hydroxy) template **6**. Interestingly, introduction of the larger guanidino group at C4 (compound **9**) results in a marked 100-fold weaker inhibition. This is in stark contrast to the C3-unsubstituted analogue, 4-deoxy-4-guanidino-Neu5Ac2en **1** (zanamivir; IC_{50} 7 nM) which binds the closed 150-loop conformation of the enzyme, and which shows significantly stronger inhibitory activity than its corresponding 4-amino analogue **7**.[7,21] The X-ray crystal structure of zanamivir **1** in complex with 'closed' group-2 sialidase N9, shows interaction of each of the three nitrogen atoms of the C4 guanidino group with one or more of the residues Glu119, Glu227 and Asp151.[7,35] The altered orientations of Glu119 and Asp151 in an open 150-loop conformation sialidase, would reduce the potential for this strong interaction. In addition, the two acidic residues Glu119 and Asp151 generally mask the positive charge related to residue Arg156 which also borders the C4 pocket. As Glu119 and Asp151 move further away, Arg156 sits in a more exposed position on the lip of the 150-cavity. Adverse interaction of the larger guanidino group at the C4 position with Arg156 could contribute to the observed weaker inhibition of guanidino derivative **9**.

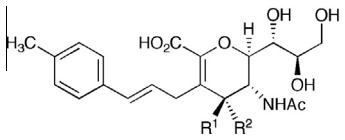


Scheme 3. Reagents and conditions: (a) NaOMe, MeOH, 5 °C to rt, 1 h (90%); (b) H_2S, pyridine, rt, 16 h (80%); (c) bisBocPCH, Et_3N, MeOH/THF, rt, 7 d (72%); (d) TFA, DCM, 5 °C to rt, 5 h, (e) aq NaOH, MeOH/H_2O (1:1), 5 °C to rt, 16 h (83% over 2 steps).



Scheme 4. Reagents and conditions: (a) Ac_2O, DMAP, pyridine, rt, 16 h (96%); (b) NaN_3, $Pd(Ph_3P)_4$, THF/H_2O, 50 °C, 16 h (82%); (c) NaOMe, MeOH, 5 °C to rt, 2 h (90%); (d) H_2S, pyridine, rt, 16 h (84%); (e) aq NaOH, MeOH/H_2O (1:1), 5 °C to rt, 16 h (68%); (f) bisBocPCH, Et_3N, MeOH/THF, rt, 3 d (85%) and (g) TFA, DCM, 5 °C to rt, 5 h; (80% over 2 steps).

Table 1
In vitro inhibition of influenza A virus N1 and N2 sialidases by 3,4-disubstituted-Neu5Ac2en derivatives **8**–**11**



Compd	R^1	R^2	IC_{50}[a] (μM)	
			N1 [b]	**N2** [c]
6	OH	H	6.5	3.2
8	NH_2	H	3.5	0.1
9	NHC(NH)NH_2	H	336	42
10	H	NH_2	465	405
11	H	NHC(NH)NH_2	730	NI [d]
5	Neu5Ac2en		0.7 [e]	1.8

[a] Results are given as means for 6 experimentally determined values. Benchmark inhibitor 4-deoxy-4-guanidino-Neu5Ac2en **1**: N1, IC_{50} 0.007 μM; N2, IC_{50} 0.005 μM.
[b] N1 [A/California/04/2009 (pdm09 H1N1)].
[c] N2 [A/R1/5+/1957 (H2N2)].
[d] No inhibition at 1 mM inhibitor concentration.
[e] N1 [A/Paris/2590/2009 (pdm09 H1N1)]; Inhibition data (K_i) from Ref. 16.

In comparison to the equatorially-substituted derivatives **8** and **9**, the corresponding axially-substituted (4-*epi*) isomers **10** and **11** show overall weak inhibitory activity. A C4 substituent in the *epi*-configuration is oriented towards the floor of the active site cavity. Previously observed sub-micromolar influenza virus sialidase inhibition by 4-*epi*-amino-4-deoxy-Neu5Ac2en,[21] has been proposed to be due to interaction of the *epi*-amino group with Glu227, which sits under the inhibitor on the floor of the active site.[21] When the 150-loop is open, Glu119 flips over and forms a very close interaction with Glu227 (less than 2 Å distant). p*K*a calculations suggest that either one or both of these normally acidic residues would be protonated, making them electrostatically neutral and reducing their interaction with a basic 4-*epi*-substituent, providing a rationale for the observed weaker affinity of 4-*epi*-amino derivative **10**. For the 4-*epi*-guanidino derivative **11**, as observed in previous modelling studies on the closed 150-loop conformation,[21] the confined space below the inhibitor in this area of the active site appears to preclude favourable binding of the larger guanidino substituent in the axial orientation; based on the inhibition values for **11** this aspect appears to be more pronounced in the N2 than in the more flexible N1 sialidase.

Interestingly, in terms of inhibition of group-1 (N1) versus group-2 (N2) sialidases, parent inhibitor **6** shows comparable inhibition of both enzymes, and equatorially C4-substituted derivatives **8** and **9** show 10-fold greater inhibitory activity against the N2 subtype. Both the pdm09 N1[13] and pdm57 N2[12] sialidases have been crystallised to date only in closed 150-loop conformations. The level of inhibition of in particular **6** and **8** against these enzymes, however, is comparable to that seen for **6** against avian (H5)N1 NA which has been shown crystallographically[4] to have a 'flexible' 150-loop. Assuming—based on this similar low micromolar level of inhibition, and the demonstrated[16] binding of **6** to the open 150-loop conformation of group-1 sialidase N8—that the compounds are binding the sialidases in similar conformations, this represents the first *in vitro* evidence, supporting in silico observations,[10,14] that group-2 sialidases may also possess some degree of flexibility in the 150-loop. It is tempting to speculate that the greater tendency for the 150-loop to remain closed in group-2 sialidases,[14] may lead to a more favourable binding environment for the amine-based C4 substituents of **8** and **9**, than does the more freely opening group-1 NA.

2.4. Conclusion

In summary, the synthesis of novel 3,4-disubstituted-Neu5Ac2en derivatives 4-amino- (**8**) and 4-guanidino- (**9**) 4-deoxy-3-(*p*-tolyl)allyl-Neu5Ac2en and their corresponding 4-*epi* analogues (**10** and **11**), as inhibitor-based probes of influenza virus sialidases, has been efficiently achieved via the key 4-*epi*-hydroxy-3-(*p*-tolyl)allyl-Neu5Ac2en derivative **19**. The equatorially-substituted derivatives **8** and **9** showed the more potent sialidase inhibition,

with the 4-amino derivative **8** in particular giving low micromolar inhibition of both group-1 (pdm09 N1) and group-2 (pdm57 N2) sialidases, and providing the first *in vitro* evidence that a group-2 sialidase may exhibit 150-loop flexibility. The intermediate 4-azido-4-deoxy-3-(*p*-tolyl)allyl-Neu5Ac2en derivative **21** is an important precursor that could lead into a range of nitrogen-based substituents—including amides, and triazoles—to further probe interactions in the sialidase C4 pocket.

3. Experimental

3.1. Chemistry

3.1.1. General information

Reagents and dry solvents purchased from commercial sources were used without further purification. Solvents used for chromatography were distilled prior to use. All anhydrous reactions were carried out under an atmosphere of nitrogen or argon, using oven-dried glassware. Reactions were monitored using TLC on aluminium plates coated with Silica Gel 60 F_{254} (E. Merck) and visualised by UV light (254 nm) where applicable, and by application of ethanolic H_2SO_4 (5% v/v) and heating. Compounds were purified by flash chromatography on Silica Gel 60 (0.040–0.063 mm). NMR spectra were recorded on a Bruker Avance 300 or 600 MHz spectrometer. ^{1}H NMR and ^{13}C NMR chemical shifts (δ) are reported in parts per million, relative to the residual solvent peak as internal reference [$CDCl_3$: 7.26 (s) for ^{1}H; 77.0 (t) for ^{13}C; D_2O: 4.67 (s) for ^{1}H; CD_3CN: 1.32 for ^{13}C; CD_3OD: 3.31 for ^{1}H; 49.0 for ^{13}C]. 2D COSY and HSQC experiments were run to support assignments. Low resolution mass spectra (LRMS) were acquired in positive or negative ion mode as indicated, on a Bruker Daltonics Esquire 3000 ESI MS; high-resolution mass spectrometry (HRMS) data was acquired at the Griffith University FTMS Facility on a Bruker Daltonics Apex III 4.7e Fourier Transform MS, fitted with an Apollo ESI source. Reversed phase (RP) HPLC purification was performed on an Agilent HP1100 series system equipped with a C18 column [Synergi 4 μm, Hydro-RP 80 Å (250 × 10 mm), Phenomenex; or, AQUA 5 μm, C18 (250 × 10 mm), Phenomenex],

N-Acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid) was purchased from Jülich Chiral Solutions GmbH (Jülich, Germany) and Carbosynth UK.

Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate (**12**)[36] and bromohydrins methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3-bromo-3,5-dideoxy-α-D-*erythro*-L-*gluco*-non-2-ulopyranosonate, and methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3-bromo-3,5-dideoxy-α-D-*erythro*-L-*mano*-non-2-ulopyranosonate (**13**)[23] were prepared according to literature procedures. Syntheses of methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-3-*C*-(prop-2′-enyl)-α/β-D-*erythro*-L-*gluco*-non-2-ulopyranosonate (**14**) was carried out as previously described.[17,22] The experimental details and physical data for new compounds are provided below. The purities of all intermediate compounds were determined to be >90% by ^{1}H and ^{13}C NMR (see Supplementary data). The purity of target compounds was ⩾95% by HPLC analysis.

3.1.2. General method for base-catalysed compound deprotection

A solution of compound (0.05 mmol) in MeOH/H_2O 1:1 (4 mL) at 5 °C is adjusted to pH 13 using aq NaOH (1 M). The solution is then stirred at room temperature and the progress of reaction is monitored by TLC analysis (EtOAc/MeOH/H_2O, 7:2:1). At completion of the reaction, Amberlite® IR-120 (H^+) resin is added to adjust to pH 3, the reaction mixture is filtered, the resin is washed with MeOH/H_2O 1:1 (25 mL), and the filtrate is concentrated to dryness under vacuum. The crude product is dissolved in water, the pH of the solution is adjusted to pH 7 using aq NaOH (1 M), and the solution is lyophilised. Target compounds were subsequently purified by reversed phase HPLC.

3.1.3. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′-enyl]-β-D-*erythro*-L-*gluco*-non-2-ulopyranosonate (15)

To a solution of allyl compound **14** (3.2 g, 6.02 mmol) in anhydrous DCM (200 mL) under N_2 was added 4-methylstyrene (7.94 mL, 60.20 mmol) at room temperature. Subsequently, ruthenium catalyst, Grubbs catalyst (2nd generation) (254 mg, 0.30 mmol, 10 mol %) was added to the reaction mixture, which was then stirred at 40 °C for 24 h. TLC analysis showed presence of starting material (~40%), then a further amount of Grubbs catalyst (2nd generation) (254 mg, 0.30 mmol, 5 mol %) was added and the reaction mixture was stirred at 40 °C for 24 h (complete disappearance of the starting material by TLC analysis). The solution was concentrated under reduced pressure and the residue was purified by flash chromatography on silica (acetone/hexane, 3:7) to afford the cross-coupled product **15** as a white solid (3.17 g, yield 85%). R_f 0.60 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.84, 1.94, 1.99, 2.07, 2.09 (5× s, 15H, NHCOCH_3, OCOCH_3 ×4), 2.15 (m, 1H, –CH_2–), 2.20 (m, 1H, H-3), 2.28 (s, 3H, *p*-tolyl CH_3), 2.60 (m, 1H, –CH_2–), 3.62 (s, 3H, COOCH_3), 4.46 (dd, *J* = 12.3, 6.9 Hz, 1H, H-9), 4.12 (dd, *J* = 10.8, 2.4 Hz, 1H, H-6), 4.21 (ddd, *J* = 10.5, 10.2, 9.9 Hz, 1H, H-5), 4.32 (dd, *J* = 12.0, 2.4 Hz, 1H, H-9′), 4.33 (d, *J* = 1.5 Hz, 1H, 2-OH, D_2O exchangeable), 5.03 (dd, *J* = 10.8, 9.9 Hz, 1H, H-4), 5.16 (m, 1H, H-8), 5.28 (dd, *J* = 6.9, 2.1 Hz, 1H, H-7), 5.41 (d, *J* = 10.2 Hz, 1H, NH), 5.91 (m, 1H, –CH=), 6.20 (d, *J* = 15.9 Hz, 1H, =C*H*-Ph), 7.04 (d, *J* = 8.1 Hz, 2H, Ph), 7.14 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.8, 20.9, 21.0, 21.2 (OCOCH_3 ×4), 23.1 (NHCOCH_3), 29.3 (*p*-tolyl CH_3), 31.5 (–CH_2–), 44.6 (C-3), 49.6 (C-5), 53.9 (COOCH_3), 62.7 (C-9), 67.8 (C-7), 70.4 (C-8), 70.6 (C-6), 73.6 (C-4), 96.4 (C-2), 125.4 (–CH=), 125.8 (Ph), 129.3 (Ph), 131.8 (=C*H*-Ph), 134.2 (Ph q carbon), 137.1 (Ph q carbon), 169.4 (C-1), 170.3, 170.4, 170.5, 170.7, 171.6 (NHC*O*CH$_3$, OC*O*CH$_3$ ×4); LRMS [$C_{30}H_{39}NO_{13}$] (+ve ion mode) (*m*/*z*): 644 [M+Na]$^+$.

3.1.4. Methyl 5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′-enyl]-β-D-*erythro*-L-*gluco*-non-2-ulopyranosonate (*16*)

A solution of compound **15** (3.1 g, 4.98 mmol) in anhydrous pyridine (22 mL) under N_2 was treated with acetic anhydride (18 mL) and DMAP (59.8 mg, 0.49 mmol). The reaction mixture was stirred at room temperature for 16 h. The progress of the reaction was monitored by TLC analysis. The reaction mixture was evaporated to dryness, taken up in EtOAc (60 mL) and the EtOAc solution was washed successively with 0.1 N HCl (20 mL), water (20 mL), and satd aq NaCl (25 mL). The organic phase was dried (Na_2SO_4), filtered and the filtrate evaporated under reduced pressure. The residue was purified by flash chromatography on silica (EtOAc/hexane, 4:1) to afford the title compound **16** as a white solid (3.13 g, yield 95%). R_f 0.50 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.83, 1.86, 2.00, 2.01, 2.12, 2.15 (6× s, 18H, NHCOCH_3, OCOCH_3 ×5), 2.20 (m, 1H, –CH_2–), 2.26 (m, 1H, H-3), 2.28 (s, 3H, *p*-tolyl CH_3), 2.56 (m, 1H, –CH_2–), 3.75 (s, 3H, COOCH_3), 3.91 (dd, *J* = 10.5, 2.4 Hz, 1H, H-6), 4.11 (dd, *J* = 12.6, 6.9 Hz, 1H, H-9), 4.20 (ddd, *J* = 10.5, 10.2, 10.2 Hz, 1H, H-5), 4.54 (dd, *J* = 12.3, 2.4 Hz, 1H, H-9′), 4.94 (m, 1H, H-8), 5.17 (dd, *J* = 10.5, 10.2 Hz, 1H, H-4), 5.26 (d, *J* = 9.6 Hz, 1H, NH D_2O exchangeable), 5.33 (m, 1H, H-7), 5.96 (m, 1H, –CH=), 6.21 (d, *J* = 15.6 Hz, 1H, =C*H*-Ph), 7.05 (d, *J* = 7.8 Hz, 2H, Ph), 7.14 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.8, 21.0, 21.1, 21.4 (OCOCH_3 ×5), 23.0 (NHCOCH_3), 29.3 (*p*-tolyl CH_3), 30.2 (–CH_2–), 45.9 (C-3), 49.3 (C-

5), 53.3 (COOCH_3), 62.1 (C-9), 68.1 (C-7), 72.3 (C-8), 72.5 (C-6, C-4), 99.5 (C-2), 125.8 (–CH_2–CH═, Ph), 129.3 (Ph), 130.9 (═CH-Ph), 134.3 (Ph q carbon), 137.0 (Ph q carbon), 165.8 (C-1), 167.9, 170.3, 170.6, 170.9, 171.3 (NH$CO$$CH_3$, O$CO$$CH_3$ ×5) LRMS [$C_{32}H_{41}NO_{14}$] (+ve ion mode) (*m*/*z*): 686 [M+Na]$^+$.

3.1.5. Methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-3,5-dideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonate (19)

To a solution of glycosyl acetate **16** (1.8 g, 2.71 mmol) in anhydrous ethyl acetate (50 mL) under N_2 was added dropwise trimethylsilyl trifluoromethanesulfonate (1.42 mL, 7.88 mmol). After the addition was complete the temperature was raised to 60 °C and the reaction was stirred for 2 h. The progress of the reaction was monitored by TLC analysis. After complete consumption of starting material the reaction mixture was allowed to cool and was poured into a vigorously stirred mixture of ice-cold saturated aq sodium hydrogen carbonate (20 mL) and solid sodium hydrogen carbonate (1 g). The layers were separated and the aqueous layer re-extracted with ethyl acetate (2 × 60 mL). The combined organic layers were evaporated and the intermediate 4,5-oxazoline compound **17** was isolated as a white foam (1.39 g, 2.56 mmol, yield 95%). The oxazoline was observed on TLC at R_f = 0.7 using ethyl acetate and product formation was confirmed by MS (LRMS [$C_{28}H_{33}NO_{10}$] (*m*/*z*): (+ve ion mode) 566 [M+Na]$^+$. It was hydrolysed without purification. The crude oxazoline **17** was dissolved in ethyl acetate (40 mL). After the addition of trifluoroacetic acid (350 μL) and water (1 mL) the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was diluted with more ethyl acetate (40 mL) and neutralised with $NaHCO_3$ solution. The organic layer was separated, dried (Na_2SO_4), filtered and evaporated. The crude product was purified by column chromatography on silica (EtOAc/hexane) to yield the title compound **19** as a white foam (1.38 g, yield 96%). R_f = 0.45 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.91 (s, 3H, NHCOCH_3), 2.04, 2.05, 2.08 (3× s, 9H, OCOCH_3 ×3), 2.30 (s, 3H, *p*-tolyl CH_3), 3.35 (dd, *J* = 14.4, 7.5 Hz, 1H, –CH_2–), 3.45 (dd, *J* = 14.4, 6.9 Hz, 1H, –CH_2–), 3.79 (s, 3H, COOCH_3), 4.09–4.17 (m, 3H, H-4, H-6, H-9), 4.32 (ddd, *J* = 11.1, 10.2, 3.9 Hz, 1H, H-5), 4.75 (dd, *J* = 12.3, 2.4 Hz, 1H, H-9′), 5.29 (m, 1H, H-8), 5.42 (m, 1H, H-7), 5.83 (d, *J* = 9.9 Hz, 1H, NH; D_2O exchanged), 6.14 (m, 1H, ═CH–), 6.43 (d, *J* = 15.9 Hz, 1H, ═C*H*-Ph), 7.08 (d, *J* = 8.1 Hz, 2H, Ph), 7.21 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.8, 20.9, 21.1 (NHCOCH_3, OCOCH_3 ×3), 23.2 (*p*-tolyl CH_3), 33.1 (–CH_2–), 46.3 (C-5), 52.2 (COOCH_3), 62.4 (C-9), 65.5 (C-6), 67.8 (C-7), 71.9 (C-8), 72.4 (C-4), 121.4 (C-3), 125.9 (–CH═), 126.1 (Ph), 129.2 (Ph), 132.1 (═CH-Ph), 134.0 (Ph q carbon), 137.2 (Ph q carbon), 140.4 (C-2), 162.8 (C-1), 170.0, 170.3, 170.5, 170.7 (NH$CO$$CH_3$, O$CO$$CH_3$ ×3); LRMS [$C_{28}H_{35}NO_{11}$] (+ve ion mode) (*m*/*z*): 584 [M+Na]$^+$.

3.1.6. Methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)aziridin-2′-methyl]-D-*glycero*-D-*galacto*-non-2-enonate (18)

To a solution of oxazoline **17** (244 mg, 0.45 mmol) in anhydrous *tert*-butyl alcohol (10 mL) under N_2 was added dropwise azidotrimethylsilane (209 mg, 1.82 mmol). After the addition was complete the temperature was raised to 80 °C and the reaction was stirred for 4 h. The progress of the reaction was monitored by TLC analysis. After complete consumption of starting material the reaction mixture was allowed to cool and was poured into a vigorously stirred mixture of ice-cold saturated aq sodium nitrite (10 mL) and then hydrochloric acid (0.1 N, 5 mL) was added over a period of 10 min. Ethyl acetate (30 mL) and water (10 mL) were then added and the organic layer was separated off and washed with water (2 × 5 mL). The combined aqueous layers were extracted with ethyl acetate (10 mL) and the combined organic layers were washed with successively with aq sodium hydrogen carbonate (5 mL) followed by brine (4 mL). The combined organic extracts were then dried (Na_2SO_4), and evaporated to dryness. The crude product was purified by column chromatography on silica (EtOAc/acetone) to yield a white solid assigned as the title aziridine derivative **18** (184 mg, yield 68%). R_f = 0.5 (EtOAc/acetone, 3:2); IR ν_{max}/cm^{-1}: 2101, 1738, 1660, 1369, 1212, 1042, 734; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.99, 2.12, 2.13, 2.32 (4× s, 12H, NHCOCH_3, OCOCH_3 ×3), 2.40 (s, 3H, *p*-tolyl CH_3), 2.83 (dd, *J* = 17.7, 3.9 Hz, 1H, –CH_2–), 3.17 dd, *J* = 17.7, 7.5 Hz, 1H, –CH_2–), 3.63 (m, 1H, –CH_2–CH═), 3.75 (m, 1H, H-5), 3.82 (s, 3H, COOCH_3), 4.11 (d. *J* = 9.9 Hz, 1H, H-4), 4.22 (dd, *J* = 12.3, 7.2 Hz, 1H, H-9), 4.33 (dd, *J* = 10.2, 1.5 Hz, 1H, H-6), 4.61 (d, *J* = 6.3 Hz, 1H, ═CH-Ph), 4.81 (dd, *J* = 12.6, 2.4 Hz, 1H, H-9′), 5.36 (m, 1H, H-8), 5.46 (dd, *J* = 4.5, 1.8 Hz, 1H, H-7), 5.73 (d, *J* = 9.0 Hz, 1H, NH), 7.24 (br s, 4H, Ph); LRMS [$C_{28}H_{35}N_5O_{10}$] (+ve ion mode) (*m*/*z*): 602 [M+H]$^+$.

3.1.7. 5-Acetamido-2,6-anhydro-3,5-dideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonic acid (20)

Compound **19** (78 mg, 0.14 mmol) was deprotected according to the general procedure for 16 h. The crude product was purified by RP-HPLC [column, Synergi; isocratic elution with 25% MeCN in water (0.05% TFA); flow rate 3 mL min^{-1}; column temperature 40 °C: retention time 16.58–19.30 min] and then lyophilised to give the title compound **20** as a white solid (52 mg, 90%). R_f = 0.4 (EtOAc/MeOH/H_2O, 7:2:1); ^{1}H NMR (300 MHz, D_2O): δ 2.07 (s, 3H, NHCOCH_3), 2.33 (s, 3H, *p*-tolyl CH_3), 3.12 (dd, *J* = 14.4, 7.2 Hz, 1H, –CH_2–), 3.48 (m, 1H, –CH_2–), 3.46–3.51 (m, 2H, H-7, H-9), 3.62–3.71 (m, 2H, H-8, H-9′), 4.18–4.21 (m, 3H, H-4, H-5, H-6), 6.29 (m, 1H, –CH═), 6.50 (d, *J* = 15.9 Hz, 1H, ═CH-Ph), 7.23 (d, *J* = 7.5 Hz, 2H, Ph), 7.38 (d, *J* = 7.5 Hz, 2H, Ph); ^{13}C NMR (125 MHz, D_2O): δ 20.1 (*p*-tolyl CH_3), 21.7 (NHCOCH_3), 34.0 (–CH_2–), 47.5 (C-5), 62.1 (C-9), 71.6 (C-6, C-7), 77.4 (C-4, C-8), 119.0 (C-3), 125.3 (–CH═), 126.1 (Ph), 129.3 (Ph), 132.0 (═C*H*-Ph), 134.3 (Ph q carbon), 137.8 (Ph q carbon), 141.7 (C-2), 171.0 (C-1), 173.3 (NH$CO$$CH_3$); LRMS [$C_{21}H_{27}NO_8$] (*m*/*z*): (+ve íon mode) 444 [M+Na]$^+$, (−ve íon mode) 420 [M−H]$^-$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{21}H_{27}NO_8Na_1$ 444.1629; found: 444.1642.

3.1.8. Methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-3-*C*-[3'-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*galacto*-non-2-enonate (21)

A mixture of 4-hydroxy derivative **19** (1.23 g, 2.2 mmol) and diphenylphosphoryl azide (0.58 mL, 2.70 mmol) were dissolved in anhydrous benzene (26 mL) under N_2. The mixture was cooled to 5–10 °C and DBU (0.4 mL, 2.70 mmol) was added under nitrogen. The reaction mixture was stirred at this temperature for 15 min and then ice bath was removed, reaction was continued at room temperature. After stirring for 5 h at room temperature, additional amounts of diphenylphosphoryl azide (120 μL, 0.54 mmol) and DBU (80 μL, 0.54 mmol) were added and stirring was continued for 2 h. To the mixture was added ethyl acetate (20 mL) and 1 N HCl (5 mL). After stirring for a few minutes, the organic layer was separated, rewashed with 1 N HCl (4 mL), dried (Na_2SO_4), filtered, evaporated to dryness. The crude product was purified by column chromatography on silica (EtOAc/hexane) to yield the title compound **21** as a white foam (1.09 g, yield 85%). R_f = 0.40 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.94, 2.02, 2.04, 2.11 (4× s, 12H, NHCOCH_3, OCOCH_3 ×3), 2.30 (s, 3H, *p*-tolyl CH_3), 3.15 (dd, *J* = 14.7, 8.1 Hz, 1H, –CH_2–), 3.76 (m, 1H, –CH_2–), 3.78 (s, 3H, COOCH_3), 4.11 (m, 2H, H-5, H-9), 4.24 (d, *J* = 8.4 Hz, 1H, H-4), 4.30 (dd, *J* = 9.6, 2.7 Hz, 1H, H-6), 4.60 (dd, *J* = 12.6, 3.0 Hz, 1H, H-9′), 5.26 (m, 1H, H-8), 5.42 (dd, *J* = 5.1, 2.7 Hz, 1H, H-7), 5.62 (d, *J* = 9.0 Hz, 1H, NH), 6.06 (m, 1H, –CH═), 6.46 (d, *J* = 15.6 Hz, 1H, ═CH-Ph), 7.08 (d, *J* = 7.8 Hz, 2H, Ph), 7.23 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.7, 20.8, 20.9, 21.1 (NHCOCH_3,

OCOCH_3 ×3), 23.2 (*p*-tolyl CH_3), 31.3 –CH_2–), 49.1 (C-5), 52.3 (COOCH_3), 62.0 (C-4), 62.1 (C-9), 67.6 (C-7), 71.0 (C-8), 75.5 (C-6), 120.7 (C-3), 125.1 (–CH=), 126.1 (Ph), 129.2 (Ph), 132.4 (=*CH*-Ph), 134.3 (Ph q carbon), 137.2 (Ph q carbon), 141.1 (C-2), 162.3 (C-1), 170.2, 170.3, 170.4, 170.5 (NH*CO*CH_3, O*CO*CH_3 ×3); LRMS [C_{28} $H_{34}N_4O_{10}$] (+ve ion mode) (*m*/*z*): 609 $[M+Na]^+$; HRMS (ESI) (*m*/*z*): $[M+Na]^+$ calcd for $C_{28}H_{34}N_4NaO_{10}$ 609.2167; found: 609.2163; ν_{max}/cm^{-1} 2100, 1743, 1660, 1370, 1215, 1040.

3.1.9. 5-Acetamido-4-amino-2,6-anhydro-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*galacto*-non-2-enonic acid (8)

To a solution of azide **21** (100 mg, 0.17 mmol) in dry tetrahydrofuran (5 mL) was added triphenylphosphine (89 mg, 0.34 mmol) at room temperature under N_2. The reaction mixture was stirred for 10 min at 50 °C. To the reaction solution were added water (2 mL) and 25% aqueous sodium hydroxide (13.6 mg, 0.34 mmol) at 50 °C, followed by stirring for 2 h at the same temperature. The reaction solution was cooled to 0 °C, concentrated hydrochloric acid (100 μL) was added and the mixture was allowed to stand for 10 min. Subsequently, the aqueous layer was separated and solvent was evaporated to dryness. To remove the formed triphenylphosphane oxide and less polar impurities, the crude product was chromatographed over 10 g of Florisil with dichloromethane/methanol/0.2 M ammonium hydroxide in water (4:4:1). The crude product was purified by RP-HPLC [column, Synergi; isocratic elution with 20% MeCN in water; flow rate 3 mL min^{-1}; column temperature 36 °C: retention time 12.50–16.48 min] and then lyophilised to give the title compound **8** as white solid (52 mg, 72% over 2 steps). R_f = 0.35 (1-propanol/H_2O/acetic acid, 15:4:0.5); ^1H NMR (300 MHz, D_2O): δ 2.04 (s, 3H, NHCOCH_3), 2.33 (s, 3H, *p*-tolyl CH_3) 3.04 (dd, *J* = 16.2, 7.2 Hz, 1H, –CH_2–), 3.40 (dd, *J* = 16.5, 5.7 Hz, 1H, –CH_2–), 3.64–3.70 (m, 2H, H-7, H-9), 3.87–3.95 (m, 2H, H-8, H-9′), 4.05 (d, *J* = 8.4 Hz, 1H, H-4), 4.26 (m, 1H, H-6), 4.40 (dd, *J* = 9.6, 8.7 Hz, 1H, H-5), 6.25 (m, 1H, –CH=), 6.57 (d, *J* = 15.9 Hz, 1H, =*CH*-Ph), 7.24 (d, *J* = 8.1 Hz, 2H, Ph), 7.39 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, D_2O): δ 20.0 (*p*-tolyl CH_3), 21.9 (NHCOCH_3), 29.6 (–CH_2–), 48.3 (C-5), 51.6 (C-4), 62.9 (C-9), 68.2 (C-7), 69.5 (C-8), 74.9 (C-6), 105.3 (C-3), 126.0 (Ph), 126.3 (–CH_2–*CH*=), 129.4 (Ph), 131.0 (=*CH*Ph), 134.2 (Ph q carbon), 137.9 (Ph q carbon), 149.5 (C-2), 170.6 (C-1), 174.7 (NH*CO*CH_3); LRMS [$C_{21}H_{28}N_2O_7$] (−ve íon mode) (*m*/*z*): 419 $[M-H]^-$; HRMS (ESI) (*m*/*z*): $[M+Na]^+$ calcd for $C_{21}H_{28}N_2O_7Na_1$ 443.1789; found: 443.1787.

3.1.10. Methyl 5-acetamido-2,6-anhydro-4-azido-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*galacto*-non-2-enonate (23)

To a solution of compound **21** (440 mg, 0.72 mmol) in anhydrous methanol (15 mL) at 5 °C was added 1 M sodium methoxide solution (0.74 mL, 0.72 mmol) under N_2. The reaction mixture was stirred at room temperature for 1 h and the progress of the reaction was monitored by TLC analysis. After complete consumption of starting material Amberlite® IR-120 (H^+) resin was added to adjust pH 6.5, the reaction mixture was filtered, the resin was washed with methanol (30 mL) and the filtrate was concentrated to dryness under vacuum. The crude product was purified by column chromatography on silica to yield the title compound **23** as a white foam (310 mg, yield 90%). R_f = 0.45 (MeOH/EtOAc, 0.5:10); ^1H NMR (300 MHz, CD_3OD): δ 2.00 (s, 3H, NHCOCH_3), 2.30 (s, 3H, *p*-tolyl CH_3), 3.16 (dd, *J* = 15.0, 8.4 Hz, 1H, –CH_2–), 3.56 (d, *J* = 9.3 Hz, 1H, H-4), 3.63–3.87 (m, 8H, COOCH_3, H-5, H-6, H-9, H-9′, 1H –CH_2–), 4.16 (m, 1H, H-8), 4.27 (m, 1H, H-7), 6.14 (m, 1H, –CH=), 6.50 (d, *J* = 15.9 Hz, 1H, =*CH*-Ph), 7.09 (d, *J* = 8.1 Hz, 2H, Ph), 7.25 (d, *J* = 8.1 Hz, 2H, Ph); LRMS [$C_{22}H_{28}N_4O_7$] (+ve íon mode) (*m*/*z*): 483 $[M+Na]^+$.

3.1.11. Methyl 5-acetamido-2,6-anhydro-4-amino-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*galacto*-non-2-enonate (24)

H_2S gas was gently bubbled into a stirred mixture of the azide compound **23** (300 mg, 0.65 mmol) in anhydrous pyridine (10 mL) at room temperature for 16 h. The mixture was flushed with nitrogen for 10 min and then evaporated to dryness keeping the bath temperature below 40 °C. The residue was purified by column chromatography on silica (EtOAc/MeOH/Et_3N, 25:10:1) to afford title compound **24** as an off-white solid (226 mg, 80%). R_f = 0.4 (MeOH/EtOAc, 3:7); ^1H NMR (300 MHz, CD_3OD): δ 2.00 (NHCOCH_3), 2.30 (s, 3H, *p*-tolyl CH_3), 3.21 (dd, *J* = 15.3, 8.1 Hz, 1H, –CH_2–), 3.54–4.04 (m, 11H, COOCH_3, H-4, H-5, H-6, H-7, H-8, H-9, H-9′, 1H –CH_2–), 6.17 (m, 1H, –CH=), 6.51 (d, *J* = 15.6 Hz, 1H, =*CH*-Ph), 7.08 (d, *J* = 8.1 Hz, 2H, Ph), 7.24 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, CD_3OD): δ 21.2 (*p*-tolyl CH_3), 22.7 (NHCOCH_3), 31.6 (–CH_2–), 52.5 (COOCH_3), 52.7 (C-5), 53.3 (C-4), 64.9 (C-9), 70.5 (C-8), 71.3 (C-7), 77.7 (C-6), 123.6 (C-3), 127.0 (Ph), 127.5 (–CH=), 129.9 (Ph), 132.7 (=*CH*-Ph), 136.0 (Ph q carbon), 138.1 (Ph q carbon), 142.5 (C-2), 165.2 (C-1), 175.1 (NH*CO*CH_3); LRMS [$C_{22}H_{30}N_2O_7$] (−ve ion mode) (*m*/*z*): 433 $[M-H]^-$; HRMS (ESI) (*m*/*z*): $[M+Na]^+$ calcd for $C_{22}H_{29}N_2O_7$ 433.1980; found: 433.1978.

3.1.12. Methyl 5-acetamido-2,6-anhydro-4-[2,3-bis(*tert*-butoxycarbonyl)guanidino]-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*galacto*-non-2-enonate (25)

A solution of **24** (120 mg, 0.28 mmol), triethylamine (108 μL, 0.86 mmol) and [(*tert*-butoxycarbonyl)imino]pyrazol-1-ylmethylcarbamic acid *tert*-butyl ester (bisBocPCH; 128 mg, 0.41 mmol) in a mixture of dry tetrahydrofuran (10 mL) and dry methanol (3 mL) was stirred under nitrogen at room temperature for 7 days. The progress of the reaction was monitored by TLC analysis. Then the reaction mixture was evaporated to dryness and the crude product was purified by column chromatography on silica (EtOAc/MeOH) to yield the title compound **25** as a white solid (134 mg, 72%). R_f = 0.8 (MeOH/EtOAc, 1:9); ^1H NMR (300 MHz, $CDCl_3$): δ 1.40, 1.48 (2× s, 18H, NBoc), 2.01 (s, 3H, NHCOCH_3), 2.30 (s, 3H, *p*-tolyl CH_3), 3.19 (dd, *J* = 14.7, 6.6 Hz, 1H, –CH_2–), 3.48 (dd, *J* = 14.7, 6.3 Hz, 1H, –CH_2–), 3.62 (d, *J* = 8.1 Hz, 1H, H-7), 3.79–3.87 (m, 4H, $COOCH_3$, H-9), 3.91-3.95 (m, 2H, H-8, H-9′), 4.07–4.17 (m, 2H, H-5, H-6), 5.24 (br s, 1H, NH), 5.41 (m, 1H, H-4), 6.09 (m, 1H, =CH–), 6.30 (d, *J* = 15.9 Hz, 1H, =*CH*-Ph), 7.07 (d, *J* = 8.1 Hz, 2H, Ph), 7.21 (d, *J* = 8.1 Hz, 2H, Ph), 8.33 (br s, 1H, NH), 8.90 (br s, 1H, NH); LRMS [$C_{33}H_{48}N_4O_{11}$] (+ve íon mode) (*m*/*z*): 699 $[M+Na]^+$.

3.1.13. 5-Acetamido-2,6-anhydro-3,4,5-trideoxy-4-guanidino-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*galacto*-non-2-enonic acid (9)

To a solution of compound **25** (60 mg, 0.09 mmol) in anhydrous dichloromethane (10 mL) at 5 °C was added trifluoroacetic acid (2 mL) under N_2. The reaction mixture was stirred at room temperature for 5 h. The progress of the reaction was monitored by TLC analysis (R_f = 0.1; MeOH/EtOAc, 3:7). The solvent was removed under vacuum. The crude product was deprotected according to the general procedure for 16 h. The crude product was purified by RP-HPLC [column, Synergi; isocratic elution with 15% MeCN in water; flow rate 3 mL min^{-1}; column temperature 36 °C: retention time 6.50–8.32 min] and then lyophilised to give the title compound (**9**) as a white solid (34 mg, 83% over 2 steps). R_f = 0.35 (1-propanol/H_2O/acetic acid, 15:4:0.5); ^1H NMR (300 MHz, D_2O): δ 2.00 (s, 3H, NHCOCH_3), 2.33 (s, 3H, *p*-tolyl CH_3), 3.00 (dd, *J* = 15.3, 8.7 Hz, 1H, –CH_2–), 3.31 (dd, *J* = 15.0, 4.2 Hz, 1H, –CH_2–), 3.62–3.68 (m, 2H, H-7, H-9), 3.86–3.93 (m, 2H, H-8, H-9′), 4.26–4.39 (m, 3H, H-4, H-5, H-6), 6.18 (m, 1H, –CH=), 6.46 (d, *J* = 15.9 Hz, 1H, =*CH*-Ph), 7.24 (d, *J* = 8.1 Hz, 2H, Ph), 7.36 (d, *J* = 8.1 Hz, 2H,

Ph); ^{13}C NMR (75.5 MHz, D_2O): δ 20.1 (*p*-tolyl CH_3), 21.8 (NHCOCH_3), 31.2 (–CH_2–), 53.1 (C-5), 62.9 (C-9), 68.1 (C-7), 69.6 (C-8), 75.1 (C-6), 106.6 (C-3), 126.0 (Ph), 126.7 (–CH_2–*CH*═), 129.3 (Ph), 131.4 (═*CH*-Ph), 134.4 (Ph q carbon), 137.7 (Ph q carbon), 148.1 (C-2), 157.0 (C-1), 170.8 (NH*CO*CH_3), (C-4 not observed). LRMS [$C_{22}H_{30}N_4O_7$] (*m*/*z*): (+ve ion mode) 463 [M+H]$^+$, (−ve ion mode) 461 [M−H]$^-$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{22}H_{30}N_4O_7Na_1$ 485.2007; found: 485.2014.

3.1.14. Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-3,5-dideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonate (26)

To a solution of compound **19** (2 g, 3.55 mmol) in anhydrous pyridine (10 mL) was added acetic anhydride (5 mL) and 4-(dimethylamino)pyridine (43.5 mg, 1 mol %) at room temperature under N_2. The reaction mixture was stirred at room temperature for 16 h (complete disappearance of starting material by TLC analysis). The reaction mixture was evaporated to dryness, taken up in ethyl acetate (50 mL) and washed successively with 0.1 N HCl, H_2O, and aq NaCl. The organic phase was dried (anhydrous Na_2SO_4), filtered and evaporated under reduced pressure, and the residue purified by flash column chromatography on silica gel (EtOAc/hexane, 4:1) to afford the title compound **26** as a white solid (2.06 g, 96%). R_f = 0.5 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.86, 2.03, 2.04, 2.06, 2.07 (5× s, 15H, NHCOCH_3, OCOCH_3 ×4), 2.29 (s, 3H, *p*-tolyl CH_3), 3.09 (dd, *J* = 15.3, 7.2 Hz, 1H, –CH_2–), 3.34 (dd, *J* = 15.3, 6.6 Hz, 1H, –CH_2–), 3.80 (s, 3H, $COOCH_3$), 4.10–4.18 (m, 2H, H-6, H-9), 4.51 (ddd, *J* = 11.1, 4.2, 3.9 Hz, 1H, H-5), 4.78 (dd, *J* = 12.3, 2.4 Hz, 1H, H-9′), 5.26-5.30 [m, 2H, H-8, NH (D_2O exchanged)], 5.46 (dd, *J* = 4.5, 2.4 Hz, 1H, H-7), 5.56 (d, *J* = 3.9 Hz, 1H, H-4), 6.05 (m, 1H, –CH═), 6.36 (d, *J* = 15.6 Hz, 1H, ═*CH*-Ph), 7.06 (d, *J* = 7.8 Hz, 2H, Ph), 7.21 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.7, 20.8, 20.9, 21.0, 21.1 (NHCOCH_3, OCOCH_3 ×4), 23.1 (*p*-tolyl CH_3), 32.8 (–CH_2–), 44.9 (C-5), 52.3 (COOCH_3), 62.3 (C-9), 67.1 (C-4), 67.6 (C-7), 71.9 (C-8), 73.0 (C-6), 118.7 (C-3), 125.2 (–CH═), 126.1 (Ph), 129.1 (Ph), 131.7 (═*CH*-Ph), 134.4 (Ph q carbon), 137.0 (Ph q carbon), 141.8 (C-2), 162.4 (C-1), 169.6, 170.0, 170.2, 170.5, 170.6 (NH*CO*CH_3, O*CO*CH_3 ×4); LRMS [$C_{30}H_{37}NO_{12}$] (+ve mode) (*m*/*z*): 626 [M+Na]$^+$.

3.1.15. Methyl 5-acetamido-7,8,9-tri-*O*-acetyl-2,6-anhydro-4-azido-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonate (27)

To a mixture of compound **26** (1.2 g, 2 mmol) in tetrahydrofuran (28.8 mL) and H_2O (12 mL) was added NaN_3 (258 mg, 4 mmol) and tetrakis(triphenylphosphine)palladium(0) (114 mg, 5 mol %) under an atmosphere of nitrogen. The resulting mixture was stirred at 50 °C for 16 h (complete disappearance of starting material by TLC analysis). The reaction mixture was concentrated to dryness, and the residue was partitioned between diethyl ether (25 mL) and H_2O (10 mL). The organic layer was washed successively with 2 N HCl (2 × 10 mL), saturated $NaHCO_3$ solution (2 × 10 mL) and H_2O (2 × 10 mL), and dried (Na_2SO_4). After solvent removal the residue was purified by column chromatography on silica (EtOAc/hexane, 3:2) to afford title compound **27** as a white solid (960 mg, 82%). R_f = 0.6 (EtOAc/hexane, 4:1); IR ν_{max}/cm^{-1}: 2100, 1732, 1370, 1213, 1043, 909, 730; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.94, 2.04, 2.05, 2.06 (4× s, 12H, NHCOCH_3, OCOCH_3 ×3), 2.30 (s, 3H, *p*-tolyl CH_3), 3.05 (dd, *J* = 15.0, 8.4 Hz, 1H, –CH_2–), 3.75 (dd, *J* = 15.0, 5.7 Hz, 1H, –CH_2–), 3.80 (s, 3H, $COOCH_3$), 4.02–4.13 (m, 3H, H-4, H-6, H-9), 4.47 (ddd, *J* = 10.8, 4.2, 4.2 Hz, 1H, H-5), 4.68 (dd, *J* = 12.3, 2.4 Hz, 1H, H-9′), 5.26 (m, 1H, H-8), 5.43 (dd, *J* = 5.1, 2.1 Hz, 1H, H-7), 5.64 (d, *J* = 10.2 Hz, 1H, NH), 6.07 (m, 1H, –CH═), 6.43 (d, *J* = 15.6 Hz, 1H, ═*CH*-Ph), 7.08 (d, *J* = 7.8 Hz, 2H, Ph), 7.23 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.7, 20.8, 20.9, 21.1 (NHCOCH_3, OCOCH_3 ×3), 23.2 (*p*-tolyl CH_3), 33.5 (–CH_2–), 45.4 (C-5), 52.4 (COOCH_3), 59.7 (C-4), 62.1 (C-9), 67.4 (C-7), 71.3 (C-8), 72.7 (C-6), 118.3 (C-3), 124.5 (–CH═), 126.2 (Ph), 129.2 (Ph), 132.8 (═*CH*-Ph), 134.0 (Ph q carbon), 137.3 (Ph q carbon), 141.1 (C-2), 162.4 (C-1), 169.8, 170.0, 170.2, 170.6 (NH*CO*CH_3, O*CO*CH_3 ×3); LRMS [$C_{28}H_{34}N_4O_{10}$] (+ve ion mode) (*m*/*z*): 609 [M+Na]$^+$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{28}H_{34}N_4NaO_{10}$ 609.2167; found: 609.2159.

3.1.16. Methyl 5-acetamido-2,6-anhydro-4-azido-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonate (28)

To a solution of compound **27** (350 mg, 0.60 mmol) in anhydrous methanol (15 mL) at 5 °C was added 1 M sodium methoxide solution (0.59 mL, 0.60 mmol) under N_2. The reaction mixture was stirred at room temperature for 2 h and the progress of the reaction was monitored by TLC analysis. After complete consumption of starting material Amberlite® IR-120 (H^+) resin was added to adjust pH 6.5, the reaction mixture was filtered, the resin was washed with methanol (30 mL) and the filtrate was concentrated to dryness under vacuum. The crude product was purified by column chromatography on silica to yield the title compound **28** as a white foam (247 mg, yield 90%). R_f = 0.45 (MeOH/EtOAc, 0.5:10); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.07 (s, 3H, NHCOCH_3), 2.31 (s, 3H, *p*-tolyl CH_3), 2.98 (dd, *J* = 15.0, 8.7 Hz, 1H, –CH_2–), 3.57 (dd, *J* = 9.0, 1.2 Hz, 1H, H-7), 3.77–3.90 (m, 6H, H-9, H-9′, 1H –CH_2–, $COOCH_3$), 3.97–4.01 (m, 2H, H-6, H-8), 4.09 (d, *J* = 4.2 Hz, 1H, H-4), 4.27 (m, 1H, H-5), 6.07 (m, 1H, –CH═), 6.19 (d, *J* = 8.4 Hz, 1H, NH), 6.42 (d, *J* = 15.9 Hz, 1H, ═*CH*-Ph), 7.09 (d, *J* = 7.8 Hz, 2H, Ph), 7.23 (d, *J* = 8.1 Hz, 2H, Ph); LRMS [$C_{22}H_{28}N_4O_7$] (+ve ion mode) (*m*/*z*): 483 [M+Na]$^+$.

3.1.17. Methyl 5-acetamido-2,6-anhydro-4-amino-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonate (29)

H_2S gas was gently bubbled into a stirred mixture of the azide compound **28** (240 mg, 0.52 mmol) in anhydrous pyridine (10 mL) at room temperature for 16 h. The mixture was flushed with nitrogen for 10 min and then evaporated to dryness keeping the bath temperature below 40 °C. The residue was purified by column chromatography on silica (EtOAc/MeOH/Et_3N, 25:10:1) to afford the title compound **29** as an off-white solid (190 mg, 84%). R_f = 0.4 (MeOH/EtOAc, 3:7); LRMS [$C_{22}H_{30}N_2O_7$] (*m*/*z*): (+ve ion mode) 457 [M+Na]$^+$, (−ve ion mode) 433 [M−H]$^-$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{22}H_{30}N_2NaO_7$ 457.1945; found: 457.1936.

3.1.18. 5-Acetamido-2,6-anhydro-4-amino-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonic acid (10)

Compound **29** (100 mg, 0.23 mmol) was deprotected according to the general procedure for 16 h. The crude product was purified by RP-HPLC [column, Synergi; isocratic elution with 25% MeCN in water (0.05% TFA); flow rate 3 mL min^{-1}; column temperature 40 °C: retention time 10.80–12.39 and 14.22–18.67 min] and then lyophilised to give the title compound **10** as a white solid (65 mg, 68%) and impurity **30** (23 mg, 24%). R_f = 0.4 (1-propanol/H_2O/acetic acid, 15:4:0.5); **10**: ^{1}H NMR (300 MHz, D_2O): δ 2.08 (s, 3H, NHCOCH_3), 2.33 (s, 3H, *p*-tolyl CH_3), 3.03 (dd, *J* = 16.2, 7.5 Hz, 1H, –CH_2–), 3.38 (dd, *J* = 16.2, 4.8 Hz, 1H –CH_2–), 3.66–3.72 (m, 2H, H-7, H-9), 3.88–4.05 (m, 3H, H-4, H-8, H-9′), 4.48 (apparent s, 2H, H-5, H-6), 6.24 (m, 1H, –CH═), 6.52 (m, 1H, ═*CH*-Ph), 7.24 (d, *J* = 8.1 Hz, 2H, Ph), 7.39 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, D_2O + CD_3CN): δ 21.7 (*p*-tolyl CH_3), 21.8 (NHCOCH_3), 31.2 (–CH_2–), 45.2 (C-5), 46.7 (C-4), 62.9 (C-9), 68.0 (C-7), 69.4

(C-8), 69.6 (C-6), 125.8 (Ph), 125.9 (Ph), 126.4 (–CH_2–*CH*═), 128.9 (Ph), 129.0 (Ph), 131.1 (═*CH*-Ph), 134.2 (Ph q carbon), 136.9 (Ph q carbon), 149.5 (C-2), 169.7 (C-1), 173.3 (NH*CO*CH_3), (C-3 not observed). LRMS [$C_{21}H_{28}N_2O_7$] (*m*/*z*): (+ve íon mode) 443 [M+Na]$^+$, (−ve íon mode) 419 [M−H]$^-$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{21}H_{28}N_2O_7Na_1$ 443.1789; found: 443.1802.

3.1.19. 4-Acetamido-2,6-anhydro-5-amino-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonic acid (30)

^{1}H NMR (600 MHz, D_2O): δ 1.84 (s, 3H, NHCO*CH*$_3$), 2.29 (s, 3H, *p*-tolyl *CH*$_3$), 3.05 (dd, *J* = 15.0, 7.8 Hz, 1H, –CH_2–), 3.15 (dd, *J* = 15.0, 6.6 Hz, 1H, –CH_2–), 3.65–3.71 (m, 3H, H-5, H-7, H-9), 3.87 (dd, *J* = 12.0, 2.4 Hz, 1H, H-9′), 3.95 (m, 1H, H-8), 4.15 (d, *J* = 10.8 Hz, 1H, H-6), 4.68 (d, *J* = 4.2 Hz, 1H, H-4), 6.18 (m, 1H, –CH═), 6.40 (d, *J* = 15.6 Hz, 1H, ═*CH*-Ph), 7.19 (d, *J* = 7.8 Hz, 2H, Ph), 7.33 (d, *J* = 7.8 Hz, 2H, Ph); ^{13}C NMR (125 MHz, D_2O + CD_3CN): δ 20.6 (*p*-tolyl C*H*$_3$), 22.1 (NHCO*CH*$_3$), 33.0 (–CH_2–), 45.9 (C-5), 49.1 (C-4), 63.1 (C-9), 67.9 (C-7), 69.9 (C-8), 70.2 (C-6), 106.1 (C-3), 126.4 (Ph), 127.3 (–CH_2–*CH*═), 129.6 (Ph), 131.1 (═*CH*-Ph), 134.9 (Ph q carbon), 137.5 (Ph q carbon), 147.9 (C-2), 170.0 (C-1), 175.0 (NH*CO*CH_3). LRMS [$C_{21}H_{28}N_2O_7$] (*m*/*z*): (+ve ion mode) 443 [M+Na]$^+$, (−ve ion mode) 419 [M−Na]$^-$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{21}H_{28}N_2O_7Na_1$ 443.1789; found: 443.1808.

3.1.20. Methyl 5-acetamido-2,6-anhydro-4-[2,3-bis(*tert*-butoxycarbonyl)guanidino]-3,4,5-trideoxy-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonate (31)

A solution of **29** (241 mg, 0.55 mmol), triethylamine (217 μL, 0.86 mmol) and [(*tert*-butoxycarbonyl)imino]pyrazol-1-ylmethylcarbamic acid *tert*-butyl ester (bisBocPCH; 344 mg, 1.11 mmol) in a mixture of dry tetrahydrofuran (10 mL) and dry methanol (3 mL) was stirred under nitrogen at room temperature for 3 days. The progress of the reaction was monitored by TLC analysis. The reaction mixture was evaporated to dryness and the crude product was purified by column chromatography on silica (EtOAc/MeOH) to yield the title compound **31** as a white solid (318 mg, 85%). R_f = 0.8 (MeOH/EtOAc, 1:9); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H, Boc), 1.45 (s, 9H, Boc), 2.04 (s, 3H, NHCO*CH*$_3$), 2.29 (s, 3H, *p*-tolyl *CH*$_3$), 3.12 (dd, *J* = 15.6, 7.8 Hz, 1H, –CH_2–), 3.46–3.54 (m, 2H, H-5, 1H –CH_2–), 3.79–3.91 (m, 5H, H-9, H-9′, $COOCH_3$), 3.98–4.13 (m, 3H, H-4, H-6, H-8), 4.65 (m, 1H, H-7), 5.67 (s, 1H, Ac*NH*), 6.06 (m, 1H, –CH═), 6.35 (d, *J* = 15.9 Hz, 1H, ═*CH*-Ph), 7.05 (d, *J* = 7.8 Hz, 2H, Ph), 7.18 (d, *J* = 8.1 Hz, 2H, Ph), 8.40 (d, *J* = 6.0 Hz, 1H, C_4–NH), 8.78 (d, *J* = 4.5 Hz, 1H, guanidine *NH*Boc); LRMS [$C_{33}H_{48}N_4O_{11}$] (+ve ion mode) (*m*/*z*): 699 [M+Na]$^+$.

3.1.21. 5-Acetamido-2,6-anhydro-3,4,5-trideoxy-4-guanidino-3-*C*-[3′-(*p*-tolyl)prop-2′enyl]-D-*glycero*-D-*talo*-non-2-enonic acid (11)

To a solution of compound **31** (100 mg, 0.14 mmol) in anhydrous dichloromethane (10 mL) at 5 °C was added trifluoroacetic acid (2 mL) under N_2. The reaction mixture was stirred at room temperature for 5 h. The progress of the reaction was monitored by TLC analysis (R_f = 0.15, MeOH/EtOAc, 3:7). The solvent was removed under vacuum. The resulting crude product was deprotected according to the general procedure for 16 h. The crude product was purified by RP-HPLC [column, Synergi; isocratic elution with 15% MeCN in water; flow rate 3 mL min^{-1}; column temperature 36 °C: retention time 5.80–7.92 min] and then lyophilised to give the title compound **11** as a white solid (54 mg, 80% over 2 steps). R_f = 0.25 (1-propanol/H_2O/acetic acid, 15:4:0.5); ^{1}H NMR (300 MHz, D_2O): δ 2.00 (s, 3H, NHCO*CH*$_3$), 2.32 (s, 3H, *p*-tolyl *CH*$_3$), 3.10 (dd, *J* = 15.6, 7.8 Hz, 1H, –CH_2–), 3.23 (dd, *J* = 15.6, 6.6 Hz, 1H, –CH_2–), 3.60–3.70 (m, 2H, H-7, H-9), 3.87–3.96 (m, 2H, H-8, H-9′), 4.11–4.18 (m, 2H, H-4, H-6), 4.41 (dd, *J* = 11.4, 4.5 Hz, 1H, H-5), 6.21 (m, 1H, –CH═), 6.48 (m, 1H, ═*CH*-Ph), 7.22 (d, *J* = 8.1 Hz, 2H, Ph), 7.37 (d, *J* = 8.1 Hz, 2H, Ph); ^{13}C NMR (75.5 MHz, D_2O): δ 19.9 (*p*-tolyl C*H*$_3$), 21.3 (NHCO*CH*$_3$), 33.1 (–CH_2–), 46.2 (C-5), 48.5 (C-4), 62.7 (C-9), 67.6 (C-7), 69.5 (C-8), 70.1 (C-6), 104.9 (C-3), 125.8 (Ph), 126.3 (–CH_2–*CH*═), 129.0 (Ph), 130.8 (═*CH*-Ph), 134.2 (Ph q carbon), 137.4 (Ph q carbon), 147.8 (C-2), 156.4 (C-10), 170.7 (C-1), 173.6 (NH*CO*CH_3); LRMS [$C_{22}H_{30}N_4O_7$] (*m*/*z*): (+ve ion mode) 463 [M+H]$^+$, (−ve ion mode) 461 [M−H]$^-$; HRMS (ESI) (*m*/*z*): [M+Na]$^+$ calcd for $C_{22}H_{30}N_4O_7Na_1$ 485.2007; found: 485.2004.

3.2. Biological evaluation

Sialidases: Recombinant influenza A virus N1 [A/California/04/2009 (pdm09 H1N1)], and N2 [A/R1/5+/1957 (H2N2)] sialidases were expressed in a baculovirus expression system as previously described,[13] from plasmids kindly provided by George F. Gao (Institute of Microbiology, Chinese Academy of Sciences, Beijing).

Sialidase inhibition assay: Compounds were assayed for their capacity to inhibit influenza virus sialidase catalysed hydrolysis of the fluorogenic substrate 4-methylumbelliferyl *N*-acetyl-α-D-neuraminide (MUN) (Sigma–Aldrich), using a modification[37] of the method of Potier et al.[38] Assays were carried-out in a 96-well plate format using the reported[19] procedure, modified by the addition of pre-incubation of the inhibitor with enzyme, before addition of the substrate. Stock solutions of the substrate MUN, inhibitor and sialidase were prepared in the reaction buffer (50 mM sodium acetate, 6 mM $CaCl_2$, pH 5.5). The reaction mixture with a final volume of 10 μL was prepared in a 96-well solid black plate on ice. All inhibition assays were done in sixplicate over an inhibitor concentration range of 1 mM to 1 nM (10-fold dilutions), with a MUN concentration of 0.1 mM. The sialidase was pre-incubated with the inhibitor for 15 min at room temperature, and then the reaction was started by the addition of the substrate MUN. The components were combined by brief centrifugation at up to 1000 rpm for approximately 10 s. Immediately after centrifugation the reaction was incubated at 37 °C with 900 rpm shaking for 30 min. After incubation at 37 °C for 30 min, the reactions were stopped by the addition of 0.25 mL of 0.2 M glycine buffered with NaOH to pH 10. Neuraminidase activity was determined by spectrofluorometric measurement of the 4-methylumbelliferone released (excitation 365 nm, emission 445 nm) in a Viktor 3 multiplate reader (Perkin Elmer); reading at 1 s per well. Sample measurements were corrected for background fluorescence that was not produced by the enzyme-catalyzed hydrolysis of the substrate, by subtracting a blank sample that contained MUN in the reaction buffer. Data analysis was carried out using SigmaPlot Enzyme Kinetics Module (Systat Software).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.054.

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