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Synthesis of functionalized cyclohexenephosphonates and their inhibitory activity towards bacterial sialidases

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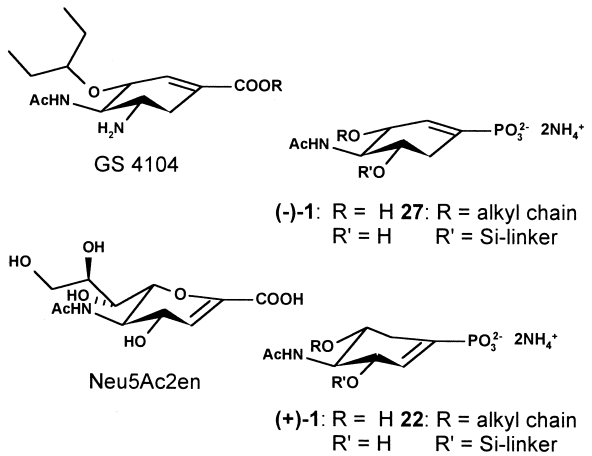
Abstract—We have synthesized a series of cyclohexenephosphonates derived from D- and L-xylose, designed as core structures for the development of high affinity mimics of sialic acid and of the sialidase reaction transition state. Extension of our syntheses to both xylose enantiomers has given us access to two series of cyclohexenephosphonates with regioisomeric double bonds. We have demonstrated the selective functionalization of the hydroxyl groups towards introduction of a glycerol side chain mimic and immobilization via a silyl linker. The inhibitory activity of a selected set of compounds towards three bacterial sialidases has been tested and moderate activity was found.

1. Introduction

The crucial role played by sialic acids in many physiological recognition events has led to increased interest in sialic acid metabolizing enzymes.[1,2] Great achievements have been made in the development of high affinity inhibitors of both the sialidase from influenza virus,[3–6] an enzyme that cleaves terminal sialic acids from the host cell glycocalix in the early stage of viral infection, and of sialyl transferases, enzymes which transfer the sialosyl cation from the natural donor CMP–Neu5Ac to the glycan chain terminus during biosynthesis.[7–9]

Inhibitors of comparable potency targeted towards sialidases from bacteria are still lacking even though some of them have been identified to be involved in infection processes.[2,10,11] Compared to viruses, bacteria have more means of interaction with the host cell glycan chains, which is also reflected in the bifunctionality of some bacterial or protozoal sialidases. These contain, as has been shown by both X-ray crystallography and sequence alignment methods, additional carbohydrate binding sites apart from the active site.[12,13]

This could, at least in principle, be exploited by designing inhibitors that allow attachment of a spacer molecule or an aglycon mimic while keeping in mind that the negative charge, known to be required for effective recognition, should be retained.[14]



Scheme 1. Structures and double bond positions in fully functionalized cyclohexenephosphonates (−)-**1** and (+)-**1** with respect to well-known sialidase inhibitors GS 4104 and Neu5Ac2en.

1.1. Synthetic approach

We have previously synthesized cyclohexenephosphonate (−)-**1**,[14] which can be seen as an analog of the well known influenza drug GS 4104 containing a phosphonate group instead of a carboxylate group (Schemes 1 and 2).

In this paper we report the synthesis of the enantiomer (+)-**1**,[†]

Keywords: sialidase inhibitors; sialic acid; cyclohexenephosphonates; *Vibrio cholerae* sialidase; bacterial sialidases.

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[†] *Numbering of D- and L-xylose derived cyclohexenephosphonates.* Whenever determined, the cyclohexenephosphonates derived from D-xylose showed positive optical rotation and were therefore termed (+)-**1**, (+)-**10**, etc. Consequently, their enantiomers derived from L-xylose showed negative optical rotation and were numbered (−)-**1**, (−)-**10**, etc.

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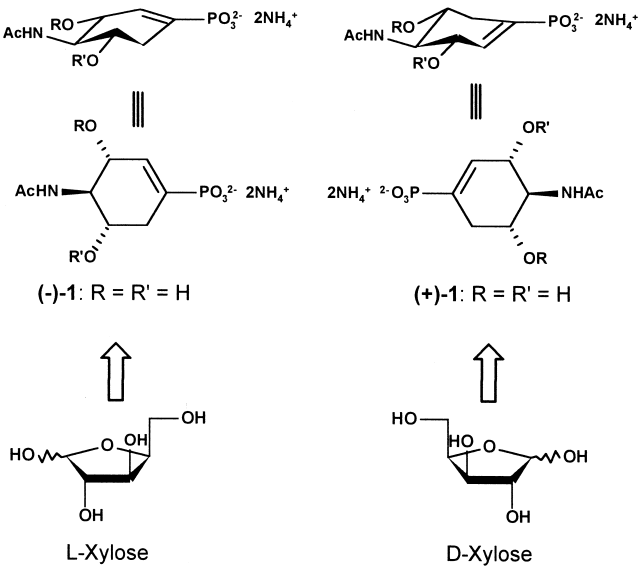
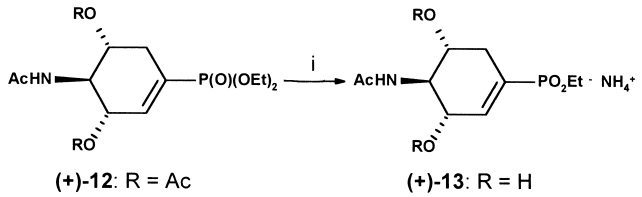


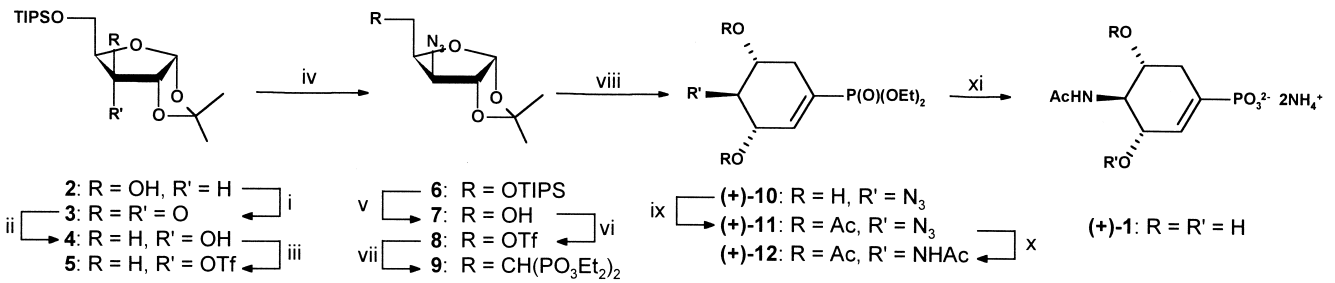
Scheme 2. Synthetic approach to enantiomeric cyclohexenephosphonates (−)-**1** and (+)-**1**, respectively.



Scheme 4. Synthesis of acetamido-cyclohexenephosphonate monoethyl ester (+)-**13**. (i) NaOEt, EtOH then NaOH, Biogel P2.

of **4** into the triflate **5** and nucleophilic substitution to give the *xylo*-configured azide **6**.

Desilylation of **6** gave the known alcohol **7**,[19] which was activated as the triflate **8** to allow substitution with the tetraethyl methylenediphosphonate anion to give the diphosphonate **9**. Removal of the isopropylidene group followed by base-mediated cyclization gave cyclohexene-phosphonate (+)-**10**, which, as well as its enantiomer, serves as a crucial intermediate for selective modifications at the hydroxyl groups (see below). To complete the synthesis of (+)-**1**, the azido-cyclohexenephosphonate (+)-**10** was acetylated ((+)-**11**), the azide moiety was reduced



Scheme 3. Synthesis of cyclohexenephosphonate (+)-**1**, employing the same strategy we reported previously for (−)-**1**. (i) $(COCl)_2$, DMSO (ii) $NaBH_4$, EtOH, (90% for two steps); (iii) Tf_2O, pyridine, CH_2Cl_2; (iv) NaN_3, EtOH, (85% for two steps); (v) TBAF, THF, (96%); (vi) Tf_2O, pyridine, CH_2Cl_2, (86%); (vii) $CH_2((P(O)(OEt)_2)_2$, $LiN(SiMe_3)_2$, DMF, (40%); (viii) IR-120 (H^+), dioxane, H_2O, then $LiN(SiMe_3)_2$ (46%), (ix) Ac_2O, pyridine, (qu); (x) H_2S, pyridine, H_2O then Ac_2O, pyridine, (70%); (xi) TMSBr, $CHCl_3$, then H_2O, Biogel P2.

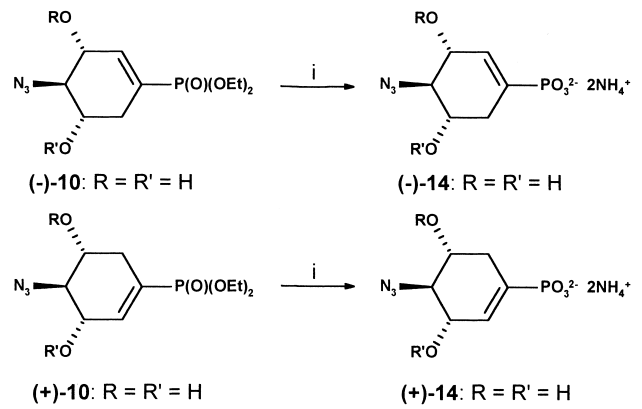
having the double bond in an orientation comparable to that in the classical sialidase inhibitor Neu5Ac2en[15] (Scheme 1). To further optimize both series of inhibitors we focused on (a) the selective introduction of an alkyl side chain mimicking the glycerol moiety in Neu5Ac, which offers possibilities for further optimization and (b) the selective silylation of the position equivalent to the 4-OH in Neu5Ac or Neu5Ac2en thus simulating immobilization on a suitable resin via a silyl linker (compounds **22** and **27**, respectively, Scheme 1). The synthesis of (+)-**1** was accomplished in a straightforward manner by simply shifting the methodology[14,16–18] of the cyclohexenphosphonate synthesis from L-xylose to D-xylose (Scheme 2).

2. Results and discussion

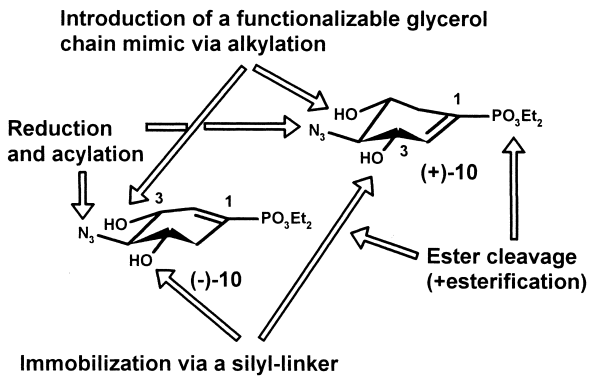
Cyclohexenephosphonate (+)-**1** was synthesized from 1,2-*O*-isopropylidene-5-triisopropylsilyl-D-xylofuranose **2** as described previously for the enantiomeric compound (−)-**1** (Scheme 3).[14,19–21] In brief, the absolute configuration at C-3 was inverted by Swern-oxidation to give the ketone **3** followed by reduction with $NaBH_4$, which furnished D-ribofuranose **4**. The azide was introduced by conversion and acetylated to give the acetamide (+)-**12**. Finally the phosphonate diester was cleaved by treatment with bromotrimethylsilane and hydrolysis which, after gel filtration, led to the ammonioum salt (+)-**1** (Scheme 3).

2.1. Synthesis of a phosphonate–monoester

It is of importance for our concept[14] to show that



Scheme 5. Synthesis of deprotected azido-cyclohexenephosphonates (−)-**14** and (+)-**14**.



Scheme 6. Regioselective modifications of key compounds (−)-**10** and (+)-**10** required to obtain potential sialidase inhibitors.

phosphonate monoesters display at least some inhibition of sialidases. We therefore deprotected and partially de-esterified acetamide (+)-**12** to give monoethyl ester (+)-**13** in almost quantitative yield (Scheme 4).

2.2. Azide-containing inhibitors

In order to assess the significance of the acetamide group, azide containing diphosphonates (+)-**10** and (−)-**10** were hydrolyzed with bromotrimethylsilane and converted into the corresponding ammonium salts (+)-**14** and (−)-**14** (Scheme 5).

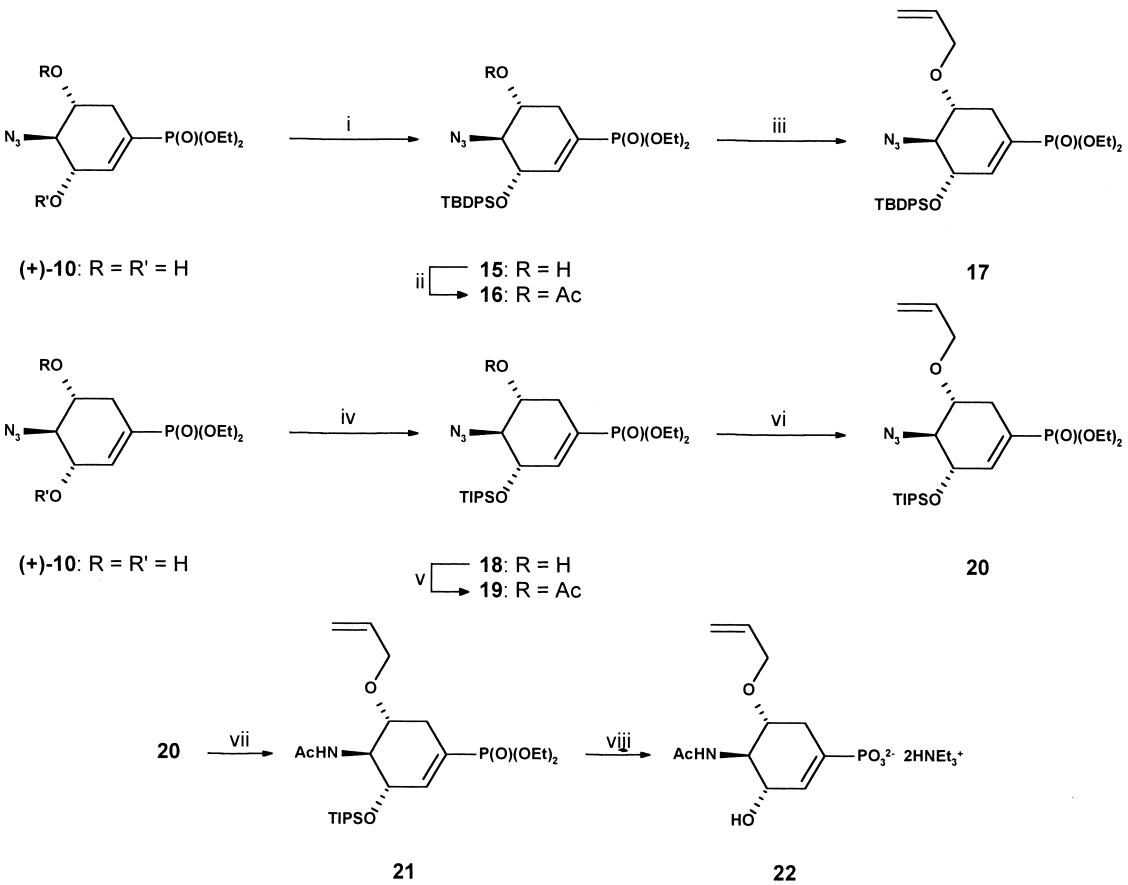
2.3. Differentiation of the hydroxyl groups in the (+)- and (−)-series

To pave the way to optimized inhibitors starting from the azides (+)-**10** and (−)-**10**, reaction sequences had to be established that would allow the selective introduction of an alkyl side chain at the position corresponding to C-6 in Neu5Ac and a silyl group simulating a linker[22] at the position corresponding to C-4 in Neu5Ac.

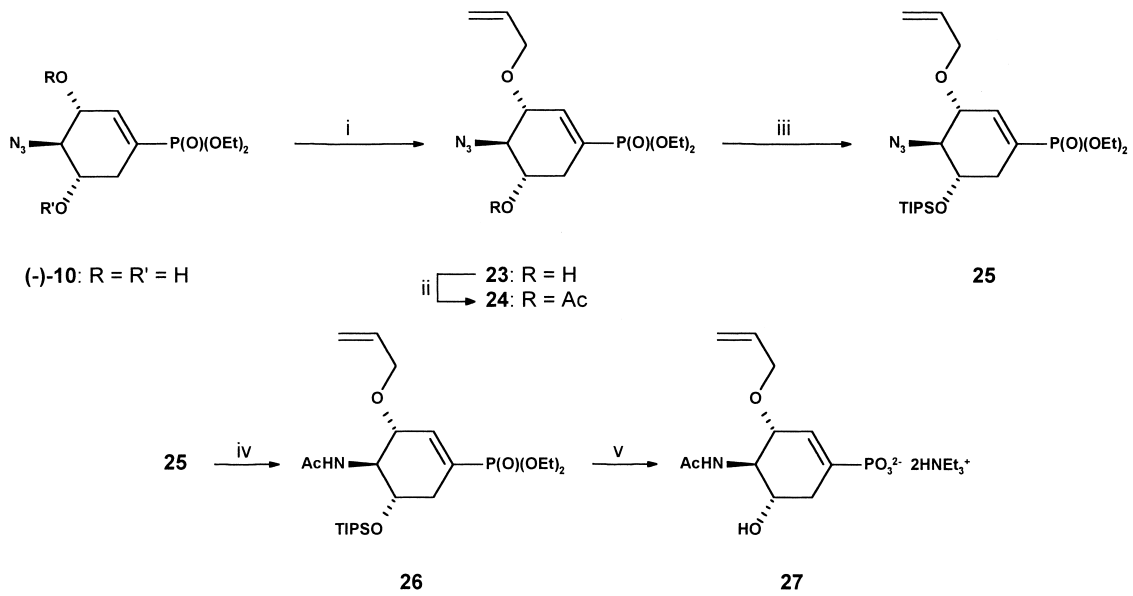
In Scheme 6, the modifications necessary to facilitate inhibitor optimization are depicted: in phosphonate (+)-**10**, the allylic hydroxyl group at C-3 corresponds to 4-OH in Neu5Ac, it therefore should be selectively silylated to allow alkylation at C-5. In the enantiomeric phosphonate (−)-**10**, the allylic hydroxyl group at C-3 occupies the position of the glycerol moiety in Neu5Ac, it therefore should be selectively alkylated followed by silylation (Scheme 6). Finally, reduction and acylation of the azide group as well as ester cleavage and, if required, esterification leads to fully optimized inhibitors. In this report, all reactions were performed in solution to find suitable reaction conditions.

2.4. Modification of azido-cyclohexenephosphonate (+)-10

Initially, we intended to employ a linker system based on an alkyldiphenylsilyl group and therefore we selectively silylated derivative (+)-**10** with *tert*-butyldiphenylsilyl



Scheme 7. Selective modifications and deprotection reactions in the (+)-series. (i) TBDPSCl, imidazole, (58%); (ii) Ac_2O, pyridine, (92%); (iii) allyl bromide, Ag_2O, (45%); (iv) TIPSCl, imidazole, (51%); (v) Ac_2O, pyridine, (94%); (vi) allyl bromide, Ag_2O, TBAI, (85%); (vii) H_2S, pyridine, H_2O then Ac_2O, pyridine (71%); (viii) TMSBr, $CHCl_3$, then H_2O, HPLC-purification.



Scheme 8. Selective modifications and deprotection reactions in the (−)-series. (i) allyl bromide, Ag_2O, TBAI, (70%); (ii) Ac_2O, pyridine, (94%); (iii) TIPSCl, imidazole, (69%); (iv) H_2S, pyridine, H_2O then Ac_2O, pyridine (81%); (v) TMSBr, $CHCl_3$, then H_2O, HPLC-purification.

chloride to give **15**, which was acetylated to confirm the position of the silyl group (Scheme 7). Allylation of **15** to give **17** proceeded in only moderate yield due to migration of the silyl group from C-3 to C-5, which led to a mixture of regioisomers (only the desired compound **17** is shown in Scheme 7).

A similar effect was observed by us previously.[14] To overcome this problem, we enhanced base stability by using the triisopropylsilyl group: silylation of (+)-**10** with triisopropylsilyl chloride gave **18** which again was acetylated to elucidate the position of the silyl group (**19**). No silyl migration was observed when **18** was allylated to give fully functionalized cyclohexenenphosphonate **20**. After reduction of the azide followed by acetylation, acetamide **21** was obtained in good yield and then further treated with bromotrimethylsilane in chloroform and then water. After purification by HPLC, target compound **22** was obtained as its triethylammonium salt.

2.5. Modification of azido-cyclohexenephosphonate (−)-10

As described above, selective alkylation should proceed silylation in the (−)-series. Fortunately, mono-allylation of (−)-**10** to give **23** was accomplished in good yield (Scheme 8), again the regioselectivity of the reaction was confirmed by acetylation of the product to furnish **24**. Although target compound **27** is, of course, directly accessible from **23** or **24**, we silylated **23** and obtained fully derivatized cyclohexenephosphonate **25**. Reduction and acetylation gave acetamide **26** which was, under the conditions described above for the (+)-series, converted into the target compound **27** and isolated as its triethylammonium salt.

2.6. Biological evaluation

We have tested a selected set of our cyclohexenephosphonates towards their ability to inhibit three commercially available sialidases from the pathogenic bacteria *Vibrio cholerae*, *Salmonella typhimurium* and *Clostridium perfringens*. Even though the substances tested still lack a glycerol side chain mimic optimized towards the binding to the respective sialidase we wanted to address several important questions immediately. (1) Does this kind of 4-acetamido cyclohexenephosphonates show activity against sialidases? (2) Is the acetamido group required for recognition by the enzymes? (3) Do the monoethyl- and diethyl esters of the phosphonate group display activity? We performed a well established sialidase inhibition assay[23,24] according to a modification by Schauer and co-workers[25] and extracted IC_{50} values (in M) from the inhibition curves (see Section 4).

The data obtained are given in Table 1.

3. Conclusions

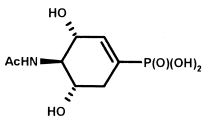
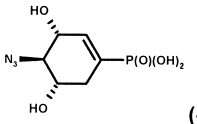
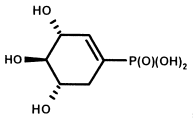
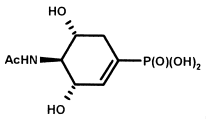
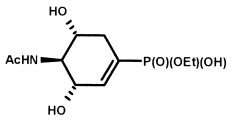
3.1. Syntheses

xylo-Configured cyclohexenephosphonates containing an acetamide group have been synthesized starting from D- and L-xylose, respectively. The molecules are designed to mimic the sialosyl cation intermediate and could therefore serve as scaffolds for the generation of optimized sialidase inhibitors. The D-xylose derived series has its double bond in a position equivalent to the well known inhibitor Neu5Ac2en while in the L-xylose derived series the double bond is in a regioisomeric position, equivalent to the carbocyclic influenza drug GS 4104. Selective alkylations, silylations and deprotection reactions of both series have been demonstrated. We therefore have, after modification of the allyl group, introduction of virtually any acylamide, ester cleavage and aglycon attachment[26] via a monoester, access to an unlimited variety of inhibitors.

3.2. Sialidase inhibition

Both enantiomeric *xylo*-cyclohexenephosphonates (+)-**1**

Table 1. Inhibition of three bacterial sialidases by a selected set of xylose-derived cyclohexenephosphonates and reference inhibitor Neu5Ac2en

Compound	Sialidase		
	V. cholerae	*S. typhimurium*	*C. perfringens*
 **(-)-1**[12]	3×10^{-3}	3×10^{-4}	2×10^{-3}
 **(-)-14**	($>10^{-2}$)	n.t.	n.t.
 **ref. 12**	n.i.	n.i.	n.i.
 **(+)-1**	2×10^{-3}	1×10^{-3}	n.i.
 **(+)-13**	3×10^{-3}	3×10^{-4}	6×10^{-4}
Neu5Ac2en	3×10^{-3}	10^{-4}	4×10^{-5}

IC_{50} values are given in M; n.i., non-inhibitory; n.t., not tested.

and (−)-**1** inhibit the sialidases from *V. cholerae* and *S. typhimurium* in the millimolar range, they can therefore be regarded as useful scaffolds for the further development of high affinity sialidase inhibitors. Only the L-*xylo* derived compound (−)-**1** showed activity towards the sialidase from *C. perfringens* in the concentration range testet. It is, however, in view of the lacking side chain too early to speculate about the reason for this selectivity. More importantly, the very weak respectively the lacking activity of azide (−)-**14** and the corresponding 4-hydroxy derivative[12] indicate that the acetamide group is an important recognition determinant, a well established fact in the field of sialic acid biology.[1] Another finding important for our concept is the activity of monoethyl ester (+)-**13**, thus allowing attachment of aglyca. The importance of a negative charge at this position is underlined by the fact that the diethyl ester corresponding to **13** does not show any inhibition (data not shown).

4. Experimental

4.1. General

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker DRX-600 and a Bruker AC-250 spectrometer, taking the chemical shift of deuterated solvent as standard, except for ^{31}P-spectra, where 85% phosphoric acid was used (0 ppm). ^{13}C chemical shifts were deduced from heteronuclear multiple quantum correlation (HMQC)-spectra. MALDI-MS were recorded on a Kratos Analytic Kompact Maldi 2, using DHB: 3,5-dihydroxybenzoic acid; HCCA: α-hydroxy-α-cyano-cinnamic acid; or ATT: azidothymidine as matrix. FAB-MS were recorded on a modified Finnigan MAT 312/AMD-5000. Reactions were monitored with plastic plates coated with silica gel $60F_{254}$. Solvents for flash chromatography (EE: ethyl acetate, Tol: toluene) were distilled before use. HPLC chromatography devices were from Knauer GmbH, Berlin, Germany equipped with an Shimadzu RI-detector, Shimadzu GmbH, Duisburg, Germany. Chemicals: TIPSCl, triisopropylsilyl chloride; TBDPSCl, *tert*-butyldiphenylsilyl chloride; TMSBr, bromotrimethylsilane; TBAF, tetrabutylammonium fluoride; TBAI, tetrabutylammonium iodide.

4.2. Compounds 2–9

D-Xylofuranose derivatives **2**–**9** were synthesized via the same sequence of reactions previously reported for the corresponding L-xylofuranose derivatives.[14] Conditions and yields are given in Scheme 3.

4.2.1. Diethyl (3*S*,4*S*,5*R*)-4-azido-3,5-dihydroxy-1-cyclohexenephosphonate (+)-10, diethyl (3*S*,4*S*,5*R*)-3,5-diacetoxy-4-azido-1-cyclohexenephosphonate (+)-11 and diethyl (3*S*,4*S*,5*R*)-3,5-di-acetoxy-4-acetamido-1-cyclohexenephosphonate (+)-12. Compounds (+)-**10**, (+)-**11** and (+)-**12** were synthesized according to our previously reported procedures,[14] conditions and yields are given in Scheme 3. NMR- and MS-data were identical to those

previously reported for the (−)-enantiomers. (+)-**10**: $[\alpha]_D^{20}$=15 (*c*=1, $CHCl_3$). (+)-**11**: $[\alpha]_D^{20}$=53 (*c*=0.2, $CHCl_3$). (+)-**12**: $[\alpha]_D^{20}$=60 (*c*=1, $CHCl_3$).

4.2.2. Ammonium [ethyl (3*S*,4*S*,5*R*)-4-acetamido-3,5-dihydroxy-1-cyclohexenephosphonate] (+)-13. Diethyl ester (+)-**12** (18 mg, 46 μmol) is dissolved in dry EtOH (3 mL) and a solution of sodium ethanoate in EtOH is added (1 M, 50 μL). The mixture is stirred overnight, neutralized with Amberlite IR-120 (H^+-form), filtered and concentrated in vacuo. The residue is dissolved in a solution of NaOH in water (3 mL, 0.1 M), strirred for another 3 h, neutralized with Amberlite IR-120 (H^+-form) and the solvent is evaporated. The residue is purified by gel filtration (Biogel P2, 0.1 M NH_4HCO_3) and lyophilized to give (+)-**13** (10 mg, 78%) as its ammonium salt. ^{1}H NMR (600 MHz, D_2O): δ=6.12 (bd, 1H, H-2, $^3J_{2,P}$=19.6 Hz), 4.14 (m, 1H, H-3), 4.15–3.62 (m, 4H, H-4, H-5, CH_2CH$_3$), 2.52 (m, 1H, H-6a), 2.11 (m, 1H, H-6b), 1.95 (s, 3H, COCH_3), 1.14–1.12 (m, 3H, CH$_2$CH_3). ^{13}C NMR (150.9 MHz, D_2O): δ=142.3 (C-2), 71.2 (C-3), 67.9 (C-5), 61.2 (*C*H$_2$CH$_3$), 58.1 (C-4), 33.8 (C-6). ^{31}P NMR (242.9 MHz, D_2O): δ=15.23 (s, 1P, PO_3Et^-). $C_{10}H_{18}NO_6P$ (M 279.1) MALDI-MS (pos. mode, DHB): 302.3 $(M+Na)^+$.

4.2.3. Ammonium (3*R*,4*R*,5*S*)-4-azido-3,5-dihydroxy-1-cyclohexenephosphonate ((−)-14) and ammonium (3*S*,4*S*,5*R*)-4-azido-3,5-dihydroxy-1-cyclohexenephosphonate ((+)-14). Diethyl esters (−)-**10**[12] or (+)-**10** (15 mg, 51 μmol) are dissolved in dry $CHCl_3$ (3 mL) and TMSBr (300 μL) is added. The mixture is stirred for 2 d at rt, evaporated and the residue is taken up in water (3 mL), stirred for additional 2 h and then lyophilized. Compounds (−)-**14** and (+)-**14** are purified by gel filtration (Biogel P2, 0.1 M NH_4HCO_3) to give the respective ammonium salts (~10 mg, 80%). ^{1}H NMR (600 MHz, D_2O): δ=5.92 (bd, 1H, H-2, $^3J_{2,P}$=18.6 Hz), 4.08 (m, 1H, H-3), 3.68 (m, 1H, H-5), 3.32 (dd, 1H, H-4, ^3J=9.5, 10.2 Hz), 2.60 (m, 1H, H-6a), 2.14 (m, 1H, H-6b). ^{13}C NMR (150.9 MHz, D_2O): δ=133.95 (C-1, $^1J_{1,P}$=168 Hz), 131.7 (C-2), 70.9 (C-3), 69.4 (C-4), 68.43 (C-5), 33.0 (C-6). ^{31}P NMR (242.9 MHz, D_2O): δ=11.50 (s, 1P, PO_3^{2-}). $C_6H_8N_3O_5P$ (M 235.2) MALDI-MS (pos. mode, DHB): 257.2 $(M+Na)^+$, (neg. mode, ATT): 234.2 $(M-H)^-$.

4.2.4. Diethyl (3*S*,4*S*,5*R*)-4-azido-5-hydroxy-3-*tert*-butyldiphenylsilyloxy-1-cyclohexenephosphonate (15). Cyclohexenephosphonate (+)-**10** (37 mg, 0.13 mmol) is dissolved in CH_2Cl_2 (3 mL) and cooled to 0°C. Imidazole (35 mg, 0.65 mmol) and TBDPSCl (36 μL, 0.14 mmol) are added, and the mixture is stirred for 1 h, another 1.1 equiv. of TBDPSCl is added and, after additional stirring for 30 min the reaction is quenched by addition of saturated NH_4Cl solution (5 mL). The layers are separated, and the aqueous layer is extracted twice with CH_2Cl_2 (5 mL). The combined organic layers are dried ($MgSO_4$), evaporated and the residue is chromatographed (EE) to yield (**15**) (40 mg, 58%). R_f=0.58 (EE); $[\alpha]_D^{20}$=34 (*c*=1, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): δ=7.73–7.33 (2m, 10H, 2C$_6$H$_5$), 6.18 (bd, 1H, H-2, $^3J_{2,P}$=21.5 Hz), 4.19 (m, 1H, H-3), 4.02–3.85 (m, 4H, 2CH_2CH$_3$), 3.66–3.48 (m, 2H, H-5, H-4), 2.60 (m, 1H, H-6a), 2.24 (m, 1H, H-6b), 1.21, 1.22 (2t, 6H, 2CH$_2$CH_3), 1.07 (s, 9H, SiC(CH_3)$_3$). $C_{26}H_{36}N_3O_5PSi$ (M 529.3) MALDI-MS (pos. mode, DHB): 568.5 $(M+K)^+$, 552.5 $(M+Na)^+$, 530.5 $(M+H)^+$.

4.2.5. Diethyl (3*S*,4*S*,5*R*)-5-acetoxy-4-azido-3-*tert*-butyldiphenylsilyloxy-1-cyclohexenephosphonate (16). Monosilylated compound (**15**) (20 mg, 38 μmol) is stirred overnight in acetic anhydride/pyridine (1:1), concentrated to dryness and the residue is chromatographed (EE) to give (**16**) (20 mg, 92%). R_f=0.75 (EE); $[\alpha]_D^{20}$=40 (*c*=1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ==7.70–7.37 (2m, 10H, 2C$_6$H$_5$), 6.25 (bd, 1H, H-2, $^3J_{2,P}$=21.4 Hz), 4.83 (ddd, 1H, H-5, $^3J_{5,4}$=~10 Hz, $^3J_{5,6}$=~10, ~6 Hz), 4.17 (m, 1H, H-3), 4.03–3.97 (m, 4H, 2CH_2CH$_3$), 3.72 (dd, 1H, H-4, ^3J=8.2, 10.9 Hz), 2.73 (m, 1H, H-6a), 2.26 (m, 1H, H-6b), 2.10 (s, 3H, COCH_3), 1.28–1.23 (2t, 6H, 2CH$_2$CH_3), 1.08 (s, 9H, SiC(CH_3)$_3$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=141.0 (C-2), 72.3 (C-3), 70.3 (C-5), 67.4 (C-4), 30.0 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=17.08 (s, 1P, P(O)(OEt)$_2$). $C_{28}H_{38}N_3O_6PSi$ (M 571.4) MALDI-MS (pos. mode, DHB): 594.6 $(M+Na)^+$, 572.4 $(M+H)^+$.

4.2.6. Diethyl (3*S*,4*S*,5*R*)-4-azido-5-(prop-2′-enyloxy)-3-*tert*-butyldiphenylsilyloxy-1-cyclohexenephosphonate (17). Compound (**15**) (30 mg, 57 μmol) is dissolved in DMF (2 mL), allyl bromide (26 μL, 285 μmol), Ag_2O (66 mg, 285 μmol) and TBAI (5 mg) are added. The mixture is stirred overnight at rt, filtered, diluted with saturated NH_4Cl solution (5 mL) and extracted with CH_2Cl_2 (3×5 mL). The solvent is evaporated and the residue is chromatographed (Tol/EE 1:1) to give (**17**) (15 mg, 45%). R_f=0.70 (EE); $[\alpha]_D^{20}$=32 (*c*=1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ=7.73–7.37 (2m, 10H, 2C$_6$H$_5$), 6.20 (bd, 1H, H-2, $^3J_{2,P}$=21.4 Hz), 5.90 (m, 1H, H-2′), 5.30–5.16 (m, 2H, H-3′a, H-3′b), 4.13–3.95 (m, 7H, H-3, H-1′a, H-1′b, 2CH_2CH$_3$), 3.60 (dd, 1H, H-4), 3.37 (m, 1H, H-5), 2.71 (m, 1H, H-6a), 2.18 (m, 1H, H-6b), 1.28–1.22 (2t, 6H, 2CH$_2$CH_3), 1.10–1.02 (s, 9H, SiC(CH_3)$_3$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=141.3 (C-2)76.2 (C-5), 72.6 (C-3), 69.3 (C-4), 30.5 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=17.73 (s, 1P, P(O)(OEt)$_2$). $C_{29}H_{40}N_3O_5PSi$ (M 569.4) MALDI-MS (pos. mode, DHB): 608.2 $(M+K)^+$, 592.2 $(M+Na)^+$.

4.2.7. Diethyl (3*S*,4*S*,5*R*)-4-azido-5-hydroxy-3-triisopropylsilyloxy-1-cyclohexenephosphonate (18). Cyclohexenephosphonate (+)-**10** (55 mg, 0.19 mmol) is dissolved in CH_2Cl_2 (5 mL), imidazole (129 mg, 1.9 mmol) and TIPSCl (204 μL, 0.95 mmol) are added and the mixture is refluxed for 16 h. The solvent is evaporated and the residue is chromatographed (Tol/EE 1:2) to yield (**18**) (43 mg, 51%). Excess starting material (10 mg, 18%) can be recovered by elution with EE:MeOH (10:1). R_f=0.55 (EE); $[\alpha]_D^{20}$=39.5 (*c*=1, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): δ=6.47 (bd, 1H, H-2, $^3J_{2,P}$=21.4 Hz), 4.32 (m, 1H, H-3), 4.13–4.00 (m, 4H, 2CH_2CH$_3$), 3.69 (m, 1H, H-5), 3.44 (dd, 1H, H-4, ^3J=9.5, 7.9 Hz), 2.66 (m, 2H, H-6a, OH), 2.29 (m, 1H, H-6b), 1.33–1.27 (2t, 6H, 2CH$_2$CH_3), 1.22–0.99 (m, 19H, 3SiCH(CH_3)$_2$). $C_{22}H_{42}N_3O_5PSi$ (M 447.4) MALDI-MS (pos. mode, DHB): 486.3 $(M+K)^+$, 470.3 $(M+Na)^+$.

4.2.8. Diethyl (3*S*,4*S*,5*R*)-5-acetoxy-4-azido-3-triisopropylsilyloxy-1-cyclohexenephosphonate (19). Monosilylated compound (**18**) (20 mg, 38 μmol) is stirred

overnight in acetic anhydride/pyridine (1:1), concentrated to dryness and the residue is chromatographed (EE) to give (**19**) (21 mg, 94%). R_f=0.70 (EE); $[\alpha]_D^{20}$=65 (c=1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ=6.47 (bd, 1H, H-2, $^3J_{2,P}$=21.4 Hz), 4.96 (m, 1H, H-5), 4.31 (m, 1H, H-3), 4.10–4.07 (m, 4H, 2CH_2CH$_3$), 3.59 (dd, 1H, H-4, ^3J=9.5, 10.5 Hz), 2.79 (m, 1H, H-6a), 2.26 (m, 1H, H-6b), 2.13 (s, 3H, COCH_3), 1.35–1.30 (2t, 6H, 2CH$_2$CH_3), 1.12–1.00 (m, 19H, 3SiCH(CH_3)$_2$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=142.3 (C-2), 125.7 (C-1, $^1J_{1,P}$=185 Hz), 72.0 (C-3), 70.3 (C-5), 67.9 (C-4), 30.1 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=17.29 (s, 1P, P(O)(OEt)$_2$). $C_{24}H_{44}N_3O_6PSi$ (M 489.4) MALDI-MS (pos. mode, DHB): 528.4 $(M+K)^+$, 512.3 $(M+Na)^+$.

4.2.9. Diethyl (3*S*,4*S*,5*R*)-4-azido-5-(prop-2′-enyloxy)-3-triisopropylsilyloxy-1-cyclohexenephosphonate (20). Mono-silylated compound (**18**) (56 mg, 0.125 mmol), allyl bromide (106 μL, 1.25 mmol), Ag_2O (290 mg, 1.25 mmol) and TBAI (23 mg, 62.5 μmol) are suspended in CH_2Cl_2 (5 mL) and the mixture is stirred overnight at rt. After filtration the solvent is evaporated and the residue is chromatographed (Tol/EE 1:4) to give (**20**) (52 mg, 85%). R_f=0.50 (Tol/EE 1:1); $[\alpha]_D^{20}$=63 (c=1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ=6.42 (bd, 1H, H-2, $^3J_{2,P}$=21.4 Hz), 5.95 (m, 1H, H-2′), 5.35–5.20 (m, 2H, H-3′a, H-3′b), 4.22–4.07 (m, 8H, H-3, H-1′a, H-1′b, 2CH_2CH$_3$), 3.88 (m, 1H, H-3), 3.82 (m, 1H, H-5), 3.50–3.46 (m, 2H, H-3, H-4), 2.77 (m, 1H, H-6a), 2.19 (m, 1H, H-6b), 1.35–1.30 (2t, 6H, 2CH$_2$CH_3), 1.16–1.03 (m, 19H, 3SiCH(CH_3)$_2$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=134.4 (C-2), 76.6 (C-5), 72.5 (C-3), 69.9 (C-4), 30.9 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=18.01 (s, 1P, P(O)(OEt)$_2$). $C_{25}H_{46}N_3O_5PSi$ (M 487.4) MALDI-MS (pos. mode, DHB): 526.5 $(M+K)^+$, 510.5 $(M+Na)^+$.

4.2.10. Diethyl (3*S*,4*S*,5*R*)-4-acetamido-5-(prop-2′-enyloxy)-3-triisopropylsilyloxy-1-cyclohexenephosphonate (21). Azide (**20**) (120 mg, 0.25 mmol) is dissolved in pyridine/water (4:1), the solution is saturated with hydrogen sulfide and stirred until TLC indicates the absence of starting material. The solvent is evaporated and the residue is taken up in pyridine/water (1:1, 5 mL), stirred for 3 h, and concentrated to dryness. Flash chromatography (EE/MeOH 10:1) yields acetamide (**21**) (85 mg, 71%). R_f=0.50 (EE); $[\alpha]_D^{20}$=29 (c=1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ=6.50 (bd, 1H, H-2, $^3J_{2,P}$=21.5 Hz), 5.86 (m, 1H, H-2′), 5.48 (bs, 1H, NH), 5.27–5.14 (m, 2H, H-3′a, H-3′b), 4.82 (m, 1H, H-3), 4.13–4.05 (m, 5H, H-1′a, 2CH_2CH$_3$), 4.02–3.95 (m, 2H, H-4, H-1′b), 3.52 (m, 1H, H-4), 2.74 (m, 1H, H-6a), 2.20 (m, 1H, H-6b), 1.98 (s, 3H, COCH_3), 1.35–1.30 (2t, 6H, 2CH$_2$CH_3), 1.12–1.00 (m, 19H, 3SiCH(CH_3)$_2$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=143.5 (C-2), 73.5 (C-5), 71.1 (C-3), 60.0 (C-4), 31.3 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=18.52 (s, 1P, P(O)(OEt)$_2$). $C_{27}H_{50}NO_6PSi$ (M 503.4) MALDI-MS (pos. mode, DHB): 542.3 $(M+K)^+$, 526.3 $(M+Na)^+$.

4.2.11. Triethylammonium (3*S*,4*S*,5*R*)-4-acetamido-3-hydroxy-5-(prop-2′-enyloxy)-1-cyclohexenephosphonate (22). Diethyl ester (**21**) (25 mg, 50 μmol) is dissolved in $CHCl_3$ (3 mL), TMSBr is added (300 μL) and the mixture is stirred until the NMR spectrum of an analytical sample indicates complete conversion of the ester moieties. The solvent is evaporated, water (3 mL) is added, the mixture is stirred for 2 h and lyophilized. The product is purified by HPLC (acetonitrile/50 mM triethylammonium hydrogen carbonate 95:5) on an Phenomenex C18, Aqua 5μ column. ^{1}H NMR (600 MHz, D_2O): δ=6.15 (bd, 1H, H-2, $^3J_{2,P}$=21.5 Hz), 5.87 (m, 1H, H-2′), 5.27–5.16 (m, 2H, H-3′a, H-3′b), 4.19 (m, 1H, H-3), 4.14, 4.02 (2m, 2H, H-3′a, H-3′b), 3.83 (dd, 1H, H-4, ^3J=9.3, 10.4 Hz), 3.60 (m, 1H, H-5), 3.15 (q, 12H, 2 N(CH_2CH$_3$)$_3$), 2.79 (m, 1H, H-6a), 2.19 (m, 1H, H-6b), 2.01 (s, 3H, COCH_3), 1.23 (t, 18H, 2 N(CH$_2$CH_3)$_3$). ^{13}C NMR (150.9 MHz, D_2O): δ=134.3 (C-2), 75.7 (C-5), 71.3 (C-3), 57.3 (C-4), 31.6 (C-6). ^{31}P NMR (242.9 MHz, D_2O): δ=12.49 (s, 1P, PO_3^{2-}). $C_{11}H_{18}NO_6P$ (M 291.2) MALDI-MS (neg. mode, ATT): 291.1 $(M-H)^-$.

4.2.12. Diethyl (3*R*,4*R*,5*S*)-4-azido-5-hydroxy-3-(prop-2′-enyloxy)-1-cyclohexenephosphonate (23). Cyclohexenephosphonate (−)-**10** (98 mg, 0.34 mmol), allyl bromide (32 μL, 0.38 mmol) and Ag_2O (395 mg, 1.7 mmol) are suspended in CH_2Cl_2 (10 mL), the mixture is cooled to 0°C and TBAI (63 mg, 0.17 mmol) is added. After 3 h of stirring EtOH (0.5 mL) is added, insoluble material is filtered off, solvent is evaporated and the residue is chromatographed (Tol/EE 1:2) to give mono-allylated compound (**23**) (70 mg, 70%). R_f=0.25 (Tol/EE 1:2); $[\alpha]_D^{20}$=−40 (c=1, $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): δ=6.59 (bd, 1H, H-2, $^3J_{2,P}$=21.7 Hz), 5.93 (m, 1H, H-2′), 5.34–5.20 (m, 2H, H-3′a, H-3′b), 4.23 (dd, 1H, H-1′a), 4.14–3.95 (m, 6H, H-1′b, H-3, 2CH_2CH$_3$), 3.66 (m, 1H, H-5), 3.50 (dd, 1H, H-4, ^3J=8.4, 10.3 Hz), 2.83 (d, 1H, OH, $^3J_{OH,5}$=2.2 Hz), 2.67 (m, 1H, H-6a), 2.19 (m, 1H, H-6b), 1.30 (2t, 6H, 2CH$_2$CH_3). $C_{13}H_{22}N_3O_5P$ (M 331.3) MALDI-MS (pos. mode, DHB): 370.4 $(M+K)^+$, 354.4 $(M+Na)^+$.

4.2.13. Diethyl (3*R*,4*R*,5*S*)-5-acetoxy-4-azido-3-(prop-2′-enyloxy)-1-cyclohexenephosphonate (24). Compound (**23**) (15 mg, 44 μmol) is stirred overnight in acetic anhydride/pyridine (1:1), concentrated to dryness and the residue is chromatographed (EE) to give (**24**) (21 mg, 94%). R_f=0.75 (EE/MeOH 10:1); $[\alpha]_D^{20}$=−35 (c=0.4, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ=6.60 (bd, 1H, H-2, $^3J_{2,P}$=21.7 Hz), 5.92 (m, 1H, H-2′), 5.34–5.24 (m, 2H, H-3′a, H-3′b), 4.96 (ddd, 1H, H-5), 4.22, 4.17 (2m, 2H, H-1′a, H-1′b), 4.10–4.05 (m, 4H, 2CH_2CH$_3$), 3.98 (m, 1H, H-3), 3.68 (dd, 1H, H-4, ^3J=8.5, 11.1 Hz), 2.76 (m, 1H, H-6a), 2.21 (m, 1H, H-6b), 2.13 (s, 3H, COCH_3), 1.35–1.30 (2t, 6H, 2CH$_2$CH_3). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=139.2 (C-2), 77.7 (C-3), 69.5 (C-5), 64.9 (C-4), 30.1 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=17.10 (s, 1P, P(O)(OEt)$_2$). $C_{15}H_{14}N_3O_6P$ (M 373.3) MALDI-MS (pos. mode, DHB): 412.3 $(M+K)^+$, 396.4 $(M+Na)^+$, 374.4 $(M+H)^+$.

4.2.14. Diethyl (3*R*,4*R*,5*S*)-4-azido-3-(prop-2′-enyloxy)-5-triisopropylsilyloxy-1-cyclohexenephosphonate (25). Compound (**23**) (90 mg, 272 μmol) is dissolved in CH_2Cl_2 (5 mL), imidazole (185 mg, 2.72 mmol) and TIPSCl (291 μL, 1.36 mmol) are added and the mixture is refluxed for 12 h. Following evaporation of the solvent the residue is chromatographed (Tol/EE 1:1) to give (**25**) (91 mg, 69%). R_f=0.75 (EE/MeOH 10:1); $[\alpha]_D^{20}$=−14 (c=1, $CHCl_3$); ^{1}H

NMR (600 MHz, $CDCl_3$): δ=6.57 (bd, 1H, H-2, $^3J_{2,P}$=21.5 Hz), 5.93 (m, 1H, H-2′), 5.34–5.20 (m, 2H, H-3′a, H-3′b), 4.23–4.05 (m, 6H, H-1′a, H-1′b, $2CH_2CH_3$), 3.88 (m, 1H, H-3), 3.82 (m, 1H, H-5), 3.47 (dd, 1H, H-4, ^3J=8.8, 9.9 Hz), 2.67 (m, 1H, H-6a), 2.21 (m, 1H, H-6b), 1.35–1.30 (2t, 6H, $2CH_2CH_3$), 1.14–0.87 (m, 19H, $3SiCH(CH_3)_2$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=139.7 (C-2), 78.5 (C-3), 70.2 (C-5), 68.7 (C-4), 34.3 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=17.69 (s, 1P, $P(O)(OEt)_2$). $C_{25}H_{46}N_3O_5PSi$ (M 487.4) MALDI-MS (pos. mode, DHB): 525.7 $(M+K)^+$, 509.8 $(M+Na)^+$.

4.2.15. Diethyl (3*R*,4*R*,5*S*)-4-acetamido-3-(prop-2′-enyloxy)-5-triisopropylsilyloxy-1-cyclohexenephosphonate (26). Azide (**25**) (30 mg, 0.06 mmol) is dissolved in pyridine/water (4:1), the solution is saturated with hydrogen sulfide and stirred until TLC indicates the absence of starting material. The solvent is evaporated and the residue is taken up in pyridine/water (1:1, 5 mL), stirred for 3 h, and concentrated to dryness. Flash chromatography (EE/MeOH 10:1) yields acetamide (**26**) 24 mg, 81%). R_f=0.3 (EE/MeOH 10:1); $[\alpha]_D^{20}$=−21 (c=1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): δ=6.64 (bd, 1H, H-2, $^3J_{2,P}$=21.5 Hz), 5.87 (m, 1H, H-2′), 5.48 (bs, 1H, NH), 5.29–5.17 (m, 2H, H-3′a, H-3′b), 4.34 (m, 1H, H-3), 4.22–4.00 (m, 7H, H-5, H-1′a, H-1′b, $2CH_2CH_3$), 3.77 (m, 1H, H-4), 2.66 (m, 1H, H-6a), 2.25 (m, 1H, H-6b), 1.99 (s, 3H, $COCH_3$), 1.35–1.30 (2t, 6H, $2CH_2CH_3$), 1.13–0.90 (m, 19H, $3SiCH(CH_3)_2$). ^{13}C NMR (150.9 MHz, $CDCl_3$): δ=140.8 (C-2), 76.3 (C-3), 68.2 (C-5), 57.5 (C-4), 34.6 (C-6). ^{31}P NMR (242.9 MHz, $CDCl_3$): δ=18.08 (s, 1P, $P(O)(OEt)_2$). $C_{27}H_{50}NO_6PSi$ (M 503.4) MALDI-MS (pos. mode, DHB): 542.3 $(M+K)^+$, 526.3 $(M+Na)^+$.

4.2.16. Triethylammonium (3*R*,4*R*,5*S*)-4-acetamido-5-hydroxy-3-(prop-2′-enyloxy)-1-cyclohexenephosphonate (27). Diethyl ester (**26**) (15 mg, 30 μmol) is dissolved in $CHCl_3$ (2 mL), TMSBr is added (200 μL) and the mixture is stirred until the NMR spectrum of an analytical sample indicates complete conversion of the ester moieties. The solvent is evaporated, water (3 mL) is added, the mixture is stirred for 2 h and lyophilized. The product (**27**) is purified by HPLC (acetonitrile/50 mM triethylammonium hydrogen carbonate 95:5) on an Phenomenex C18, Aqua 5μ column. ^{1}H NMR (600 MHz, D_2O): δ=6.17 (bd, 1H, H-2, $^3J_{2,P}$=19.6 Hz), 5.80 (m, 1H, H-2′), 5.23–5.13 (m, 2H, H-3′a, H-3′b), 4.10–4.06 (m, 2H, H-1′a, H-3), 3.96 (dd, 1H, H-1′b), 3.81 (dd, 1H, H-4, ^3J=9.2, 10.7 Hz), 3.69 (m, 1H, H-5), 3.08 (q, 12H, 2 $N(CH_2CH_3)_3$), 2.60 (m, 1H, H-6a), 2.18 (m, 1H, H-6b), 1.96 (s, 3H, $COCH_3$), 1.17 (t, 18H, 2 $N(CH_2CH_3)_3$). ^{13}C NMR (150.9 MHz, D_2O): δ=78.4 (C-3), 67.9 (C-5), 56.1 (C-4), 33.6 (C-6). ^{31}P NMR (242.9 MHz, D_2O): δ=12.89 (s, 1P, PO_3^{2-}). $C_{11}H_{18}NO_6P$ (M 291.2) MALDI-MS (pos. mode, DHB): 314.4 $(M+Na)^+$, 292.5 $(M+H)^+$.

4.2.17. Ammonium (3*S*,4*S*,5*R*)-4-acetamido-3,5-dihydroxy-1-cyclohexenephosphonate ((+)-1). Diethyl phosphonate (+)-**12** is cleaved as described[14] before in the synthesis of (−)-**1**. NMR- and MS-spectra are identical to those described for the (−)-enantiomer. $[\alpha]_D^{20}$=14 (c=0.5, H_2O).

4.3. Inhibition assay

The inhibitory potencies (IC_{50} values) of the different inhibitors were determined in a spectrofluorimetric assay using (4-methylumbelliferyl-)-α-D-*N*-acetylneuraminic acid (4-MU-NANA) as substrate according to Schauer and co-workers.[25] The sialidases from *S. typhimurium* and *C. perfringens* were from Sigma (Taufkirchen, Germany) and the sialidase from *V. chloreae* was from Boehringer Mannheim (Mannheim, Germany). 4-MU-NANA was from Fluka (Buchs, Switzerland). The assay in brief: Incubations were carried out in a final volume of 100 μL containing 0.2 mU of sialidase (20 μL of a stock solution of 0.1 U in 10 mL of 0.1 M acetate buffer of pH 5.5, 0.5 mM $CaCl_2$, 0.01% (w/v) NaN_3, 0.1 mg/mL bovine serum albumine (Serva)), a final $CaCl_2$ concentration of 0.5 mM, 0.1 M acetate buffer (pH 5.5), 20 μL of a solution of inhibitor in water (50–0.5 mM) by finally (after 10 min of incubation) adding 20 μL of 4-MU-NANA in water (1 mM). Enzymatic reactions were stopped by rapidly adding 900 μL of cold glycine buffer of pH 10 (0.06 M NaCl, 0.042 M Na_2CO_3, 0.133 M glycine) and liberated 4-methylumbelliferone (MU) determined on Wallac Victor[2] fluorescence spectrophotometer using a wavelength of 460 nm for emission and 365 nm for excitation. Blanks were run without enzyme, controls were run without inhibitor, benchmark inhibitor Neu5Ac2en[15] (ammonium salt) was included in every assay for comparison.

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