Parainfluenza Virus

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The Catalytic Mechanism of Human Parainfluenza Virus Type 3 Haemagglutinin-Neuraminidase Revealed**

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Abstract: *Human parainfluenza virus type 3 (hPIV-3) is one of the leading causes for lower respiratory tract disease in children, with neither an approved antiviral drug nor vaccine available to date. Understanding the catalytic mechanism of human parainfluenza virus haemagglutinin-neuraminidase (HN) protein is key to the design of specific inhibitors against this virus. Herein, we used ^{1}H NMR spectroscopy, X-ray crystallography, and virological assays to study the catalytic mechanism of the HN enzyme activity and have identified the conserved Tyr530 as a key amino acid involved in catalysis. A novel 2,3-difluorosialic acid derivative showed prolonged enzyme inhibition and was found to react and form a covalent bond with Tyr530. Furthermore, the novel derivative exhibited enhanced potency in virus blockade assays relative to its Neu2en analogue. These outcomes open the door for a new generation of potent inhibitors against hPIV-3 HN.*

Viruses and their associated diseases constitute one of the greatest health threats to mankind. The *Paramyxoviridae* family includes viruses of significant medical and veterinary importance and among them the human parainfluenza viruses (hPIVs) are a leading cause of severe lower respiratory tract illness in infants and young children. Human parainfluenza virus type 3 (hPIV-3) is responsible for 7 to 11 %[1,2] of all lower respiratory tract illness hospitalizations in children below the age of five, second only to human respiratory syncytial virus (RSV).[3,4] Furthermore, hPIV-3 infections are of particular concern for both elderly and immunocompromised patients, with high mortality rates, reaching nearly 50 %.[5,6] Infections attributed to hPIV-3 represent a substantial socioeconomic burden[7] and despite ongoing efforts,[8,9] there are currently neither vaccines nor specific antiviral therapy for prevention or treatment of infection.

The hPIV-3 haemagglutinin-neuraminidase (HN) glycoprotein plays a major role in the virus' life cycle, encompassing three critical functions: virus–host-cell recognition, involvement in fusion, and release of virion progeny.[10] This multifunctional role makes HN an ideal target for anti-parainfluenza drug design. Despite the large number of Neu5Ac2en (**1**)-based inhibitors designed to target this essential protein,[11–14] such as BCX2798 (**2**),[15–17] none of these inhibitors has progressed to the clinic. Elucidation of the, as yet undescribed, catalytic mechanism of this critical protein is an important step towards the design of more specific and potent inhibitors of hPIV-3 infection.

Unlike the distantly related influenza A virus (IAV) neuraminidase (sialidase, NA), the catalytic mechanism of the whole paramyxovirus family HN has not been structurally investigated. Thus, understanding the catalytic mechanism of hPIV-3 HN will also provide, for the first time, a model for the catalytic mechanism of the glycoside hydrolase 83 family,[18] that includes paramyxovirus HN. In studies on both the IAV NA[19] and human neuraminidase 2 (*h*Neu2)[20] catalytic mechanism, sialosyl fluorides have been utilized to form a relatively stable covalent adduct between the inhibitor and the NA protein. Herein the 2,3-difluorosialic acid analogues (**3**–**5**) of the potent hPIV-3 HN inhibitor **2** have been synthesized to probe the catalytic mechanism of hPIV-3 HN. Our multidisciplinary study clearly supports the existence of a covalent intermediate between the HN protein and inhibitor **5** through the C2 atom of inhibitor **5** and the phenolic oxygen atom of a key catalytic amino acid, Tyr530.

To investigate the catalytic mechanism of hPIV-3 HN, the 2,3-difluoro analogues (**3**–**5**) of the previously reported potent hPIV inhibitor BCX2798 (**2**), have been synthesized following the procedures illustrated in Figure 1 b. The synthesis of the target compounds started with the known[21,22] 4-azido-4-deoxy-5-isobutyramido-Neu2en intermediate **6**. The halohydrins **7** and **8** were obtained in very good overall yields of 58 % and 35 %, respectively, and in a short period of time by heating intermediate **6** with Selectfluor in an aqueous nitromethane solution by microwave (MW) irradiation for 2 h at

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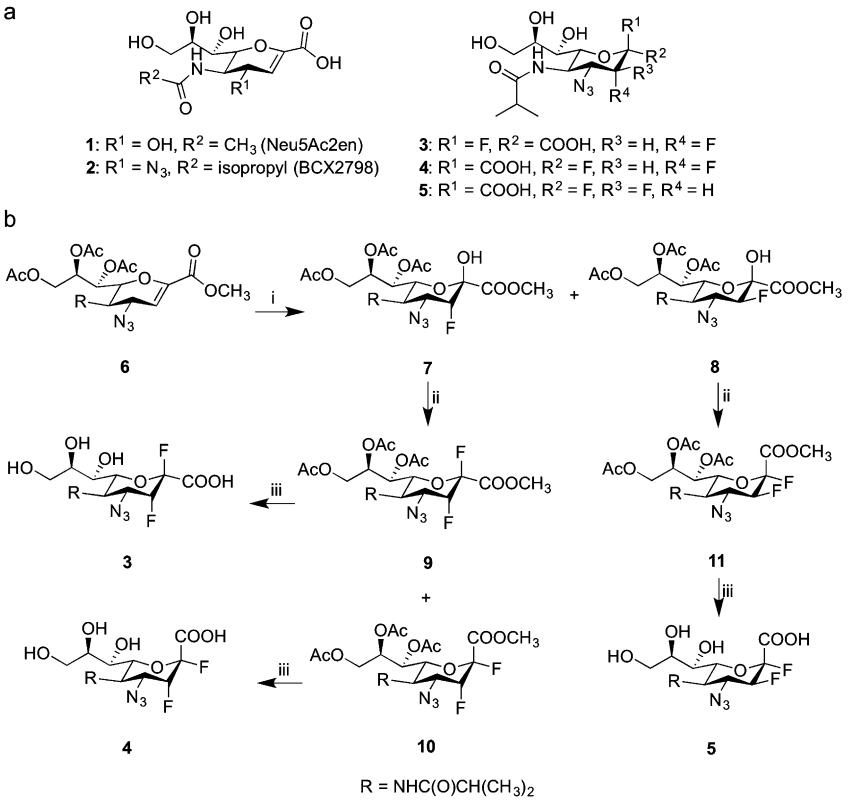

Figure 1. a) Structures of the reference inhibitors Neu5Ac2en (**1**) and BCX2798 (**2**), and the novel 2,3-difluoro analogues of BCX2798 (**3**–**5**). b) Synthesis of inhibitors **3**–**5**. i) Selectfluor, nitromethane/H_2O (9:1), microwave, 80 °C, 2 h, 93% yield (**7**, 58%; **8**, 35%); ii) DAST, CH_2Cl_2, −40 °C to −10 °C, 30 min (**9**, 21%; **10**, 66%; **11**, 90%); iii) NaOH, MeOH/H_2O (1:1), room temperature, overnight, H^+ (**3**, 78%; **4**, 91%; **5**, 82%).

80 °C. This procedure provides a significant improvement over the original procedure[19] in which a mixture of the corresponding Neu2en intermediate and Selectfluor is stirred at room temperature for 5 days. Following this reported procedure, compounds **7** and **8** were obtained in 49% and 17% yields, respectively (66% overall yield compared with a 93% overall yield from our MW-assisted methodology). Chromatographic separation of each of the halohydrins **7** and **8**, followed by fluorination with (diethylamino)sulfurtrifluoride (DAST) in CH_2Cl_2 at −40 °C resulted in the conversion of the C2 alcohol groups into the corresponding fluorides **9**–**11**. In the case of the 3-fluoro halohydrin **8**; a clean inversion at C2 was found with the production of the 2,3-difluoro derivative **11** as the only product in 90% yield. On the other hand, when the same reaction conditions were applied to the 3-fluoro halohydrin **7**, a mixture of the 2,3-difluoro derivatives **9** and **10** were obtained, in yields of 21% and 66%, respectively. The deprotection of the 2,3-difluoro derivatives **9**–**11**, by treatment with 50% aq methanol adjusted to pH 13–14 using NaOH at room temperature for 24 h, yielded the final products **3** (78%), **4** (91%) and **5** (82%).

The synthesized 2,3-difluoro derivatives (**3**–**5**) were then evaluated for their capacity to inhibit hPIV-3 HN neuraminidase function using a fluorescence-based assay that employs the substrate 4-methylumbelliferyl α-D-*N*-acetylneuraminide (MUN).[21–23] The results of the NA inhibition assays against hPIV-3 HN confirmed the 2,3-difluorosialic acid derivative **5** as the most potent among the three synthesized isomers, with an IC_{50} value of 4.8 μM. A significant gap in potency was observed between inhibitor **5** and the other two 2,3-difluoro isomers (**3** and **4**), where **5** was found to be 180 and 250 times more potent than **4** and **3**, respectively. This very critical influence in the stereochemistry of the C2 and C3 fluorides on inhibitor efficacy is consistent with what has been observed for IAV NA[19] and for *h*Neu2.[20] Thus, in general, it appears that α-sialosyl fluorides (e.g. **4** and **5**) bearing an equatorial (eq) instead of an axial (ax) C3 fluoro-substituent are more potent neuraminidase inhibitors, irrespective of the enzyme's origin.

To gain a deeper insight into the hPIV-3 HN mechanism, we have determined if HN is a retaining or an inverting enzyme. Using ^{1}H NMR spectroscopy, the initial stereochemistry around the anomeric center (C2) of the released product, *N*-acetyl-D-neuraminic acid (Neu5Ac), was determined by monitoring the products from hPIV-3 HN action on the substrate MUN over 55 min. After 5 min of incubation at 37 °C, the hydrolysis of MUN showed only peaks corresponding to the $H3_{ax}$ and $H3_{eq}$ atoms of the α-Neu5Ac anomer (Figure 2). The β-anomer, the mutarotated product characterized by $H3_{ax}$ and $H3_{eq}$ resonances at $\delta = 1.7$ and 2.1 ppm, respectively was initially observed after 25 min of incubation. As seen in Figure 2, the enzymatic reaction was much faster than the rate of mutarotation as the substrate was completely consumed before mutarotation reached equilibrium. These studies support the notion that hPIV-3 HN-catalyzed substrate hydrolysis occurs with overall retention of configuration and that hPIV-3 HN is a retaining glycohydrolase, consistent with all other wild type neuraminidases.[19,20,24]

Following the characterization of hPIV-3 HN as a retaining glycohydrolase, inhibitor **5** was used to further study the catalytic mechanism of hPIV-3 HN. In an initial assay, the ability of inhibitor **5** to block the hPIV-3 HN neuraminidase activity for a prolonged time was evaluated. Therefore, **5** and the benchmark inhibitor BCX2798 (**2**) were investigated at equimolar concentrations (20 μM) in an NMR-spectroscopy-based neuraminidase inhibition assay that enabled direct monitoring of substrate (MUN) turnover (Figure 3). In the presence of BCX2798 (**2**), the intensity of the $H3_{eq}$ resonance ($\delta = 2.63$ ppm) of the product α-Neu5Ac, was found to steadily increase over time. This indicates a continuous turnover of MUN, with the reaction almost complete after incubation for 1.5 h. In contrast, in the presence of inhibitor **5**, only a small increase in the α-Neu5Ac $H3_{eq}$ peak intensity was observed within the first 25 min of incubation, indicating little

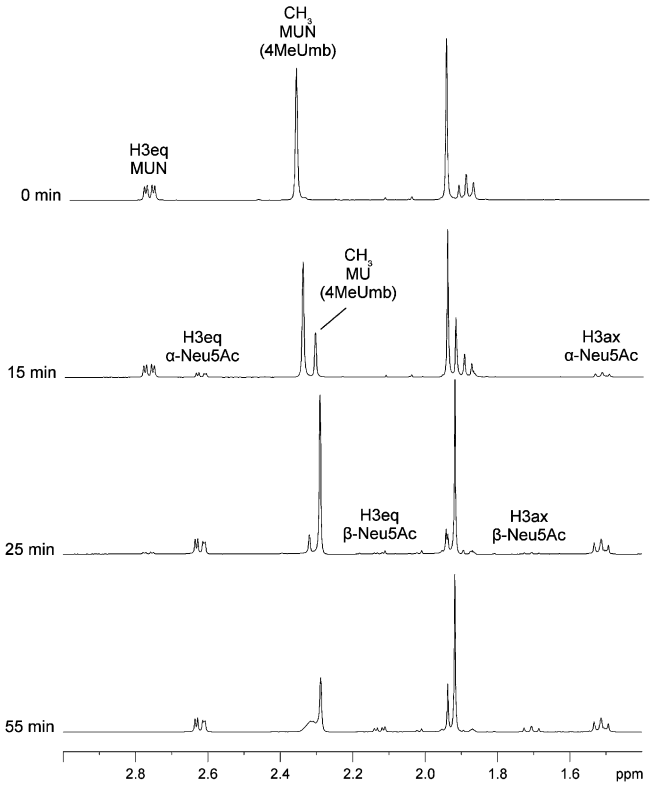

Figure 2. An NMR-based time course study of MUN turnover in the presence of hPIV-3 HN. The reaction was performed with 5 mM MUN in the presence of 12 μg hPIV-3 HN at 298 K in 0.2 mL deuterated NA reaction buffer (0.05 M sodium acetate buffer pD 4.6, 5 mM $CaCl_2$). eq = equatorial, ax = axial.

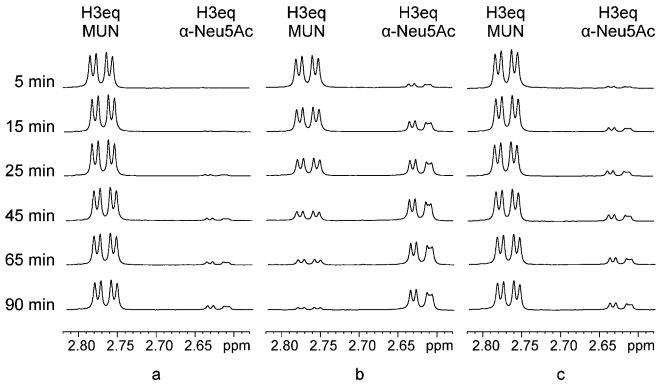

Figure 3. An NMR-spectroscopy-based time course study of MUN turnover in the presence and absence of inhibitors **2** and **5**. All reactions were conducted at 298 K in 0.2 mL deuterated NA reaction buffer with the following set up: a) 5 mM MUN without hPIV-3 HN (control); b) 12 μg hPIV-3 HN in presence of 20 μM of inhibitor **2** and 5 mM MUN; c) 12 μg hPIV-3 HN in presence of 20 μM of inhibitor **5** and 5 mM MUN. eq = equatorial.

cleavage of MUN (less than 10%) during this time. No further product release was observed in subsequent spectra, indicating a prolonged blockade of hPIV-3 HN by inhibitor **5** (Figure 3).

To further explore the capacity of **5** to inhibit hPIV-3 HN activity for longer times, hPIV-3 HN protein was pre-incubated with **5** for 1 h and then any residual inhibitor was washed out from the reaction mixture using the NMR reaction buffer with an Amicon filtration step (Supporting Information). MUN was added and substrate turnover monitored by ^{1}H NMR spectroscopy over 24 h. Under these experimental conditions we found only trace amounts of cleavage products over the 24 h incubation period, indicating tight and prolonged binding of the inhibitor to hPIV-3 HN (see Supporting Information). On the other hand, and as expected, in the negative control (no inhibitor) a complete consumption of the substrate was observed within the 24 h reaction period.

Based on our presented data and previous reports,[19,20,25] we hypothesized that hPIV-3 HN forms a covalent intermediate with the substrate by nucleophilic attack at the anomeric carbon (C2) of the Neu5Ac glycoside. The phenolic oxygen of the key catalytic amino acid Tyr530 acts as the nucleophile. In all wild-type neuraminidases a Tyr residue, commensurate with Tyr530 in hPIV-3 HN, appears to be strictly conserved,[26] and therefore can be targeted by C2 functionalized sialic acid-based inhibitors that are susceptible to a nucleophilic displacement reaction to form a covalent adduct with inverted configuration (α→β) around the anomeric center. To investigate this hypothesis, we have undertaken protein X-ray crystallography studies using pre-formed hPIV-3 HN crystals, soaked with inhibitor **5**. The three-dimensional structure of the hPIV-3 HN-**5** complex (protein data bank (pdb) accession code: 4XJR) supports the conclusion that hPIV-3 HN covalently bound **5** as a 3-fluorosialyl-enzyme intermediate. Indeed, continuous electron density extending from the inhibitor towards Tyr530 is indicative of a covalent bond of 1.39 Å between the phenolic oxygen atom of Tyr530 and C2 of the 3-eq-fluoro sialic acid derivative **5** in binding site A and B (Figure 4). The well characterized triarginyl cluster of HN (Arg192, Arg502, Arg424) is extended towards the carboxylic group of **5**, as seen in the co-complex with Neu5Ac2en.[26] Furthermore, the equatorial C3 fluoride of **5** makes an interaction (2.65 Å and 2.3 Å in binding site A and B, respectively) with a terminal nitrogen atom (N2) of Arg192, similar to that observed between a covalently bound 3-fluorosialic acid derivative and Arg21 of *h*Neu2[20] or Arg118 of IAV N9.[19] Although the structural resolution of the data for the hPIV-3 HN-**5** complex is not sufficient to allow definite positioning of the azido moieties within chains A and B with certainty, the difference in the orientation of the group is unequivocal and derives from its flexibility, solvent access, and lack of coordination by the enzyme's active site residues. Thus, the C4 azido moiety was found to occupy two distinct orientations in the two binding sites A and B (Figure 4). While, most amino acid side chain orientations in the binding pocket were found to be similar in both the hPIV-3 HN-**5** complex and the *apo* structure (code: 4XJQ), some reorientation of both Arg192 and Glu549 side chains appears to occur. Upon complexation of inhibitor **5**, a major reorientation of the Tyr530 side chain is observed. The key Tyr530 residue was found to be directed away from the active site in the *apo* structure (in a similar orientation to a previously reported *apo* structure).[26] Complexation of **5** in the active site results in Tyr530 reorienting

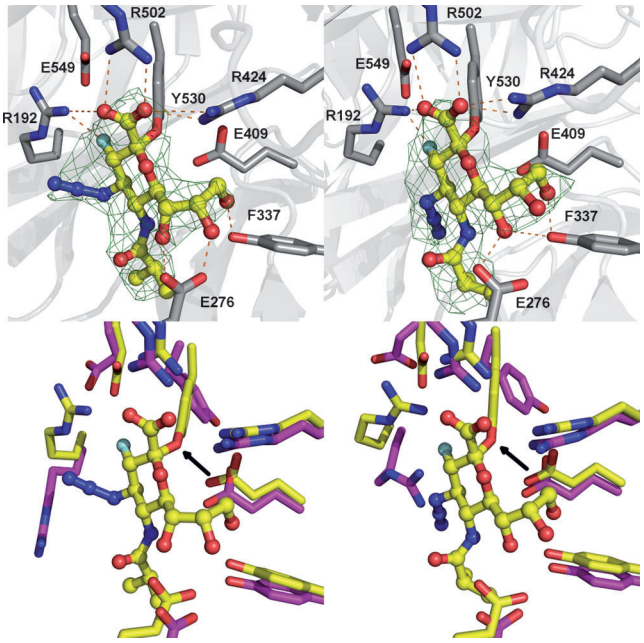

Figure 4. X-ray structures of the hPIV-3 HN-**5** complex (pdb: 4XJR). Top: X-ray structure of the hPIV-3 HN-**5** complex in binding site A (left panel) and binding site B (right panel). Bottom: hPIV-3 HN-**5** complex (4XJR, yellow) and the *apo* hPIV-3 HN structure (4XJQ, magenta) that shows binding site A (left panel) and binding site B (right panel). The arrow indicates the newly formed covalent bond between the phenolic oxygen of Tyr530 and the C2 of **5**. O red, N blue, F light blue.

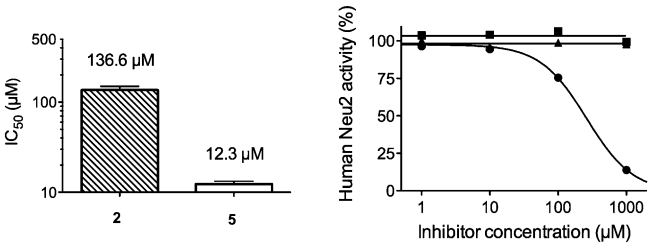

Figure 5. A virus infectivity blockade study of inhibitors **2** and **5.** Left: Virus growth inhibition IC_{50} values were evaluated by an in situ ELISA technique using the human cell line A549. IC_{50} values of 136.6 ± 18.8 μM and 12.3 ± 1.3 μM were determined for **2** and **5**, respectively. These values were evaluated from at least two independent experiments performed in triplicate and error bars correspond to the calculated standard deviation. Right: Activity of the human neuraminidase **2** was tested in the presence of inhibitors **2** (■) and **5** (▲) as well as the reference compound Neu5Ac2en, **1** (●).

towards the binding site and enables covalent bond formation through the residue's phenolic oxygen atom with the C2 of 3-eq-fluoro sialic acid derivative **5** (Figure 4).

As our neuraminidase inhibition screening data of the three 2,3-difluoro analogues **3**–**5** of BCX2798 (**2**) revealed **5** to be a potent inhibitor, we thought it important to extend our study to investigate the potential of inhibitor **5** to block virus infection and propagation in a whole-cell-based assay and compare it with the Neu2en parent analogue BCX2798 (**2**). Accordingly, both compounds were evaluated following our well-established in situ ELISA technique (see Supporting Information)[21] using the human respiratory cell line A549 (human lung adenocarcinoma epithelial cells). The results of this whole-cell-based assay were consistent with the neuraminidase inhibition screening evaluation. Thus, the 2,3-difluoro derivative **5** displayed significantly enhanced virus blockade relative to its parent Neu2en inhibitor **2** with calculated IC_{50} values of 12.3 μM and 136.6 μM for **5** and **2**, respectively (Figure 5).

In addition, the specificity of the inhibitors was assessed using *h*Neu2 as a representative of human neuraminidases. Neu5Ac2en (**1**), an established moderate *h*Neu2 inhibitor,[27] has been utilized as a reference in the enzymatic neuraminidase inhibition assay with minor modifications to the method.[27] No inhibition of *h*Neu2 enzymatic activity was displayed for compounds **5** and **2**, whereas Neu5Ac2en was found to inhibit the protein with an IC_{50} of 292.4 μM (Figure 5). The observed selectivity of **5** and **2** for hPIV-3 HN is not surprising as *h*Neu2 cannot accommodate a more bulky C4 substituent on the Neu5Ac2en template, as shown in a published structural study.[27]

In summary, we have successfully synthesized 2,3-difluorosialic acid analogues of the hPIV-3 HN inhibitor BCX2798 (**2**) and utilized them as probes to explore the catalytic mechanism of hPIV-3 HN. We have also verified hPIV-3 HN as a retaining glycohydrolase that acts by a nucleophilic mechanism to form a covalent adduct between substrate and enzyme. This appears to be a general mechanism for influenza[19,25] and mammalian[20] neuraminidases. Furthermore, our study provides new insights in targeting the key catalytic amino acid Tyr530 of hPIV-3 HN by a mechanism-based inhibition. The 2,3-difluoro analogue **5** showed not only prolonged inhibition of hPIV-3 HN, but also superior potency in virus blockade assays and therefore represents a promising new lead in anti-parainfluenza virus inhibitor design.

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[1] G. A. Weinberg, C. B. Hall, M. K. Iwane, K. A. Poehling, K. M. Edwards, M. R. Griffin, M. A. Staat, A. T. Curns, D. D. Erdman, P. G. Szilagyi, *J. Pediatr.* **2009**, *154*, 694–699.e1.
[2] K. J. Henrickson, *Clin. Microbiol. Rev.* **2003**, *16*, 242–264.
[3] R. M. Chanock, *Pediatr. Res.* **1990**, *27*, S39–43.
[4] B. R. Murphy, *Bull. W. H. O.* **1988**, *66*, 391–397.
[5] M. Zambon, T. Bull, C. J. Sadler, J. M. Goldman, K. N. Ward, *J. Clin. Microbiol.* **1998**, *36*, 2289–2293.
[6] R. T. Maziarz, P. Sridharan, S. Slater, G. Meyers, M. Post, D. D. Erdman, T. C.-T. Peret, R. A. Taplitz, *Biol. Blood Marrow Transplant.* **2010**, *16*, 192–198.
[7] M.-S. Lee, R. E. Walker, P. M. Mendelman, *Hum. Vaccines* **2004**, *1*, 5–11.
[8] J. A. Englund, R. A. Karron, C. K. Cunningham, P. LaRussa, A. Melvin, R. Yogev, E. Handelsman, G. K. Siberry, B. Thumar, E. Schappell, et al., *Vaccine* **2013**, *31*, 5706–5712.
[9] A. Moscona, M. Porotto, S. Palmer, C. Tai, L. Aschenbrenner, G. Triana-Baltzer, Q.-X. Li, D. Wurtman, S. Niewiesk, F. Fang, *J. Infect. Dis.* **2010**, *202*, 234–241.

[10] A. Moscona, *J. Clin. Invest.* **2005**, *115*, 1688 – 1698.
[11] K. Ikeda, S. Kitani, K. Sato, T. Suzuki, C. Hosokawa, Y. Suzuki, K. Tanaka, M. Sato, *Carbohydr. Res.* **2004**, *339*, 1367 – 1372.
[12] D. J. Tindal, J. C. Dyason, R. J. Thomson, T. Suzuki, H. Ueyama, Y. Kuwahara, N. Maki, Y. Suzuki, M. von Itzstein, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1655 – 1658.
[13] R. Nishino, K. Ikeda, T. Hayakawa, T. Takahashi, T. Suzuki, M. Sato, *Bioorg. Med. Chem.* **2011**, *19*, 2418 – 2427.
[14] R. Nishino, T. Hayakawa, T. Takahashi, T. Suzuki, M. Sato, K. Ikeda, *Chem. Pharm. Bull.* **2013**, *61*, 69 – 74.
[15] I. V. Alymova, G. Taylor, T. Takimoto, T.-H. Lin, P. Chand, Y. S. Babu, C. Li, X. Xiong, A. Portner, *Antimicrob. Agents Chemother.* **2004**, *48*, 1495 – 1502.
[16] I. V. Alymova, M. Watanabe, K. L. Boyd, P. Chand, Y. S. Babu, A. Portner, *Antiviral Ther.* **2009**, *14*, 891 – 898.
[17] M. Watanabe, V. P. Mishin, S. A. Brown, C. J. Russell, K. Boyd, Y. S. Babu, G. Taylor, X. Xiong, X. Yan, A. Portner, et al., *Antimicrob. Agents Chemother.* **2009**, *53*, 3942 – 3951.
[18] AFMB-CNRS-Université d'Aix-Marseille, "CAZy – GH83", can be found under http://www.cazy.org/GH83_all.html, **2014**.
[19] J.-H. Kim, R. Resende, T. Wennekes, H.-M. Chen, N. Bance, S. Buchini, A. G. Watts, P. Pilling, V. A. Streltsov, M. Petric, et al., *Science* **2013**, *340*, 71 – 75.
[20] S. Buchini, F.-X. Gallat, I. R. Greig, J.-H. Kim, S. Wakatsuki, L. M. G. Chavas, S. G. Withers, *Angew. Chem. Int. Ed.* **2014**, *53*, 3382 – 3386; *Angew. Chem.* **2014**, *126*, 3450 – 3454.
[21] P. Guillon, L. Dirr, I. M. El-Deeb, M. Winger, B. Bailly, T. Haselhorst, J. C. Dyason, M. von Itzstein, *Nat. Commun.* **2014**, *5*, 5268.
[22] I. M. El-Deeb, P. Guillon, M. Winger, T. Eveno, T. Haselhorst, J. C. Dyason, M. von Itzstein, *J. Med. Chem.* **2014**, *57*, 7613 – 7623.
[23] M. Potier, L. Mameli, M. Bélisle, L. Dallaire, S. B. Melançon, *Anal. Biochem.* **1979**, *94*, 287 – 296.
[24] D. L. Zechel, S. G. Withers, *Curr. Opin. Chem. Biol.* **2001**, *5*, 643 – 649.
[25] C. J. Vavricka, Y. Liu, H. Kiyota, N. Sriwilaijaroen, J. Qi, K. Tanaka, Y. Wu, Q. Li, Y. Li, J. Yan, et al., *Nat. Commun.* **2013**, *4*, 1491.
[26] M. C. Lawrence, N. A. Borg, V. A. Streltsov, P. A. Pilling, V. C. Epa, J. N. Varghese, J. L. McKimm-Breschkin, P. M. Colman, *J. Mol. Biol.* **2004**, *335*, 1343 – 1357.
[27] L. M. G. Chavas, R. Kato, N. Suzuki, M. von Itzstein, M. C. Mann, R. J. Thomson, J. C. Dyason, J. McKimm-Breschkin, P. Fusi, C. Tringali, et al., *J. Med. Chem.* **2010**, *53*, 2998 – 3002.

