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Aglycone-focused randomization of 2-difluoromethylphenylsialoside-type suicide substrates for neuraminidases

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ABSTRACT

A selective and potent inhibitor of neuraminidases, a hydrolase that is responsible for processing sialylated glycoconjugates, is a promising drug candidate for various infective diseases. The current study demonstrates that the use of an aglycone-focused library of 2-difluoromethylphenyl α -sialosides is an effective technique to find potent and selective mechanism-based labeling reagents for neuraminidases. The focused library was constructed from a 4-azide-2-difluoromethylphenyl sialoside (**2**) and an alkyne-terminated compound library by a click reaction. The focused library showed different inhibition patterns for two neuraminidases, *Vibrio cholerae* neuraminidase (VCNA) and human neuraminidase 2 (*hNeu2*), and the most potent inhibitors for each neuraminidase were selected. A kinetic analysis of the selected inhibitors demonstrated that the modification of the aglycone moiety improved the K_I value with little change in the $t_{1/2}$ value of the enzyme activity relative to the basic skeleton (**2**).

1. Introduction

Neuraminidases (NAs; EC 3.2.1.18), an important family of glycoside hydrolases, cleave the glycosidic linkages of sialic acid (Neu5Ac; *N*-acetylneuraminic acid) and regulate the interactions between a cell and the extracellular world during infection, development, inflammation, antigenicity, and cell-cell adhesion.¹ Thus, inhibition of neuraminidases is a potential target for therapeutic reagents. Since the first report in 1969, 2,3-dehydroneuraminic acid (DANA; Figure 1)² has been utilized as a transition state mimetic inhibitor of neuraminidases, and DANA-based inhibitors have occupied the main stream of neuraminidase inhibitor design. During the 1990s, highly potent and selective inhibitors for influenza virus neuraminidases, Zanamivir (Relenza) and GS4071 (Tamiflu), were successfully developed and approved as anti-influenza drugs (Figure 1)^{3,4}; however, similar inhibitors have not been developed for the pathogenesis of other diseases, such as cholera, in which neuraminidases are involved. The difficulty in developing a selective and potent inhibitor for

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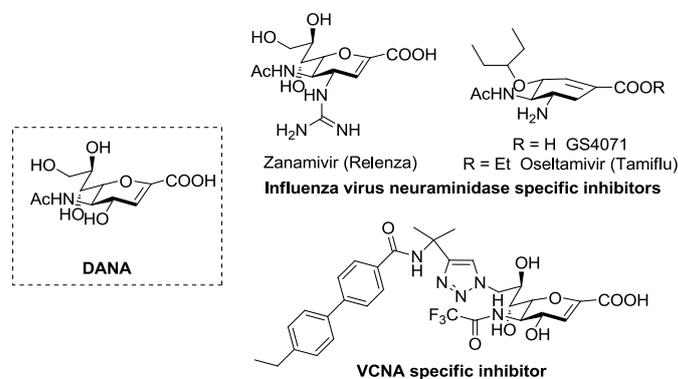


Figure 1. DANA and DANA-based inhibitors.

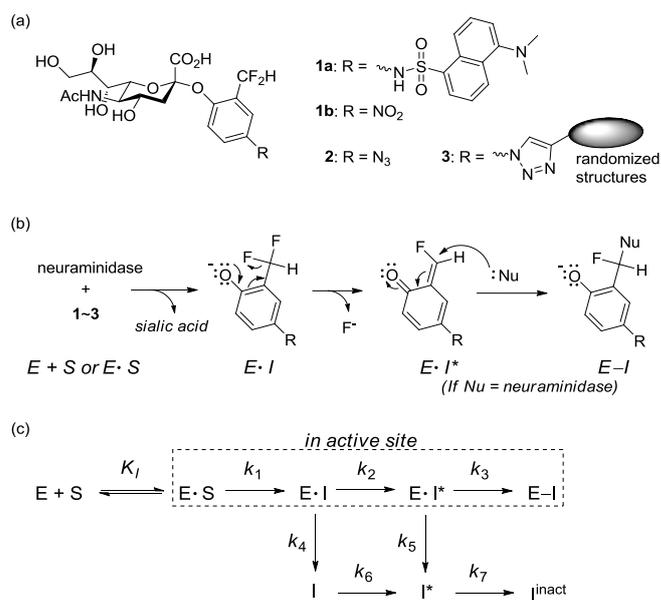


Figure 2. Structure and mechanism of suicide substrates; (a) Structures of the reported suicide substrate **1**, the *p*-azide derivative **2**, and the aglycone-focused library **3**; (b) plausible reaction mechanism of the selective activation and covalent-bond formation of the aglycone moiety in the presence of neuraminidases; (c) plausible kinetics of the inactivation by the 2-difluoromethylphenyl sialosides **1-3**.

neuraminidases might be due to the flexibility of the cavities around the active sites of neuraminidases.^{5,6} The cavities of neuraminidases are primarily composed of a loop moiety on a six-bladed β -propeller fold, and despite their low sequence identities, this structural feature is preserved among every species among eukaryote, prokaryote, and virus.^{7,8} The flexibility of the loop structure makes it difficult to design an inhibitor by a structure-based drug-design strategy using only the static information available from crystal structures. Previously, we reported a strategy to develop a potential inhibitor (Figure 1) for *Vibrio cholerae* neuraminidase (VCNA) using labeling information that was obtained from a dansyl-modified 2-difluoromethylphenyl-type sialoside, **1a** (Figure 2a).^{9,10} A focused library was prepared from 9-N₃-substituted DANA, and an alkyne-terminated compound library was designed based on the labeling information. A tailored inhibitor for VCNA that is selective and potent ($K_I = 73$ nM) was successfully identified. The use of a focused library to probe a suitable structure for the inhibition of the hydrolytic function of neuraminidases is a potential method of finding a potent and selective inhibitor for each neuraminidase.⁹

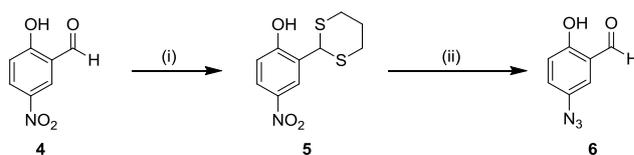
2-Difluoromethylphenyl-type sialosides, which are mechanism-based inactivators for neuraminidases such as the probe **1a**, also provide an attractive alternative to the DANA-based competitive inhibitors. The 2-difluoromethyl-type sialoside is activated by the hydrolytic cleavage of a phenolic glycoside bond. Following fluoride ion desorption, the activated sialoside forms a Michael reaction acceptor, which forms a covalent bond with nucleophiles, and the activated acceptor moiety binds to nucleophilic amino acid side chains to inhibit the enzymatic activity (Figure 2b). The function-specific activation by a neuraminidase (sialidase) and the irreversible labeling ability of the 2-difluoromethylphenyl sialoside have potential as novel drug and diagnostic tools. This 2-difluoromethylphenyl-type glycoside was first reported in 1990 by Danzin *et al.* as a glucosidase inhibitor,¹¹ and it has been applied to various glycosidases, such as galactosidases,¹² *N*-acetyl glucosaminidase¹³; other hydrolases, such as phosphatases,¹⁴ sulfatase,¹⁵ and proteases¹⁶; and neuraminidases.^{10,17-19} Recently, we reported that another 2-difluoromethylphenyl-type sialoside (**1b**) (Figure 2a) inhibits the sialidase activity of a *Trypanosoma cruzi* *trans*-sialidase (TcTS) and that a host cell infection by *T. cruzi* is also inhibited by treatment with compound **1b**.²⁰ A point mutation of an amino acid residue that is detected by the labeling study with **1a** almost completely abolishes the activity of TcTS. Although there are advantages to the 2-difluoromethylphenyl-type sialoside, a relatively high concentration (mM range) of this glycoside is required to inhibit the target enzymes. This problem may be due to the basic architecture of this type of compound; only a glycan moiety can assume the potency and specificity of this type of glycoside to be recognized by target enzymes. To overcome this problem, we sought to design a focused library consisting of the 2-difluoromethylphenyl sialoside skeleton (**2**) to which we could add the aglycone moiety with potent and specific affinity to each target neuraminidase by the so-called [2+3] cyclization click

reaction²¹ as demonstrated by the most potent inhibitor for VCNA.⁹ This modification of **2** at the aglycone moiety is expected to strengthen its interaction with the target neuraminidase not only as the sialoside for hydrolysis [E·S] but also as the hydrolyzed aglycone [E·I] and the activated aglycone [E·I*] to form a covalent bond with the target enzyme [E-I] (Figures 2b, 2c).^{22,23} Previous research has indicated that the activation rate of the aglycone moiety (k_2) is slower than its dissociation rate from an active site on the target enzyme (k_4).^{15,19} The aglycone-focused library, **3**, prepared from **2** might also improve this problem. Neuraminidases must have sufficient space in the active site to recognize large glycoconjugates, such as glycoproteins and glycolipids, and the structurally randomized aglycone moiety of the mechanism-based inhibitor **2**. We now report the preparation of both compound **2** and the focused library **3** and the evaluation of **3** regarding the inhibition potency and selectivity for VCNA and human neuraminidase 2 (*hNeu2*).

2. Results and Discussion

2.1. Preparation and biochemical evaluation of 4-azide-2-difluoromethylphenyl sialoside **2**

The azide-equipped aglycone moiety (**6**) was prepared from 5-nitrosalicylaldehyde (**4**) as shown in Scheme 1. The direct reduction of the nitro group of **4** yielded a complex mixture due to the instability of the amine and intermolecular imine formation. To avoid this instability, the carbonyl group of **4** was converted to a dithioacetal to yield **5**. The nitro group of **5** was then converted to an azide group by treatment with zinc, sodium nitrite, and sodium azide to yield **6**.²⁴ The deprotection of the dithioacetal group²⁵ at the 2-position and the formation of a diazonium salt at the 4-position were simultaneously accomplished by treatment with 4 equivalents of sodium nitrite.

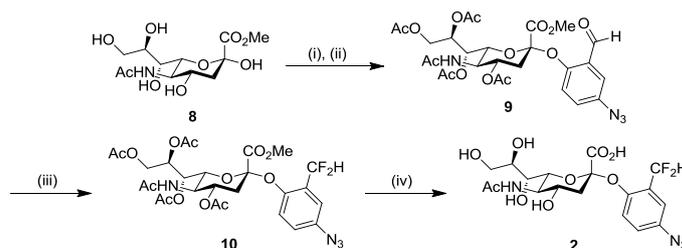


Scheme 1. Preparation of 5-azidesalicylaldehyde (**6**). Reagents and conditions: (i) 1,3-propanedithiol (1.2 eq), $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (1.2 eq), CH_2Cl_2 , r.t., 30 min, 95%, (ii) a: Zn (5.0 eq), AcOH, r.t., 1 h, b: NaNO_2 (4.0 eq), AcOH, 0 °C, 2 h, c: NaN_3 , AcOH, 0 °C \rightarrow r.t., 30 min, 65% (in 3 steps).

The azide-equipped skeleton of the suicide substrate **2** was prepared by an established procedure^{10,11,17} as shown in Scheme 2. The methyl ester of sialic acid **8** was coupled with **6** in a one-pot reaction²⁶ via a glycosyl chloride intermediate to yield the α -sialoside **9**. The aldehyde group of **9** was converted to a difluoromethyl group by treatment with diethylamino sulfur trifluoride (DAST) to yield **10**. The two-step deprotection of **10**, i.e., the sodium methoxide-catalyzed *O*-acyl

replacement in methanol followed by the saponification of the methyl neuramate, yielded the basic skeleton **2**.

The kinetic constants of the time-dependent inhibition (K_I , k_{inact} , and $t_{1/2}$) of VCNA and *h*Neu2 by **2** were explored according to a method of an irreversible inhibitor reported by Kitz and Wilson.^{11,22,23} Each enzyme was preincubated with **2**, and an aliquot of the solution was added to a solution of 2- α -(4-methylumbelliferyl)-*N*-acetylneuraminic acid (4-MU-NANA) to determine the residual activity in 5-min intervals. As shown in Table 1, the mM range of the obtained K_I values suggests that a high concentration of **2** is required to inactivate the neuraminidases because of the poor interaction between the enzymes and the aglycone moiety. Compared to VCNA, *h*Neu2 requires a higher concentration of **2** to be inactivated. The higher K_I value for *h*Neu2 is not surprising, because the K_m value of *h*Neu2 is generally higher than that of VCNA with respect to a common substrate.⁹



Scheme 2. Preparation of **2**. Reagents and Conditions: (i) AcCl, r.t., 24 h, (ii) **6** (1.0 eq), DIEA (3.0 eq), MeCN, r.t., 24 h, 61%, (iii) DAST (2.5 eq), CH₂Cl₂, r.t., 3 h, 72%, (iv) a: sodium methoxide, MeOH, r.t., 30 min, b: 1 N NaOH (1.2 eq), H₂O, r.t., 30 min, 79%.

Table 1. K_I , k_{inact} , and $t_{1/2}$ values of compound **2** for VCNA and *h*Neu2.

Neuraminidase	K_I (mM)	k_{inact} (min ⁻¹)	$t_{1/2}$ (min)
VCNA	2.7	0.11	5.9
<i>h</i> Neu2	20	0.12	5.7

2.2. First screening of the focused library **3** prepared by the click reaction

For the first screening, we adapted a direct analysis of the crude solution of the click reaction as previously reported.⁹ The azide-equipped skeleton **2** was coupled with a commercially available alkyne compound library (230 compounds, Figure SI-10) in the presence of

tris-(benzyltriazolylmethyl)amine (TBTA),²⁷ copper sulfate, and ascorbic acid in 10% dimethyl sulfoxide (DMSO) to afford the aglycone randomized suicide substrate library **3**. Before the first screening, the effect of the reagents of the click reaction on the *h*Neu2 activity was evaluated. In the case of VCNA,⁹ its NA activity was only slightly affected by the reagents. Itzstein *et al.* reported that *h*Neu2 require calcium ion for their NA activity, and the NA activity decreases by one-half by the addition of 7 mM copper salt.²⁸ As shown in Figure 3a, the NA activity of *h*Neu2 disappeared in the presence of a copper salt even at a very low copper concentration (50 μ M CuSO₄) in the presence of 4 mM calcium salt. To negate the effect of the copper salt, the effect of a chelating agent for the copper ion, 2-({2-[bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino) acetic acid (EDTA) was evaluated. Although Itzstein *et al.* reported that 1 mM EDTA also decreased the NA activity of *h*Neu2 by half,²⁸ addition of 1 equivalent of EDTA recovered the NA activity of *h*Neu2 completely, and a small excess of EDTA did not affect the activity (Figure 3). The reported inhibition effect in the presence of 1 mM EDTA might be due to the absence of external addition of calcium ion.²⁸ Based on these results, 2.5 equivalents of EDTA were added to the assay solution to bind the copper ion for the first screening of *h*Neu2.

The yield of the click reaction in each crude solution was roughly estimated from the ratio of **2** and **3** obtained from the product ion signal of the ESI-MS analysis.^{9,29} For 90 of the compounds, no product was determined from the ESI-MS analysis, whereas for 45 of the compounds, the yields of the product were less than 50%. Among these compounds, those possessing a thiourea group and an imine group tended to produce low yields, and this tendency differs from the results obtained using an aliphatic azide.⁹ A yield of greater than 70% was obtained for 78 reaction solutions in the ESI-MS analysis, and these compounds were used for the first screening with VCNA and *h*Neu2 (Figure 4a, SI-11).

The levels of neuraminidase activity that remained after treatment with each click reaction solution of **3** with VCNA and *h*Neu2 are shown in Figure 4b (and Figure SI-12) as the result of the first screening. The triazole derivatives of **3** (30 μ M for VCNA, 100 μ M for *h*Neu2; calculated as though the click reactions produced a 100% yield) were preincubated with VCNA or *h*Neu2 for 15 min and diluted with 2 mM of 4-MU-NANA, and the remaining NA activity was analyzed using a fluorometric assay method.³⁰ A 125- μ M solution of EDTA was also added to the preincubation solution of *h*Neu2, which also contained 50 μ M CuSO₄.

As shown in Figure 4b, the different inhibition patterns of focused library **3** for VCNA and *h*Neu2 clearly demonstrated the potency of our strategy to give the mechanism-based labeling reagent selectivity and inhibition potency for each of the neuraminidase.

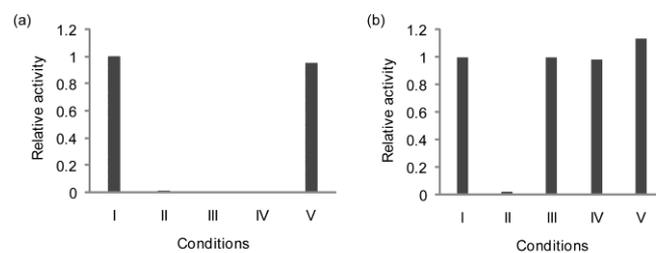


Figure 3. Optimization of the assay conditions for the first screening of *hNeu2*; (a) effect of the reagents in the click reaction solution on the NA activity of *hNeu2*. I: control (no additive), II: CuSO_4 , III: CuSO_4 and TBTA, IV: CuSO_4 , TBTA, and ascorbic acid, V: TBTA and ascorbic acid; (b) effect of EDTA in the presence of $50 \mu\text{M}$ CuSO_4 . I: control (without CuSO_4 or EDTA), II: without EDTA, III: with $50 \mu\text{M}$ EDTA, IV: with $125 \mu\text{M}$ EDTA, V: with $250 \mu\text{M}$ EDTA.

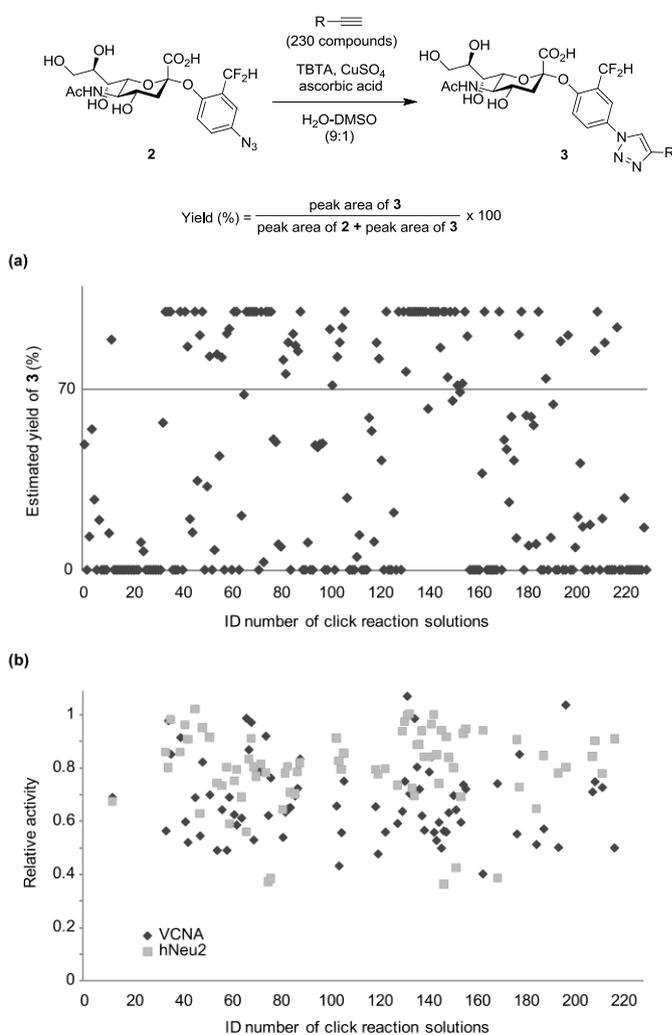


Figure 4. Construction and evaluation of the focused library 3; (a) estimated yield of 3; (b) relative NA activity of VCNA and *hNeu2* remaining after preincubation with each click solution.

2.3. Determination and evaluation of the best structure of **3** as an inhibitor of VCNA and *hNeu2*

To determine the best structure from the focused library **3** to inhibit VCNA, the results in Figure 4b were classified into two groups using 55% of the remaining NA activity as the borderline of the first screening. The compounds that exhibited less than 55% remaining NA activity were prepared as purified compounds. Among the compounds that passed the first screening, the 11 candidates from the focused library **3** shown in Figure 5a could be isolated and used for the second screening. For the second screening, a 50 μM solution of each compound was preincubated with VCNA for 15 min, and the remaining NA activity was analyzed in 2 mM 4-MU-NANA. Compound **3a** (Figure 5b, click ID: 140) was determined to be the most potent inhibitor for VCNA in the focused library **3**. The kinetic constants of the time-dependent inhibition (K_I , k_{inact} , and $t_{1/2}$) of VCNA by compound **3a** were extrapolated^{22,23} to be 61 μM , 0.091 min^{-1} and 7.6 min, respectively.

For *hNeu2*, the results in Figure 4b were classified into two groups using 60% of the remaining activity as the borderline of the first screening. The seven candidates shown in Figure 6a were isolated and used for the second screening. For the second screening, a 100 μM solution of each compound was preincubated with *hNeu2* for 15 min, and the remaining NA activity was analyzed in 1 mM 4-MU-NANA. Compound **3b** (Figure 6b, click ID: 60) was determined to be the most potent inhibitor for *hNeu2* among the focused library **3**. The extrapolated kinetic constants of the time-dependent inhibition (K_I , k_{inact} , and $t_{1/2}$) of *hNeu2* by compound **3b** were determined to be 215 μM , 0.054 min^{-1} and 12.6 min, respectively.

A comparison of the kinetic constants of compounds **2** and **3a** for VCNA and compounds **2** and **3b** for *hNeu2* indicated that the K_I value changed by approximately one-hundredth owing to the modifications in both cases. These differences in binding properties between the basic skeleton and the hit compounds are larger than that of the DANA-based inhibitor, which was reported previously to be approximately one-tenth.⁹ This result indicates that the modification of the aglycone moiety via the triazole linker greatly contributed to make sialoside **3** a good substrate to bind to the active site of the target enzyme, as we expected. In contrast to the K_I values, the change in $t_{1/2}$ for both neuraminidases was slightly increased. This result indicates that the modification of the aglycone moiety did not contribute to the change of the rate of hydrolysis (k_1 , Figure 2c), the activation rate of the released aglycone (k_2), or the covalent bond formation that inactivated the enzyme (k_3). Rather, the substituent on the difluoromethylphenyl moiety is thought to decrease the k_1 value by inhibiting the ability of the enzyme to hydrolyze the substrate, as we observed for our DANA-based inhibitors.⁹ Replacement of the azide functional group on the difluoromethylphenyl group with 1,2,3-triazol might also affect the kinetics (k_{1-3}) based on the electrostatic change at the phenolate moiety.¹¹

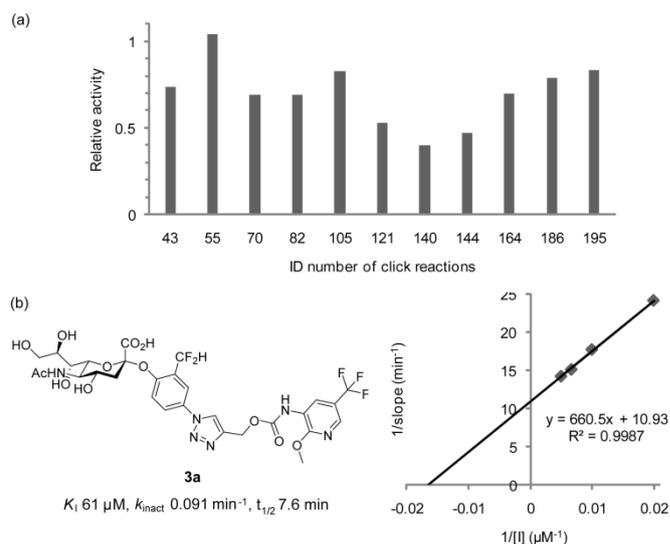


Figure 5. Determination and evaluation of the best inhibitor for VCNA from the focused library **3**. (a) Relative remaining activity of VCNA after incubation with the 11 isolated compounds selected from the first screening. (b) Structure and the kinetic constants of the most potent inhibitor, **3a**, for VCNA among the click library **3**, and the reciprocal plot to determine the kinetic constants.

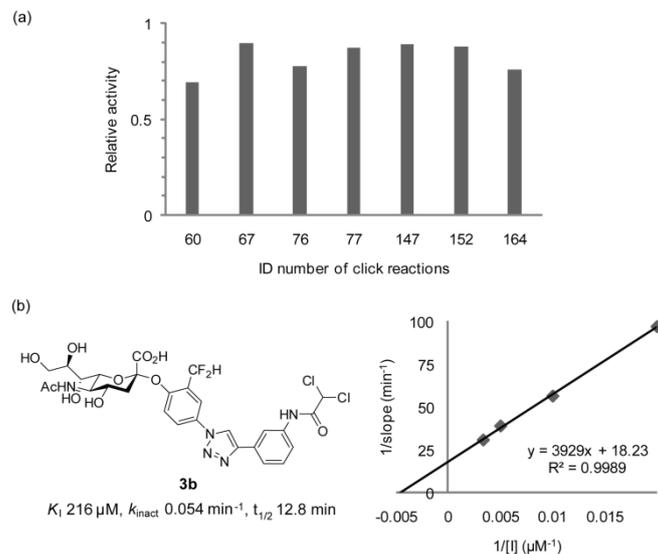


Figure 6. Determination and evaluation of the best inhibitor for *hNeu2* from the focused library **3**. (a) Relative remaining activity of *hNeu2* after incubated with 7 isolated compounds selected by the first screening. (b) Structure and the kinetic constants of the most potent inhibitor, **3b**, for *hNeu2* among click library **3**, and the reciprocal plot to determine the kinetic constants.

3. Conclusions

An aglycone-focused library of 2-difluoromethylphenyl sialoside (**3**) was efficiently constructed from the azide-equipped skeleton **2** and an alkyne-terminated compound library using the click strategy. Two new lead structures, **3a** and **3b**, were determined to be potent and selective inhibitors for VCNA and *hNeu2*, respectively, by a two-step screening, i.e., the crude click reaction mixture was used for the first screening, and the isolated compounds were used for the second screening. Although *hNeu2* was found to be deactivated almost completely by copper ions (μM concentration range) during the click reaction of the first screening, nullification of this effect was achieved by the addition of a small excess of EDTA. Although it tended to decrease the rate of inactivation, the substituent on the aglycone moiety greatly contributed to the binding properties of the sialoside **3** in terms of both potency and selectivity.

Further studies to elucidate the customized lead structure for other targets, such as the influenza virus neuraminidase and *Trypanosoma cruzi trans-sialidase*, are now in progress.

4. Experimental

4.1. General

2- α -(4-methylumbelliferyl)-*N*-acetylneuraminic acid (4-MU-NANA) was synthesized according to published methods.³¹ The alkyne compounds were purchased from Thermo Fisher Scientific Inc. (Cornwall, UK) (230 compounds). The ID number of each alkyne compound assigned to the click reaction solution is shown in Figure SI-10. *Vibrio cholerae* neuraminidase (VCNA) was purchased from Sigma-Aldrich Co. (N6514, St. Louis, MO, USA). Human neuraminidase 2 (*hNeu2*), which was prepared as reported procedure,^{5,9} was a gift from Dr. X.-D. Gao and Mr. R. Miyoshi. The solvents and other reagents for the chemical syntheses were purchased from Sigma-Aldrich Co., Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification unless otherwise noted. Thin-layer chromatography (TLC) was performed on 0.25 mm pre-coated silica gel plates (Merck 60 F-254) and visualized by UV light charring with a methanol solution of 5% H_2SO_4 . Column chromatography was performed using silica gel (Kanto 60 N, $\phi = 40\text{-}50\ \mu\text{m}$; Kanto Chemical Co., Inc.) with air flashing. The ^1H NMR (500 MHz or 600 MHz) and ^{13}C NMR (125 MHz or 150 MHz) spectra were recorded on a BRUKER ADVANCE 500 or a BRUKER ADVANCE 600 spectrometer (Bruker BioSpin Co., Germany) with CDCl_3 , D_2O , and CD_3OD as solvents. The chemical shifts are reported in δ (ppm), and the coupling constants (J) are in hertz (Hz). The following abbreviations were used for signal multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet. The FAB-MS and HR-MS spectra were obtained on a JEOL JMS-HX110 spectrometer (JEOL, Japan) at the

Center for Instrumental Analysis, Hokkaido University. The reverse-phase HPLC was performed using a HITACHI L-6200 HPLC system equipped with an Inertsil-ODS3 column (20 mm x 250 mm, GL Sciences Inc.) and a HITACHI L-7405 UV-Vis detector with a shallow linear gradient at room temperature. The activity of neuraminidase was measured via fluorescence spectrometry in the presence of 4-MU-NANA as a substrate and using a microplate reader (SpectraMax M5, Molecular Devices Co., Sunnyvale, CA).

4.2. Synthesis of compound 5, 6, 9, 10, and 2

4.2.1. 2-(1,3-dithian-2-yl)-4-nitrophenol (5)

$\text{BF}_3 \cdot \text{Et}_2\text{O}$ (12.1 mL, 48 mmol) was gradually added to a solution of 2-hydroxy-5-nitrobenzaldehyde (**4**) (6.7 g, 40 mmol) and propane-1,3-dithiol (4.88 mL, 48 mmol) in dry CH_2Cl_2 (200 mL), and the mixture was stirred under a N_2 gas atmosphere at room temperature. After 2 h, the reaction was quenched by MeOH, and the solvent was removed under reduced pressure followed by recrystallization from ethyl acetate-hexane to yield a light yellow powder (9.78 g, 95%). The product decomposed at 202 °C. ^1H NMR (500 MHz, CDCl_3 , 300 K): δ 8.26 (d, 1H, J = 2.5 Hz, H-3), 8.13 (dd, 1H, J = 2.6, 9.0 Hz, H-5), 7.43 (s, 1H, OH), 6.98 (d, 1H, J = 9.0 Hz, H-6), 5.43 (s, 1H, PhCH), 3.09 (dd, 2H, J = 12.4 Hz, S-C(H_{eq}) H_{ax} - CH_2), 2.97 (ddd, 2H, J = 3.2, 3.6, 14.0 Hz, S-C(H_{ax}) H_{eq} - CH_2), 2.23 (dtt, 1H, J = 2.1, 2.2, 14.3 Hz, CH_2 -C(H_{ax}) H_{eq} - CH_2), 1.97 (dtt, 1H, J = 2.0, 13.2 Hz, CH_2 -C(H_{eq}) H_{ax} - CH_2); ^{13}C NMR (125 MHz, CDCl_3 , 300 K): δ 160.5, 141.2, 126.1, 125.8, 124.1, 118.0, 46.7, 31.2, 24.5. HRMS (ESI) Calcd. for $\text{C}_{10}\text{H}_{10}\text{NO}_3\text{S}_2$ [$\text{M}-\text{H}$] 256.0108, found 256.0111.

4.2.2. 5-azido-2-hydroxybenzaldehyde (6)

Zn powder (5.6 g, 86 mmol) was added to a solution of 2-(1,3-dithian-2-yl)-4-nitrophenol (**5**) (4.4 g, 17 mmol) in AcOH (100 mL), and the mixture was stirred at room temperature for 1 h. The residue was filtered through a celite pad, and the pad was washed with EtOAc. The combined solutions were removed under reduced pressure. The residue was dissolved in glacial acetic acid (100 mL) and cooled on an ice bath to < 5 °C. While stirring, a solution of sodium nitrate (1.72 g, 69 mmol) dissolved in a minimum amount of water was added dropwise, and mixing was continued for 2 h. Sodium azide (1.70 g, 26 mmol) was dissolved in a minimal amount of water and added dropwise to the mixture. After 30 min, the glacial acetic acid was roughly removed under reduced pressure. The residue was extracted with EtOAc, and the organic phases were washed with sat. NaHCO_3 , brined, dried over MgSO_4 , filtered, and concentrated. Purification by flash column chromatography on a silica gel (hexane:EtOAc = 6:1) yielded **6** as an orange solid (1.83 g, 65%). ^1H NMR (600 MHz, CDCl_3 , 300 K): δ 10.83 (s, 1H, OH), 9.86 (s, 1H, CHO), 7.20 (m, 2H, H-arom.), 7.00 (t, 1H, H-arom.); ^{13}C NMR (150 MHz, CDCl_3 , 300 K): δ 195.7, 158.8, 132.1, 127.9, 122.6, 120.8, 119.4. HRMS (ESI) Calcd. for $\text{C}_7\text{H}_5\text{N}_3\text{O}_2\text{Na}$ [$\text{M}+\text{Na}$] $^+$ 186.0274, found 186.0274.

4.2.3. Methyl [4-azido-2-formylphenyl

5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranoside]onate (9)

A suspension of methyl 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosonate (**8**) (969 mg, 3.0 mmol) in acetyl chloride (10 mL) was vigorously stirred under a N₂ gas atmosphere at room temperature for 36 h. The resulting solution was concentrated and co-evaporated with toluene three times. Diisopropylethylamine (1.57 mL, 9.0 mmol) was added dropwise to a suspension of the residue and 5-azido-2-hydroxybenzaldehyde (**6**) (490 mg, 3.0 mmol) in acetonitrile (30 mL), and the mixture was stirred under a N₂ gas atmosphere at room temperature for 24 h. After the solvent was evaporated in vacuo, the residue was purified by flash column chromatography on a silica gel (hexane:acetone = 7:4) to yield compound **9** as a yellow powder (1.16 g, 61%). ¹H NMR (600 MHz, CDCl₃, 300 K) δ 10.35 (s, 1H, CHO), 7.49 (d, 1H, J = 3.0 Hz, H-arom.), 7.31 (d, 1H, J = 4.2 Hz, H-arom.), 7.19 (dd, 1H, H-arom.), 5.35 (m, 3H, NH, H-7, H-8), 4.99 (ddd, 1H, J = 4.8, 10.8, 12.0 Hz, H-4), 4.46 (d, 1H, J = 10.8 Hz, H-6), 4.26 (dd, 1H, J = 2.4, 11.4 Hz, H-9a), 4.06-4.12 (m, 2H, H-5, H-9b), 3.65 (s, 3H, COOMe), 2.79 (dd, 1H, J = 4.8, 12.6 Hz, H-3a), 2.28 (t, 1H, J = 12.0 Hz, H-3b), 2.15, 2.14, 2.06, 2.05 (s, 3H each, OAc), 1.93 (s, 3H, NHAc); ¹³C NMR (150 MHz, CDCl₃, 300 K) δ 188.5, 170.9, 170.8, 170.3, 170.1, 170.0, 167.4, 152.9, 136.9, 129.1, 128.5, 128.2, 126.1, 122.5, 117.7, 100.6, 73.5, 68.5, 68.3, 67.1, 62.1, 53.2, 49.5, 38.2, 23.2, 21.0, 20.82, 20.75. HRMS (ESI) Calcd. for C₂₇H₃₂N₄O₁₄Na[M+Na]⁺ 659.1807, found 659.1806.

4.2.4. Methyl [2-difluoromethyl-4-azidophenyl

4,7,8,9-tetra-O-acetyl-5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranoside]onate (10)

Diethylamino sulfur trifluoride (530 μ L, 3.88 mmol) was added dropwise to a solution of **9** (1.01 g, 1.55 mmol) in dichloromethane (15 mL), and the reaction mixture was stirred under a N₂ gas atmosphere at room temperature for 3 h. MeOH was added to the reaction mixture at 0 °C, and the mixture was concentrated in vacuo. The residue was purified by flash column chromatography on a silica gel (hexane:acetone = 7:4) to yield compound **10** as a light yellow powder (738 mg, 72%). ¹H NMR (500 MHz, CDCl₃, 300 K) δ 7.34 (d, 1H, J = 8.4 Hz, H-arom.), 7.21 (d, 1H, J = 2.4 Hz, H-arom.), 7.07 (dd, 1H, J = 2.4, 8.4 Hz, H-arom.), 6.88 (t, 1H, J = 55.2 Hz, CHF₂), 5.43 (d, 1H, J = 10.2 Hz, NH), 5.34-5.38 (m, 2H, H-7, H-8), 4.94 (m, 1H, H-4), 4.44 (d, 1H, J = 10.8 Hz, H-6), 4.28 (dd, 1H, J = 2.4, 12.8 Hz, H-9a), 4.13 (dd, 1H, J = 4.8, 12.6 Hz, H-9b), 4.09 (q, 1H, J = 10.2 Hz, H-5), 3.66 (s, 3H, COOMe), 2.74 (dd, 1H, J = 4.8, 12.6 Hz, H-3a), 2.24 (t, 1H, J = 6.6 Hz, H-3b), 2.15, 2.14, 2.06, 2.05 (s, 3H each, OAc), 1.92 (s, 3H, NHAc); ¹³C NMR (125 MHz, CDCl₃, 300K) δ 179.2, 170.9, 170.7, 170.4, 167.8, 148.9, 136.7, 127.5, 127.4, 127.2, 122.7, 121.7, 117.1, 112.6, 111.1, 109.5, 101.0, 73.8, 69.0, 68.8, 67.5, 62.5, 53.5, 49.8, 38.5, 23.5, 21.3, 21.1. HRMS (ESI) Calcd. for C₂₇H₃₂N₄O₁₃F₂Na[M+Na]⁺ 681.1826, found 681.1827.

4.2.5. 2-difluoromethyl-4-azidophenyl

5-acetoamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosidonic acid (2)

A solution of 5 M sodium methoxide in MeOH (450 μ L) was added dropwise to a solution of **10** (620 mg, 0.94 mmol) in MeOH (10 mL), and the reaction mixture was stirred under a N₂ gas atmosphere at room temperature for 2 h. To neutralize the excess sodium methoxide, 1% acetic acid was added, and the mixture was concentrated in vacuo. The residue was dissolved in H₂O (8.5 mL), and 1 N NaOH (1.5 mL) was added dropwise to the mixture, which was then stirred at room temperature for 1 h. The reaction mixture was neutralized with 1% acetic acid, and the residue was purified by reverse-phase chromatography (Wakogel 50C18, H₂O:acetonitrile = 3:1) and lyophilized to yield compound **2** as a beige solid (354 mg, 79%). ¹H NMR (500 MHz, D₂O, 300 K) δ 7.25 (d, 1H, J = 8.9 Hz, H-arom.), 7.21 (d, 1H, J = 1.8 Hz, H-arom.), 7.05 (d, 1H, J = 8.8 Hz, H-arom.), 6.96 (t, 1H, J = 55.2 Hz, CHF₂), 3.61-3.81 (m, 5H, H-4, 5, 6, 9a), 3.46-3.51 (m, 2H, H-7, 9b), 2.78 (dd, 1H, J = 1.8, 12.6 Hz, H-3a), 1.91 (s, 3H, NHAc), 1.84 (t, 1H, J = 12.5 Hz, H-3b); ¹³C NMR (125 MHz, CDCl₃, 300K) δ 175.1, 171.9, 136.3, 122.9, 122.1, 116.4, 113.4, 111.6, 109.7, 103.0, 73.5, 71.6, 68.1, 68.0, 62.6, 51.6, 40.3, 22.0. HRMS (ESI) Calcd. for C₁₈H₂₂N₄O₉F₂Na[M+Na]⁺ 499.1247, found 499.1249.

4.3. Construction of the libraries 3 for first screening

Aqueous solutions of compound **2** (100 mM), sodium ascorbate (66.7 mM), and CuSO₄ (100 mM) and dimethylsulfoxide solutions of TBTA (100 mM) and the alkyne compounds (100 mM) were prepared. Each alkyne compound (5 μ L) was added to CuSO₄ (2.5 μ L), TBTA (2.5 μ L), compound **2** (5 μ L), and sodium ascorbate (35 μ L), and the mixture was incubated for 8 h. The yields of the triazole compounds were roughly estimated by comparison between the ESI-MS signal intensity of compound **2** and that of each triazole product **3**. The solutions with a greater than 70% yield of the triazole product **3** were used for the following screening assay.

4.4. General procedure for preparation of click compound 3

Solutions of compound **2** (100 mM), sodium ascorbate (250 mM), and CuSO₄ (100 mM) in H₂O and solutions of TBTA (100 mM) and the alkyne compounds (100 mM) in dimethylsulfoxide were prepared. Each alkyne compound (150 μ L) was added to CuSO₄ (75 μ L), TBTA (75 μ L), compound **2** (150 μ L), and sodium ascorbate (150 μ L), and the mixture was incubated for 18 h. The resulting mixture was purified by reverse-phase HPLC using an H₂O-acetonitrile linear gradient system and then lyophilized to yield **3**.

4.4.1. 4-[[2-methoxy-5-(trifluoromethyl)pyridin-3-ylcarbamoyloxy]methyl]-1H-1,2,3-triazol-1-yl-2-(difluoromethyl)phenyl

5-acetoamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosidonic acid (3a)

White solid (64%). ^1H NMR (500 MHz, CD_3OD , 300 K) δ 8.66 (s, 1H, H-triazole), 8.52 (s, 1H, H-arom.), 8.14 (s, 1H, H-arom.), 8.03 (s, 1H, H-arom.), 7.91 (d, J = 8.6 Hz, H-arom.), 7.78 (d, J = 8.8 Hz, H-arom.), 7.18 (t, 1H, J = 55.3 Hz, CHF_2), 5.42 (s, 2H, CH_2), 4.07 (s, 3H, OMe), 3.95 (d, 1H, J = 6.2 Hz, H-6), 3.82-3.90 (m, 4H, H-4, 5, 8, 9a), 3.65 (dd, 1H, J = 5.8, 11.6 Hz, H-9b), 3.57 (d, 1H, J = 9.1 Hz, H-7), 3.02 (d, 1H, J = 10.6 Hz, H-3a), 2.05 (s, 3H, NHAc), 1.99 (t, 1H, J = 11.4 Hz, H-3b); ^{13}C NMR (125 MHz, CD_3OD , 300 K) δ 174.1, 153.8, 136.8, 132.4, 123.4, 123.2, 122.8, 122.0, 120.3, 120.0, 119.7, 117.7, 111.2, 74.4, 71.6, 68.8, 67.5, 63.3, 57.7, 53.5, 52.5, 41.2, 21.2. HRMS (ESI) Calcd. for $\text{C}_{29}\text{H}_{30}\text{N}_6\text{O}_{12}\text{F}_5$ $[\text{M}-\text{H}]^-$ 749.1847, found 749.1851.

4.4.2. 4-[3-(2,2-dichloroacetamido)phenyl]-1H-1,2,3-triazol-1-yl-2-(difluoromethyl)phenyl

5-acetoamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosidonic acid (3b)

White solid (46%). ^1H NMR (500 MHz, CD_3OD , 300 K) δ 8.95 (s, 1H, H-triazole), 8.13 (s, 1H, H-arom.), 8.08 (s, 1H, H-arom.), 7.96 (d, 1H, J = 8.5 Hz, H-arom.), 7.79 (d, 1H, J = 8.9 Hz, H-arom.), 7.73 (d, 1H, J = 7.7 Hz, H-arom.), 7.68 (d, 1H, J = 8.1 Hz, H-arom.), 7.48 (dd, J = 7.9 Hz, H-arom.), 7.17 (t, 1H, J = 55.1 Hz, CHF_2), 6.43 (s, 1H, CHCl_2), 3.94 (d, 1H, J = 9.7 Hz, H-6), 3.78-3.89 (m, 4H, H-4, 5, 8, 9a), 3.64 (dd, 1H, J = 5.4, 11.2 Hz, H-9b), 3.56 (d, 1H, J = 4.5 Hz, H-7), 3.00 (dd, 1H, J = 4.1, 12.7 Hz, H-3a), 2.03 (s, 3H, NHAc), 1.96 (dd, 1H, J = 11.9 Hz, H-3b); ^{13}C NMR (125 MHz, CD_3OD , 300 K) δ 174.1, 173.3, 163.1, 147.7, 138.1, 132.5, 131.0, 129.4, 123.0, 122.6, 122.3, 120.2, 119.3, 117.3, 74.4, 71.5, 68.8, 67.5, 66.8, 63.3, 52.5, 41.2, 21.2. HRMS (ESI) Calcd. for $\text{C}_{28}\text{H}_{28}\text{N}_5\text{O}_{10}\text{F}_2\text{Cl}_2$ $[\text{M}-\text{H}]^-$ 702.1187, found 702.1199.

4.5. Biological assay

4.5.1. First and second screening (Vibrio cholerae neuraminidase; VCNA)

For the VCNA pre-incubation experiment, VCNA (8.3 mU) and inhibitor **3** (30 μM for 1st screening, 50 μM for 2nd screening; final concentration) were pre-incubated in 100 mM MES buffer (pH 6.5) with 4 mM CaCl_2 at 25 $^\circ\text{C}$ in a final volume of 50 μL . At 5-min intervals (0-15 min), aliquots (3 μL) of the pre-incubated enzyme solution were transferred into the assay medium and assayed for residual activity.

For the VCNA residual activity assay, the VCNA activity was measured in 100 mM MES buffer (pH 6.5) containing 4 mM CaCl_2 and 2 mM 4-MU-NANA at 37 $^\circ\text{C}$. The enzyme reaction was initiated by the addition of the pre-incubated enzyme solution in a final volume of 60 μL . The VCNA activity was estimated by monitoring the increase in the fluorescence due to the hydrolysis of

4-MU-NANA using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The residual activity was estimated by comparison with the control, which was obtained in the absence of the inhibitor.

4.5.2. Determination of K_I and k_{inact} and $t_{1/2}$ values (VCNA)

For the VCNA pre-incubation experiment, VCNA (8.3 mU) and several concentrations of inhibitor **3** (10-200 μ M) were pre-incubated in 100 mM MES buffer (pH 6.5) with 4 mM CaCl_2 at 25 °C in a final volume of 50 μ L. At 5-min intervals (0-15 min), aliquots (3 μ L) of the pre-incubated enzyme solution were transferred into the assay medium and assayed for residual activity.

For the VCNA residual activity assay, the VCNA activity was measured in 100 mM MES buffer (pH 6.5) containing 4 mM CaCl_2 and 2 mM 4-MU-NANA at 25 °C. The enzyme reaction was initiated by the addition of the pre-incubated enzyme solution in a final volume of 60 μ L. The VCNA activity was estimated by monitoring the increase in the fluorescence due to the hydrolysis of 4-MU-NANA using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The residual activity was estimated by comparison with the control, which was obtained in the absence of the inhibitor.

4.5.3. First and second screening (Human neuraminidase 2; *hNeu2*)

For the Neu2 pre-incubation experiment, Neu2 (10.6 mU), inhibitor**3** (final concentration 100 μ M for 1st and 2nd screening), EDTA (final concentration 0.25 mM), and BSA (13.2 μ g) were pre-incubated in 33 mM MES buffer (pH 6.5), 4 mM CaCl_2 at 25 °C in final volume of 32 μ L. At 5-min intervals (0 ~ 15 min), aliquots (4 μ L) of the pre-incubation enzyme solution were transferred into assay medium and assayed for the residual activity.

For the Neu2 residual activity assay, Neu2 activity was measured in 33 mM MES buffer (pH 6.5), 4 mM CaCl_2 and 1 mM 4-MU-NANA at 37 °C. The enzyme reaction was initiated by addition of pre-incubation enzyme solution in a final volume of 60 μ L. The Neu2 activity was estimated by monitoring the increase of fluorescence due to hydrolysis of 4-MU-NANA using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The residual activity was estimated by comparison with control, which was obtained without inhibitor.

4.5.4. Determination of K_I and k_{inact} and $t_{1/2}$ values (*hNeu2*)

For the *hNeu2* pre-incubation experiment, *hNeu2* (10.6 mU), several concentrations of inhibitor **3** (50-500 μ M), and BSA (20 μ g) were pre-incubated in 33 mM MES buffer (pH 6.5) with 4 mM CaCl_2 at 25 °C in a final volume of 50 μ L. At 5-min intervals (0-15 min), aliquots (4 μ L) of the pre-incubated enzyme solution were transferred into the assay medium and assayed for residual activity.

For the *hNeu2* residual activity assay, the *hNeu2* activity was measured in 33 mM MES buffer (pH 6.5) containing 4 mM CaCl₂ and 1 mM 4-MU-NANA at 25 °C. The enzyme reaction was initiated by the addition of the pre-incubated enzyme solution in a final volume of 60 μL. The *hNeu2* activity was estimated by monitoring the increase in the fluorescence due to the hydrolysis of 4-MU-NANA using an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The residual activity was estimated by comparison with the control, which was obtained in the absence of the inhibitor.

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References and notes

1. Varki, A. *Nature* **2007**, *446*, 1023–1029.
2. Meindl, P.; Tuppy, H. *Monatsh. Chem.* **1969**, *100*, 1295–1306.
3. von Itzstein, M.; Wu, W. Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Van Phan, T.; Smythe, M. L.; White, H. F.; Oliver, S. W.; Colman, P. M.; Varghese, J. N.; Ryan, D. M.; Woods, J. M.; Bethell, R. C.; Hotham, V. J.; Cameron, J. M.; Penn, C. R. *Nature* **1993**, *363*, 418–423.
4. Kim, C. U.; Lew, W.; Williams, M. A.; Liu, H. T.; Zhang, L. J.; Swaminathan, S.; Bischofberger, N.; Chen, M. S.; Mendel, D. B.; Tai, C. Y.; Laver, W. G.; Stevens, R. C. *J. Am. Chem. Soc.* **1997**, *119*, 681–690.
5. Chavas, L. M. G.; Tringali, C.; Fusi, P.; Venerando, B.; Tettamanti, G.; Kato, R.; Monti, E.; Wakatsuki, S. *J. Biol. Chem.* **2005**, *280*, 469–475.
6. Russell, R. J.; Haire, L. F.; Stevens, D. J.; Collins, P. J.; Lin, Y. P.; Blackburn, M.; Hay, A. J.; Gamblin, S. J.; Skehel, J. J. *Nature* **2006**, *443*, 45–49.
7. Crennell, S.; Garman, E.; Laver, G.; Vimr, E.; Taylor, G. *Structure* **1994**, *2*, 535–544.
8. Buschiazzo, A.; Alzari, P. M. *Curr. Opin. Chem. Biol.* **2008**, *12*, 565–572.

9. Hinou, H.; Miyoshi, R.; Takasu, Y.; Kai, H.; Kuroguchi, M.; Arioka, S.; Gao, X. D.; Miura, N.; Fujitani, N.; Omoto, S.; Yoshinaga, T.; Fujiwara, T.; Noshi, T.; Togame, H.; Takemoto, H.; Nishimura, S.-I. *Chem. Asian. J.* **2011**, *6*, 1048–1056.
10. Hinou, H.; Kuroguchi, M.; Shimizu, H.; Nishimura, S.-I. *Biochemistry* **2005**, *44*, 11669–11675.
11. Halazy, S.; Berges, V.; Ehrhard, A.; Danzin, C. *Bioorg. Chem.* **1990**, *18*, 330–344.
12. Kuroguchi, M.; Nishimura, S.-I.; Lee, Y. C. *J. Biol. Chem.* **2004**, *279*, 44704–44712.
13. Ichikawa, M.; Ichikawa, Y. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1769–1773.
14. Wang, Q. P.; Dechert, U.; Jirik, F.; Withers, S. G. *Biochem. Biophys. Res. Commun.* **1994**, *200*, 577–583.
15. Ahmed, V.; Liu, Y.; Taylor, S. D. *ChemBioChem* **2009**, *10*, 1457–1461.
16. Zhu, Q.; Girish, A.; Chattopadhyaya, S.; Yao, S. Q. *Chem. Commun.* **2004**, 1512–1513.
17. Driguez, P.-A.; Barrere, B.; Chantegrel, B.; Deshayes, C.; Doutheau, A.; Quash, G. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1361–1366.
18. Lu, C.-P.; Ren, C.-T.; Lai, Y.-N.; Wu, S.-H.; Wang, W.-M.; Chen, J.-Y.; Lo, L.-C. *Angew. Chem. Int. Ed.* **2005**, *44*, 6888–6892.
19. Kwan, D. H.; Chen, H.-M.; Ratananikom, K.; Hancock, S. M.; Watanabe, Y.; Kongsaree, P. T.; Samuels, A. L.; Withers, S. G. *Angew. Chem. Int. Ed.* **2011**, *50*, 300–303.
20. Carvalho, S.; Sola-Penna, M.; Oliveira, I.; Pita, S.; Goncalves, A.; Neves, B.; Sousa, F.; Freire-de-Lima, L.; Kuroguchi, M.; Hinou, H.; Nishimura, S.-I.; Previato, J.; Todeschini, A. *Glycobiology* **2010**, *20*, 1034–1045.
21. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2001**, *40*, 2004–2021.
22. Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245–3249.
23. Copeland, R. A. *Evaluation of enzyme inhibitors in drug discovery: A guide for medicinal chemist and pharmacologists*; Wiley-Interscience: Hoboken, NJ, 2005; pp 214–248.
24. Ritchie, C. D.; Wright, D. J. *J. Am. Chem. Soc.* **1971**, *93*, 2429–2432.
25. Khan, A. T.; Mondal, E.; Sahu, P. R. *Synlett* **2003**, 377–381.
26. Kuboki, A.; Sekiguchi, T.; Sugai, T.; Ohta, H. *Synlett* **1998**, 479–482.
27. Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853–2855.
28. Albouz-Abo, S.; Turton, R.; Wilson, J. C.; von Itzstein, M. *FEBS Lett.* **2005**, *579*, 1034–1038.
29. Hosoguchi, K.; Maeda, T.; Furukawa, J.-i.; Shinohara, Y.; Hinou, H.; Sekiguchi, M.; Togame, H.; Takemoto, H.; Kondo, H.; Nishimura S.-I. *J. Med. Chem.* **2010**, *53*, 5607–5619.
30. Palese, P.; Bucher, D.; Kilbaume, E. D. *Appl. Microbiol.* **1973**, *98*, 195–201.
31. Myers, R. W.; Lee, R. T.; Lee, Y. C.; Thomas, G. H.; Reynolds, L. W.; Uchida, Y. *Anal. Biochem.* **1980**, *101*, 166–174.