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One-Bead–One-Inhibitor–One-Substrate Screening of Neuraminidase Activity

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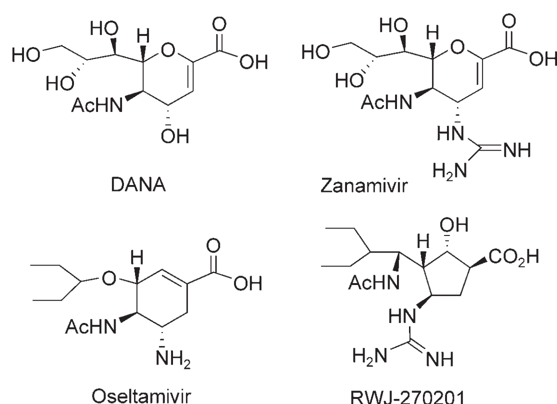
Given the eminent threat of a 21st century flu pandemic, the search for novel antiviral compounds is an increasingly important area of research. Recent developments in antiviral research have established the viability of targeting viral neuraminidase (NA), an enzyme that cleaves sialic acid from the cell-surface-mediating passage of the virus in the respiratory tract. N*-acetyl neuraminic acid (NeuAc) is the substrate for NA, and analogues of this core structure have been commercialized as antiviral therapeutics. Recent studies have established that this system is well suited for combinatorial approaches to drug discovery. An important step in the process is to develop solid-phase screening technologies. The feasibility of performing competitive solid-phase NA assays is reported herein. Initially, a fluorogenic NeuAc substrate was immobilized on solid support, and the ability of three NAs (*Clostridium perfringens, Salmonella typhimurium, *and* Vibrio cholerae*) to cleave the substrate was shown to be analogous to solution-phase assays. The solid support was then bifunctionalized with the fluorogenic NeuAc substrate and one of two known inhibitors (DANA and Zanamivir). The ability of NA to cleave NeuAc from the solid support when simultaneously presented with an inhibitor was shown to be enzyme dependent. As expected, simultaneous presentation of NeuAc and DANA, a nonspecific inhibitor, led to diminished activity for all three enzymes tested. In contrast, dual presentation of NeuAc and the selective inhibitor Zanamivir only showed significant activity against* Vibrio cholerae.

Introduction

Influenza causes considerable disease burden each year. Although vaccination is the first line of defense against influenza A and B viruses, antiviral therapy can aid in controling the impact of the disease.[1] Influenza neuraminidase (NA) is a tetrameric protein embedded in the viral coat that acts in concert with hemagglutinin (HA) to mediate passage of the virus to and from sites in the respiratory tract.[2] HA recognizes host-associated *N*-acetyl neuraminic acid (NeuAc) in the initial stages of infection. As new viral particles emerge, influenza NA cleaves NeuAc to facilitate migration of the virus. Loss of NA activity is correlated with reduced infectivity; this has prompted the development of specific inhibitors as anti-influenza drugs.[3]

The crystal structure of neuraminidase complexed with the inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA, Scheme 1) indicates that a tri-arginyl cluster (R118, R292, R370) complexes with the carboxylate of DANA; D151 and R152 hydrogen bond to the C-4 hydroxyl, and the *N*-acetyl group interacts with W178. DANA is a nonselective NA inhibitor that exerts its action on bacterial, viral, and mammalian enzymes but had no efficacy in animal modes of viral infection.[4] This finding suggested that selective inhibitors of influenza NA were needed, and, since the active-site amino acids of NA are conserved among all types and subtypes of influenza viruses, the DANA core structure served as a platform for discovery. These endeavors led to the development of Zanamivir[5] and Oseltamivir,[6] the two most recently commercialized anti-influenza drugs (Scheme 1). Additionally, the synthesis and biological evaluation of a functionalized cyclopentane analogue have been reported (RWJ-270201).[7] This compound is a potent inhibitor of wild-type NA and some Zanamivir- and Oseltamivir-resistant influenza A and B virus variants.[8]



Scheme 1. Transition-state analogue NA inhibitors.

Success in achieving selective NA inhibition by modifying the glycerol side chain to increase hydrophobic interactions led to several other studies of Zanamivir analogues. Honda

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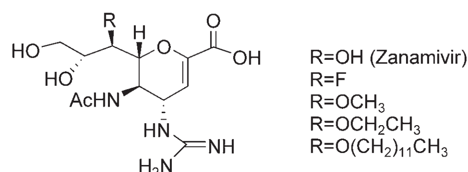
et al. published a series of papers describing the synthesis and biological evaluation of 7-*O*-alkylated Zanamivir analogues. Their first studies showed that the replacement of 7-hydroxyl with fluorine gave an improved activity profile (Scheme 2). Methylation of the 7-hydroxyl led to slightly diminished anti-



Scheme 2. Potent C-7 analogues of Zanamivir.

NA activity, but the ethyl ether was actually more active than Zanamivir in the NA assay, and both ethers showed increased activity in plaque-reduction assays.[9] In general, compounds with alkyl ethers of less than 12 carbons showed potent (nM) inhibition of NA and improved activity relative to Zanamivir in cell-culture assays.[10] These combined studies indicate that modifications of the glycerol side chain of Zanamivir are tolerated and often beneficial.

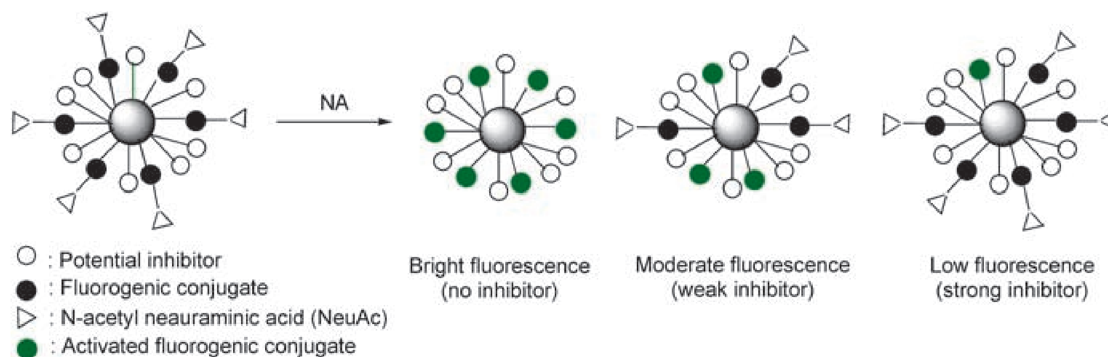
Combinatorial chemistry has developed into a useful method for the rapid identification of lead compounds for drug discovery.[11] A critical step in the process is the facile identification of positive hits from a large collection of compounds. When libraries are prepared in solution,[12] NA activity can be monitored by using a synthetic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid, which is cleaved to yield a fluorescent product (4-methylumbelliferone) that can be quantified fluorometrically.[13] Our combinatorial studies typically involve one-bead–one-compound strategies,[14] in which split-and-mix methods generate millions of beads, each presenting a single compound. Solution-phase assays are not applicable to one-bead–one-compound libraries because it is impossible to identify the bead providing the activity. To circumvent this problem, we have developed an on-bead assay of NA that allows simultaneous monitoring of substrate cleavage and inhibitor efficiency. This is a critical step toward developing combinatorial platforms for the discovery of NA inhibitors.

The current system was inspired by Meldal's FRET-substrate assay, which utilizes one-bead–two-compound constructs for solid-phase screening.[15] In the basic NA assay design (Scheme 3), each bead from the combinatorial library contains a fluorogenic NeuAc substrate and a potential inhibitor. When the resin is incubated with neuraminidase, those beads having ligands that do not inhibit neuraminidase quickly undergo cleavage of NeuAc from the fluorogenic substrate, and the bead shows strong fluorescence. In contrast, beads with potent inhibitors do not fluoresce and can be selected as positive hits for structural characterization. Two critical features of the assay include the design of a NeuAc substrate with a fluorogenic indicator that maintains activity when conjugated to the solid support and the confirmation that inhibitors bound to the solid support are still active. We report here our studies establishing the feasibility of monitoring NA activity on a solid support by using a modified 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid substrate in combination with known NA inhibitors in a one-bead–two-compound assay.

Results and Discussion

Initial studies focused on preparing a fluorogenic NeuAc substrate for conjugation to solid support. As noted above, 2′-(4-methylumbelliferyl)-α-D-acetyl-neuraminic acid is commonly used in solution-phase assays, so we designed a fluorogenic substrate functionalized for immobilization on solid support based upon this model (Scheme 4). Resorcinol (**1**) was first converted to 7-hydroxycoumarin-4-acetic acid methyl ester (**2**).[16] Glycosylation of 2-deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid methyl ester[17] with **2** gave the protected substrate **3**, deprotection followed to yield **4**, which was immobilized on PL-PEGA[18] resin by standard amide coupling.

To test the activity of resin-bound fluorogenic substrate (**5**), four known viral neuraminidase inhibitors (Figure 1, top) were preincubated with three different bacterial neuraminidases (*Clostridium perfringens*, *Salmonella typhimurium*, and *Vibrio cholerae*) individually. In a control experiment, no inhibitor was added. Immobilized **5** was then added to the solution, and the fluorescence intensity was measured after 20 min incubation with a fluorescence plate reader. As shown in Figure 1, **5**



Scheme 3. Illustration of one-bead–two-compound screening of neuraminidase inhibitors. The bead is functionalized with a potential inhibitor and NeuAc conjugated to a fluorophore that is activated when NeuAc is cleaved. When incubated with NA, beads with no inhibitors are bright whereas beads with strong inhibitors only weakly fluoresce.

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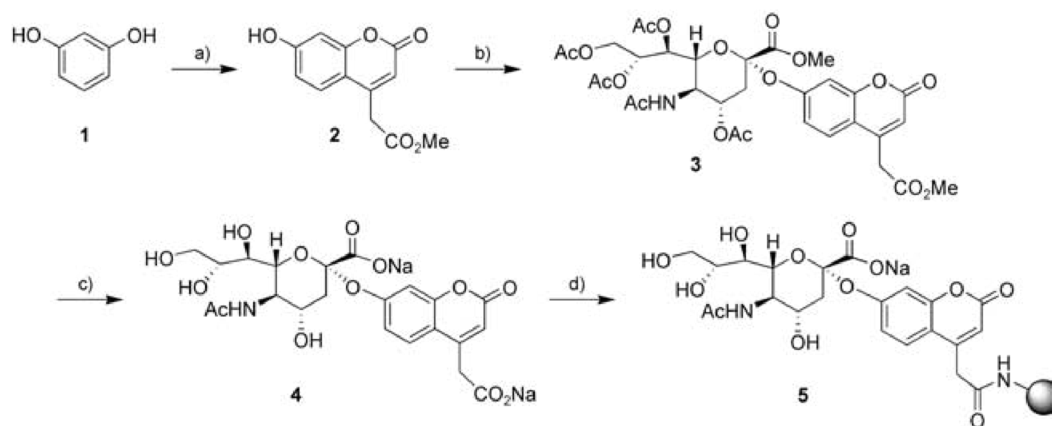

Scheme 4. Synthesis of resin-bound fluorogenic NeuAc. a) 1,3-Acetonedicarboxylic acid, 70% H_2SO_4; $SOCl_2$, MeOH; b) 2-Deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-*N*-acetylneuraminic acid, Ag_2CO_3, CH_3CN; c) NaOMe, MeOH; aq. NaOH; d) PL-PEGA resin, HOBt, DIC, Bu_3N, DMF.

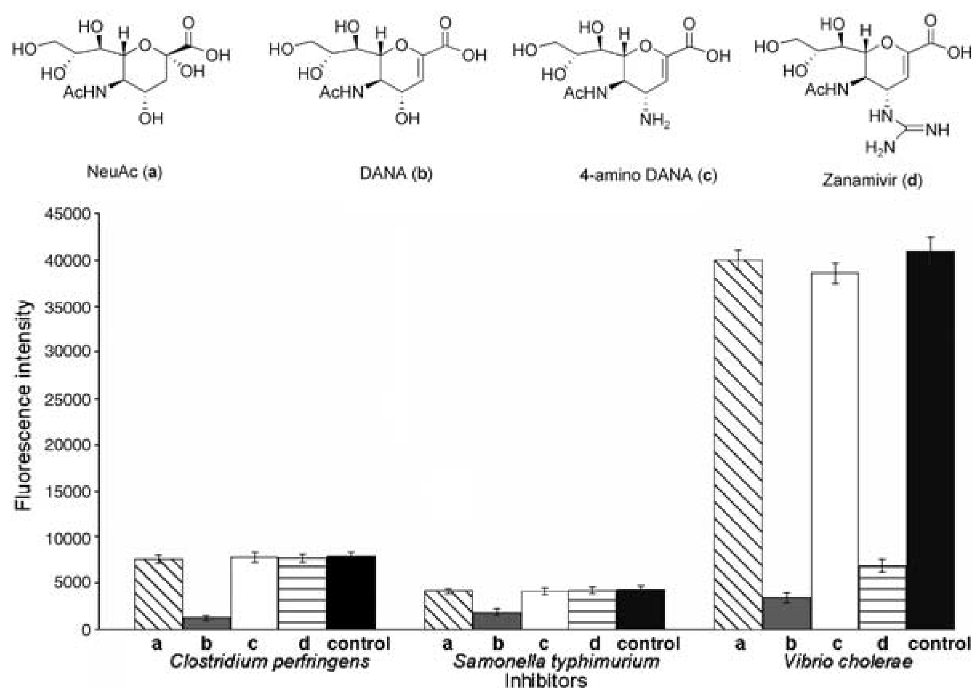

Figure 1. Inhibition activity of NA inhibitors (**a**–**d**) using resin-bound substrate **5**. The inhibitors were preincubated with the indicated enzyme and **5** was subsequently added. In the control, no inhibitor was added. After 20 min incubation time, fluorescence intensity was measured with a plate reader.

showed variable activity depending on the inhibitor and enzyme. The strong fluorescence observed for NeuAc (**a**) and 4-amino DANA (**c**) when tested against *Vibrio cholerae* is comparable to the control; this indicates rapid substrate turnover and that these compounds do not inhibit the enzyme. In contrast, DANA (**b**) and Zanamivir (**d**) both inhibited *Vibrio cholerae*. Neuraminidase activity is lowest for *Salmonella typhimurium* and only DANA inhibits this enzyme. Likewise, DANA is the only compound among the four that inhibits *Clostridium perfringens*.

In order to directly compare the solid- and solution-phase assays, we determined IC_{50} values using soluble substrate and soluble inhibitors. The standard fluorimetric assay was used to measure NA-inhibition activity.[13] Compounds **a**–**d** were preincubated with NA at various concentrations, as described. The enzymatic reaction was initiated by the addition of the soluble substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid. After incubation for 0.5–2 h, depending on the turnover rates of the different neuraminidases, the reaction was terminated by adding aqueous NaOH. Fluorescence intensity was recorded with a fluorescence plate reader, and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting the percent inhibition of NA activity versus the inhibitor concentration. The results are shown in Table 1. As expected, NeuAc (**a**) does not effectively inhibit the bacterial neuraminidases. In contrast, DANA (**b**) is a good inhibitor for all three neuraminidases, consistent with the on-bead assay. The low inhibition for *Salmonella typhimurium* is attributed to fast turnover ($2700\,s^{-1}$) compared to viral neuraminidase ($9\,s^{-1}$), and the

Table 1. IC_{50} values of NA inhibition in a standard solution-phase assay.

Inhibitor	*Clostridium perfringens*	*Salmonella typhimurium*	*Vibrio cholerae*
a	>2 mM	>2 mM	>2 mM
b	4 μM	0.31 mM (0.35 mM)[19]	20 μM (30 μM)[25]
c	>2 mM	>2 mM	>2 mM
d	>2 mM	>2 mM	0.2 mM

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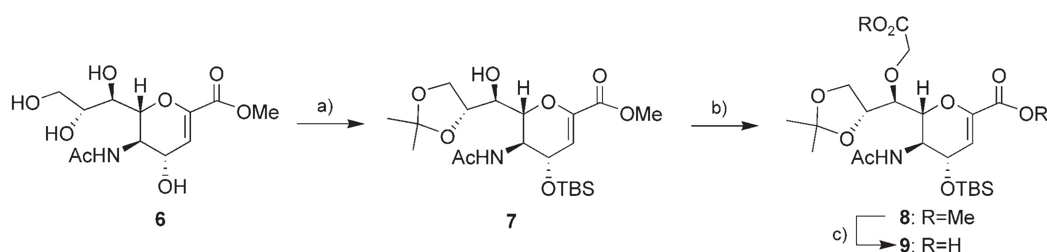
value is consistent with earlier reports.[19] Converting the C-4 hydroxyl group of DANA to an amino group, as in compound **c**, diminished inhibition for all three bacterial neuraminidases. As was observed in the solid-phase assay, the C-4 guanidine group in Zanamivir (**d**) also reduces bacterial NA activity relative to DANA, as **d** does not inhibit *Clostridium Perfringens* or *Salmonella typhimurium*, and only weakly inhibits *Vibrio cholerae*.

Having demonstrated that the NA activities of resin-bound substrate correlate with solution-phase assays, we next turned our attention to preparing one-bead–two-compound systems, which required functionalization of DANA and Zanamivir for conjugation to solid support. Derivatives **9** and **13** were chosen because Honda and co-workers have shown that substitution at the C-7 position is tolerated and even desirable (vide supra). As shown in Scheme 5, the 8,9-dihydroxy groups of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid methyl ester[17] were selectively protected with an isopropylidene protecting group, followed by protection of the 4-hydroxy group with *tert*-butyldimethylsilyl chloride (TBSCl) to give the intermediate **7**.[20] The 7-hydroxy group of **7** was then alkylated with methyl bromoacetate to afford **8**.[21] Finally, both of the methyl esters were removed by aqueous NaOH to yield **9**.

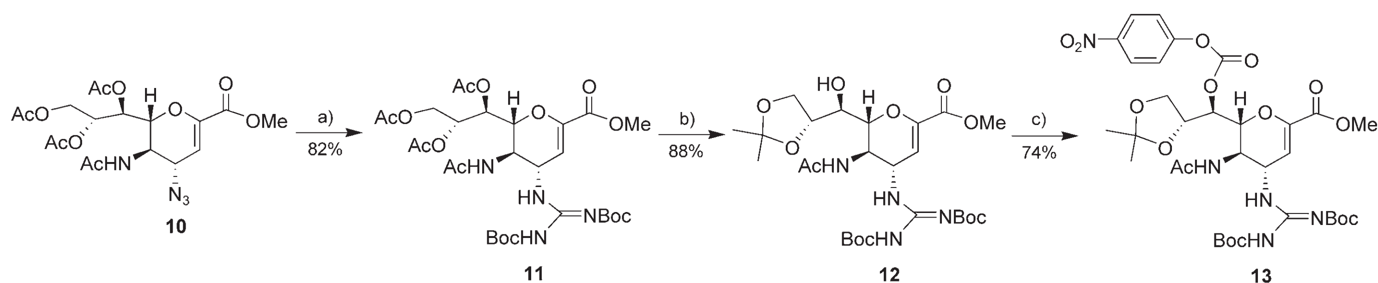
In the preparation of **13** (Scheme 6), azide **10** was subjected to hydrogenation in the presence of Lindlar catalyst, followed by protection of the 4-amino group by guanidinylation with *N*,*N*′-bis-*tert*-butoxycarbonyl-1*H*-pyrazole-1-carboxamidine (BisBocPCH) to produce the protected guanidine **11**.[22] After removal of the acetate protecting group with catalytic sodium methoxide in methanol, the 8,9-dihydroxy groups were selectively protected by using 2,2-dimethoxy propane and catalytic *p*-toluenesulfonic acid in acetone to give the 8,9-isopropylidene protected intermediate **12**.[20] Compound **12** was then treated with 4-nitrophenyl chloroformate and DMAP in dry pyridine to generate the active ester **13** in high yield.[3e]

In order to achieve differential functionalization of the solid support, PEGA resin was functionalized with orthogonal protecting groups by coupling with a mixture of 9-fluorenylmethoxycarbonyl (Fmoc) -protected glycine and *tert*-butyloxycarbonyl (Boc) -protected glycine (9:1; Scheme 7). We and others[14,15,23] have shown that a 9:1 ratio routinely achieves differential functionalization that can be quantified with UV spectroscopy by measuring dibenzofulvene release after piperidine treatment.[24] After removal of the Fmoc protecting group with 20% piperidine in DMF, compound **9** was coupled to the beads by using 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC). The TBS group was deprotected by using tetrabutyl ammonium fluoride (TBAF) in THF, and subsequent treatment with trifluoroacetic acid (TFA) in dichloromethane (DCM) unmasked the amine, which was coupled with **4** to give **14**. In a similar fashion, compound **13** was also immobilized on orthogonally protected resin to afford **15**. As a control, orthogonally protected resin was also conjugated with substrate **4** after acylation providing **16**.

In a proof-of-concept study, the functionalized beads (**14**–**16**) were incubated with the bacterial neuraminidases and monitored on a fluorescence plate reader, in analogy to the solution-phase assays. As shown in Figure 2, the on-bead assays are completely consistent with the solution-phase assays (Table 1). Resin-functionalized **16** served as a control showing no inhibition of the three bacterial neuraminidases; the NeuAc substrates attached to these beads underwent facile cleavage of the α-ketosidic linkage of sialic acid to result in strong fluorescence (Figure 3c). In contrast, DANA functionalized beads



Scheme 5. Synthesis of DANA-analogue for conjugation to solid support. a) 2,2-Dimethoxy propane, acetone, *p*-TsOH; TBSCl, imidazole, DMF; b) Methyl bromoacetate, NaH, DMF; c) aq NaOH, THF.



Scheme 6. Synthesis of a Zanamivir analogue for conjugation to solid support. a) Lindlar cat., H_2, EtOH; *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, TEA, THF; b) NaOMe, MeOH; 2,2-dimethoxy propane, acetone, *p*-TsOH; c) *p*-$NO_2C_6H_4OCOCl$, DMAP, pyridine.

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Scheme 7. Synthesis of one-bead–two-compound constructs with DANA or Zanamivir serving as potential inhibitors. a) Fmoc-Gly/Boc-Gly 9:1, HOBt, DIC, DMF; b) 20% piperidine in DMF; **9**, HOBt, DIC, DMF; c) TBAF, THF; 50% TFA in DCM; d) **4**, HOBt, DIC, Bu_3N, DMF; e) 20% piperidine in DMF; **13**, DMAP, pyridine; f) aq NaOH, MeOH; 50%TFA in DCM; g) 20% piperidine in DMF; Ac_2O, pyridine; h) 50%TFA in DCM.

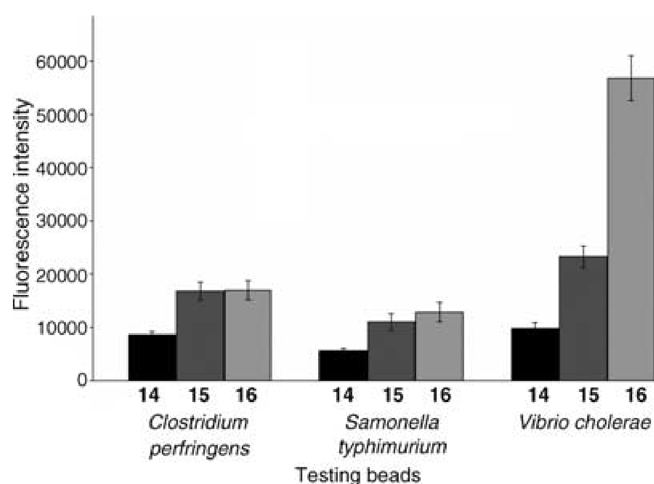

Figure 2. Inhibitory activity of one-bead–two-compound constructs **14**–**16**. The bifunctionalized beads were incubated with the bacterial neuraminidases and monitored on a fluorescence plate reader. Control experiments (**16**) indicate that the enzymes have different turnover rates, which is consistent with the data shown in Figure 1 and Table 1. Beads functionalized with DANA (**14**) inhibit all three enzymes, whereas beads functionalized with Zanamivir (**15**) inhibit *Vibrio cholerae* and only weak activity is seen with *Salmonella typhimurium*.

(**14**) inhibited all three bacterial neuraminidases, as evidenced by weak fluorescence intensity as compared to the control (**16**) (Figure 3a). Beads with Zanamivir showed no inhibition of *Clostridium perfringens* and *Salmonella typhimurium* neuraminidases and only weak inhibition of *Vibrio cholerae* neuraminidase (Figure 3b); again this is completely consistent with the solution assays in Table 1 and the one-bead–one-substrate assay (Figure 1).

Conclusion

In summary, synthetic protocols have been established for the conjugation of fluorogenic neuraminidase substrates to solid support. Enzymatic assays confirmed that resin-bound compounds are susceptible to enzymatic cleavage to the same extent as in solution-phase protocols. In a proof-of-concept study, two known neuraminidase inhibitors, DANA and Zanamivir, were immobilized on bifunctionalized beads that were subsequently treated with the fluorogenic NeuAc substrate; this allowed simultaneous presentation of one-bead–one-inhibitor–one-substrate constructs to the enzyme in solution.

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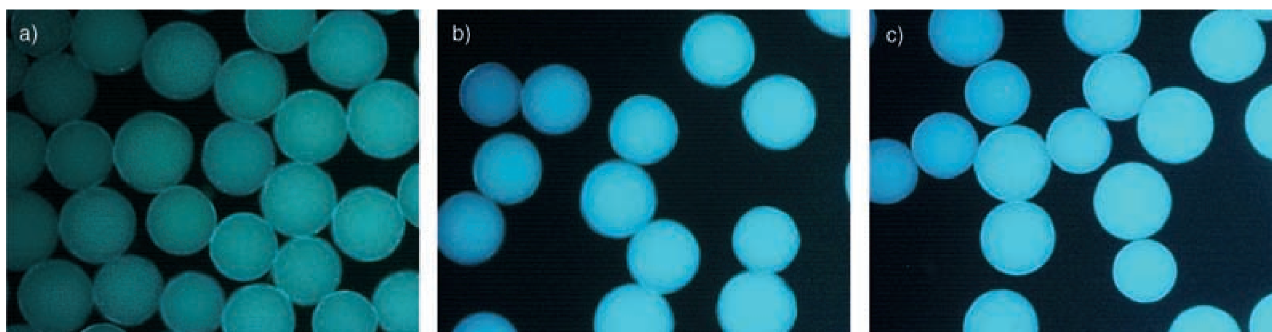

Figure 3. a) PEGA functionalized beads **14** after incubation with *Vibrio cholerae*. b) PEGA functionalized beads **15** after incubation with *Vibrio cholerae*. c) PEG functionalized beads **16** after incubation with *Vibrio cholerae*.

The results of these experiments were completely consistent with solution-phase assays; this demonstrated the feasibility of on-bead neuraminidase screening with competing inhibitors. Future investigations in our laboratory include applying this technology to combinatorial-library screening.

Experimental Section

All chemicals were used as supplied without further purification. Solvents (MeOH 99.8%, CH_2Cl_2 99.8%, CH_3CN 99.8%, DMF 99.8%) were purchased in anhydrous Sure/Seal bottles from Aldrich, used without further purification, and stored under argon. Bacterial neuraminidases (*Clostridium perfringens*, *Salmonella typhimurium*, *Vibrio cholerae*) were purchased from Sigma. PL-PEGA resin (0.4 mmol g^{-1}, 150–300 μm) was purchased from Polymer Laboratories. Dowex 50WX8 (200 mesh) acidic resin was purchased from Aldrich, washed copiously with methanol, and used without further purification. NaOMe/MeOH (0.5 M) was purchased from Aldrich. Glass-backed EM Science TLC plates (silica gel 60 with a 254 nm fluorescent indicator) were purchased from VWR International, cut into 2 cm × 5 cm portions, used without further manipulation, and stored over dessicant. Developed TLC plates were visualized under a short wave UV lamp, stained with a cerium-molybdate solution and charred. Column chromatography was conducted with flash silica gel (32–63 μm) available from Scientific Adsorbends and solvents purchased from EM Science. NMR experiments (1D and 2D) were conducted on Bruker DRX 500 MHz spectrometers at 298 K. RP-HPLC preparative separations were carried out on a Vydac C18 column (10 × 250 mm). Solvents: A) H_2O and B) CH_3CN with UV detection at 220 and 254 nm. Fluorescence intensity was measured with a Perkin–Elmer fluorescence plate reader.

4-Methyl acetate-umbelliferyl-4,7,8,9-tetra-*O*-acetyl-α-D-*N*-acetyl-neuraminic acid methyl ester (3): 2-Deoxy-2-chloro-4,7,8,9-tetra-*O*-acetyl-α-D-*N*-acetyl-neuraminic acid methyl ester (1.10 g, 2.12 mmol) was dissolved in a mixture of **2**[10] (0.54 g, 2.33 mmol), Ag_2CO_3 (0.58 g, 2.12 mmol), and activated molecular sieves (1.8 g) in anhydrous acetonitrile (30 mL). The mixture was stirred under argon at room temperature in the dark for 24 h, filtered, and evaporated. The residue was purified by column chromatography (ethyl acetate) to give **3** as pale yellow solid (0.66 g, 44%). TLC (ethyl acetate): $R_f = 0.18$, m.p. 105 °C; $[\alpha]_D^{26} = +25.6°$ ($c = 0.5$, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): $\delta = 1.92$, 2.04, 2.14 (3 s, 15 H, 4-OAc, 1-NAc), 2.25 (m, 1 H, H-3a), 2.72 (dd, $J = 4.5$, 13.0 Hz, 1 H, H-3e), 3.70 (s, 3 H, CO_2CH_3), 3.74 (s, 3 H, CO_2CH_3), 4.10 (m, 4 H, H-5, H-9a, CH_2), 4.29 (m, 1 H, H-9b), 4.51 (m, 1 H, H-6), 5.00 (m, 1 H, H-4), 5.37 (m, 3 H, H-7, H-8, NH), 6.29 (s, 1 H, C=C*H*), 7.04 (m, 2 H), 7.49 (d, $J =$ 9.0 Hz, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 20.86$, 20.95, 21.11, 23.34, 37.92, 38.24, 49.60, 52.84, 53.45, 62.18, 67.46, 68.51, 69.19, 73.84, 99.96, 108.02, 115.15, 115.51, 116.13, 125.78, 147.83, 154.79, 156.92, 160.54, 168.06, 169.20, 170.22, 170.53, 170.79, 170.99; ESIMS calcd for $C_{32}H_{37}O_{17}NNa$ $[M+Na]^+$ 730.2, found 730.0.

4-Acetic acid umbelliferyl-α-D-*N*-acetyl-neuraminic acid disodium salt (4): NaOMe (0.1 mL, 0.5 M, 0.05 mmol) was added slowly under argon to a stirred solution of **3** (200 mg, 0.28 mmol) in dry methanol (10 mL). The resulting mixture was stirred for 0.5 h. The methanolic solution was acidified with Dowex 50WX8 (H^+) resin, filtered, washed with methanol, and evaporated to dryness. The residue was dissolved in water (5 mL), and NaOH (5.9 mL, 0.1 M, 0.59 mmol) was added. The mixture was stirred for 1 h at room temperature, and freeze-dried to give a yellow solid, which was purified by RP-HPLC (H_2O/CH_3CN 90:10 → 70:30) to afford **4** as a white solid (100 mg, 64%). m.p. 170 °C (decomposed); $[\alpha]_D^{24} = +45.3°$ ($c = 0.7$, H_2O); ^1H NMR (500 MHz, D_2O): $\delta = 2.08$ (m, 4 H, NAc, H-3a), 2.89 (dd, $J = 4.0$, 12.5 Hz, 1 H, H-3e), 3.66 (m, 2 H), 3.88 (m, 6 H), 4.13 (m, 1 H), 6.37 (s, 1 H, C=C*H*), 7.23 (m, 2 H), 7.71 (d, $J = 8.5$ Hz, 1 H); ^{13}C NMR (125 MHz, D_2O/CD_3OD): $\delta = 22.86$, 42.25, 53.39, 64.0, 68.91, 69.72, 72.85, 74.91, 103.83, 109.33, 113.82, 116.55, 119.0, 127.05, 154.89, 155.05, 159.05, 164.76, 173.09, 175.87; ESIMS calcd for $C_{22}H_{24}O_{13}NNa_2$ $[M+H]^+$ 556.1, found 556.1.

Conjugation of 4 on beads (5): PL-PEGA resin (40 mg, 0.4 mmol g^{-1}, 150–300 μm) was swollen in DMF overnight. Compound **4** (27 mg, 48 μmol), HOBt (48 μL, 1.0 M), Bu_3N (10 μL), and DIC (8 μL) were added to the resin. The resin was shaken for 3 h (monitored by Kaiser test) and washed with DMF (5 × 3 mL), CH_2Cl_2 (5 × 3 mL), MeOH (5 × 3 mL), and H_2O (5 × 3 mL).

5-Acetamido-4-(*tert*-butyldimethylsilanyloxy)-8,9-*O*-(1-methylethylidene)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-non-2-enonic acid methyl ester (7): 2,2-Dimethoxypropane (0.45 mL, 3.67 mmol) was added slowly to a stirring solution of **6**[13] (187 mg, 0.61 mmol) in dry acetone (10 mL) under argon. *p*-Toluenesulfonic acid (12 mg, 0.06 mmol) was added to the solution. The resulting mixture was stirred overnight at room temperature. The mixture was evaporated and dissolved in DMF (10 mL) and TBSCl (110 mg, 0.73 mmol), and imidazole (42 mg, 0.61 mmol) was added. The solution was stirred under argon for 6 h at room temperature. The mixture was evaporated, extracted with ethyl acetate, and purified by column chromatography (ethyl acetate/hexane 2:1) to give **7** as a white solid (208 mg, 74%). TLC (ethyl acetate/hexane 3:1): $R_f =$ 0.35, m.p. 245 °C; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 0.17$ (s, 3 H, CH_3), 0.19 (s, 3 H, CH_3), 0.91 (s, 9 H, 3 CH_3), 1.37 (s, 3 H, CH_3), 1.41 (s, 3 H, CH_3), 2.04 (s, 3 H, NAc), 3.60 (m, 1 H), 3.81 (s, 3 H, OCH_3), 4.07 (m, 2 H), 4.16 (t, $J = 8.5$ Hz, 1 H), 4.24 (d, $J = 7.0$ Hz, 1 H), 4.34 (m, 2 H),

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4.48 (m, 1H, OH), 5.54 (d, $J=7.5$ Hz, 1H), 5.89 (d, $J=3.5$ Hz, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=-4.46$, -4.27, 18.10, 23.36, 25.46, 25.77, 27.20, 51.70, 52.63, 66.26, 67.51, 72.12, 74.63, 76.70, 109.42, 109.92, 144.97, 162.75, 171.46; ESIMS calcd for $C_{21}H_{37}NO_8SiNa$ $[M+Na]^+$ 482.2, found 482.3; elemental analysis calcd (%) for $C_{21}H_{37}NO_8Si$: C 54.88, H 8.11, N 3.05, found: C 54.82, H 8.19, N 3.03.

5-Acetylamino-4-(*tert*-butyldimethylsilanyloxy)-6-[(2,2-dimethyl-[1,3]dioxolan-4-yl)-methoxycarbonylmethoxy-methyl]-5,6-dihydro-4*H*-pyran-2-carboxylic acid methyl ester (8): Sodium hydride (26 mg, 1.09 mmol) was added in portions to a stirred solution of **7** (250 mg, 0.54 mmol) and methyl bromoacetate (0.1 mL, 1.09 mmol) in dry DMF (10 mL). The resulting mixture was stirred overnight at room temperature. Then, the mixture was evaporated, extracted with ethyl acetate, and purified by column chromatography (ethyl acetate/hexane 2:1) to give **8** as a white solid (205 mg, 71%); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=0.03$ (s, 3H, CH_3), 0.05 (s, 3H, CH_3), 0.85 (s, 9H, $3CH_3$), 1.27 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.91 (s, 3H, NAc), 3.49 (m, 1H, H-5), 3.70 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 4.07 (m, 2H), 4.17 (t, $J=9.0$ Hz, 1H), 4.26 (t, $J=7.0$ Hz, 1H), 4.36 (m, 1H), 4.57 (m, 1H), 4.66 (dd, $J=2.0$, 10.0 Hz, 1H, H-6), 5.23 (dd, $J=2.0$, 8.5 Hz, 1H, H-4), 5.78 (d, $J=2.5$ Hz, 1H, H-3), 7.02 (d, $J=6.5$ Hz, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=-4.75$, -4.58, 18.04, 23.71, 24.63, 25.64, 25.81, 26.35, 51.99, 52.25, 54.0, 64.46, 64.62, 68.33, 76.07, 76.26, 78.81, 107.79, 113.61, 143.01, 162.60, 171.28, 172.39; ESIMS calcd for $C_{24}H_{41}NO_{10}SiNa$ $[M+Na]^+$ 554.3, found 554.3; elemental analysis calcd (%) for $C_{24}H_{41}NO_{10}Si$: C 54.22, H 7.77, N 2.63, found: C 54.14, H 7.98, N 2.61.

5-Acetylamino-4-(*tert*-butyldimethylsilanyloxy)-6-[carboxymethoxy-(2,2-dimethyl-[1,3]dioxolan-4-yl)-methyl]-5,6-dihydro-4*H*-pyran-2-carboxylic acid (9): Sodium hydroxide (5 mL, 0.1 M, 0.5 mmol) was added slowly to a stirred solution of **8** (100 mg, 0.19 mmol) in THF (5 mL). The resulting mixture was stirred for 2 h at room temperature. The organic solvent was evaporated and the aqueous solution was acidified with HCl to pH 2, extracted with ethyl acetate, and concentrated to give **9** as a white solid (66 mg, 70%). m.p. 130 °C; $[\alpha]_D^{26}=+35.1°$ ($c=1.2$, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD): $\delta=0.11$ (s, 3H, CH_3), 0.14 (s, 3H, CH_3), 0.91 (s, 9H, $3CH_3$), 1.32 (s, 3H, CH_3), 1.39 (s, 3H, CH_3), 2.0 (s, 3H, NAc), 3.66 (s, 2H, OCH_2), 3.98 (m, 2H), 4.14 (t, J = 8.0 Hz, 1H), 4.20 (t, J = 8.0 Hz, 1H), 4.27 (m, 1H, H-6), 4.33 (m, 1H), 4.63 (m, 1H, H-4), 5.84 (s, 1H, H-3); ^{13}C NMR (125 MHz, CD_3OD): $\delta=-4.65$, -4.45, 18.81, 23.19, 25.43, 26.18, 26.61, 30.89, 51.97, 66.47, 68.13, 68.79, 70.76, 77.71, 78.38, 78.68, 109.35, 113.37, 144.93, 164.97, 173.24, 173.61; ESIMS calcd for $C_{22}H_{37}NO_{10}SiNa$ $[M+Na]^+$ 526.3, found 526.3; elemental analysis calcd (%) for $C_{22}H_{37}NO_{10}Si$: C 52.47, H 7.41, N 2.78, found: C 52.38, H 7.48, N 2.64.

5-Acetylamino-4-[2,3-bis(*tert*-butoxycarbonyl)guanidine]-6-(1,2,3-triacetoxy-propyl)-5,6-dihydro-4*H*-pyran-2-carboxylic acid methyl ester (11): Lindlar catalyst (1 g) was added to a stirred solution of **10** (5.0 g, 11.0 mmol) in anhydrous ethanol (80 mL), and the flask was flashed with argon. Hydrogen gas was then bubbled through the vigorously stirring solution for 10 h. The reaction mixture was then filtered through celite, and the filtrate was concentrated. The residue was dissolved in dry THF (50 mL). *N,N'*-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine (3.42 g, 11.0 mmol), and triethylamine (1.9 mL, 13.2 mmol) were then added. The mixture was stirred vigorously overnight at room temperature. The reaction mixture was then concentrated and purified by column chromatography (hexane/ethyl acetate 1:1) to give **11** as colorless oil (6.1 g, 82%). TLC (hexane/ethyl acetate, 1:1): $R_f=0.20$; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=1.50$ (s, 18H, $6CH_3$), 1.86 (s, 3H, NAc), 2.04, 2.06, 2.08 (3 s, 12H, 3OAc), 3.79 (s, 3H, OCH_3), 4.28 (m, 1H), 4.70 (dd, $J=2.0$ Hz, 10.5 Hz, 1H) 5.13 (m, 1H), 5.30 (m, 1H), 5.51 (d, $J=4.0$ Hz, 1H), 5.86 (d, $J=1.5$ Hz, 1H, H-3), 6.37 (s, 1H, NH), 7.15 (d, $J=9.0$ Hz, 1H), 7.69 (s, 1H), 8.51 (d, $J=9.0$ Hz, 1H, NH), 11.42 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=20.80$, 20.86, 20.94, 21.04, 23.00, 28.06, 28.31, 47.60, 49.19, 52.45, 60.40, 62.38, 67.89, 71.69, 77.96, 79.90, 83.90, 105.12, 109.79, 145.21, 152.69, 157.33, 161.75, 162.96, 170.22, 170.32, 170.58, 170.87; FABHRMS calcd for $C_{29}H_{44}N_4O_{14}Na$ $[M+Na]^+$ 695.2751, found 695.2750.

5-Acetylamino-4-[2,3-bis(tert-butoxycarbonyl)guanidine]-6-[(2,2-dimethyl-[1,3]dioxolan-4-yl)-hydroxy-methyl]-5,6-dihydro-4*H*-pyran-2-carboxylic acid methyl ester (12): NaOMe (2 mL, 0.5 M, 1 mmol) was added to a stirred solution of **11** (3.0 g, 4.46 mmol) in anhydrous methanol (60 mL). The reaction mixture was then stirred for 30 min. Dowex 50WX8 (H^+) resin was added to neutralize the reaction mixture and filtered. The filtrate was concentrated and dissolved in dry acetone (50 mL). 2,2-Dimethoxypropane (4.5 mL, 36.7 mmol) and *p*-toluenesulfonic acid (80 mg, 0.4 mmol) were added to the reaction mixture. The resulting mixture was stirred overnight at room temperature. The reaction mixture was then concentrated and purified by column chromatography (hexane/ethyl acetate 1:1) to give **12** as a white solid (2.3 g, 88%). TLC (hexane/ethyl acetate 1:3): $R_f=0.47$, m.p. 126 °C; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=1.37$ (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.50 (s, 9H, $3CH_3$), 1.52 (s, 9H, $3CH_3$), 2.02 (s, 3H, NAc), 3.50 (m, 1H, H-7), 3.79 (s, 3H, OCH_3), 3.97 (m, 1H, H-5), 4.03 (m, 1H, H-6), 4.10 (m, 1H, H-9), 4.10 (m, 1H, H-9'), 4.40 (m, 1H, H-8), 5.15 (m, 1H, H-4), 5.28 (d, $J=4.0$ Hz, 1H, OH), 5.80 (d, $J=2.0$ Hz, 1H, H-3), 8.00 (d, $J=5.5$ Hz, 1H, NH), 8.64 (d, $J=7.5$ Hz, 1H, NH), 11.37 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=22.98$, 25.25, 27.12, 28.04, 28.24, 48.46 (C-4), 52.13 (OCH_3), 52.41 (C-5), 67.50 (C-9), 69.75 (C-7), 74.01 (C-8), 78.53, 80.09 (C-6), 84.38, 106.67 (C-3), 109.19, 146.96 (C-2), 152.70, 157.66, 161.99, 162.35, 174.01; FABHRMS calcd for $C_{26}H_{43}N_4O_{11}$ $[M+H]^+$ 587.2928, found 587.2951; elemental analysis calcd (%) for $C_{26}H_{42}N_4O_{11}$: C 53.23, H 7.22, N 9.55, found: C 53.20, H 7.26, N 9.51.

5-Acetylamino-4-[2,3-bis(*tert*-butoxycarbonyl)guanidine]-6-[(2,2-dimethyl-[1,3]dioxolan-4-yl)-(4-nitro-phenoxycarbonyloxy)-methyl]-5,6-dihydro-4*H*-pyran-2-carboxylic acid methyl ester (13): 4-Dimethylaminopyridine (149 mg, 1.22 mmol) and 4-nitrophenylchloroformate (245 mg, 1.22 mmol) were added to a solution of **12** (286 mg, 0.49 mmol) in dry pyridine (10 mL). The reaction mixture was stirred vigorously overnight at room temperature. The solution was then concentrated and the residue was extracted with ethyl acetate and purified by column chromatography (hexane/ethyl acetate 1:1) to give **13** as a white solid (271 mg, 74%). TLC (hexane/ethyl acetate 1:3): $R_f=0.69$, m.p. 150 °C; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=1.39$ (s, 3H, CH_3), 1.42 (s, 3H, CH_3), 1.49 (s, 18H, $6CH_3$), 1.94 (s, 3H, NAc), 3.82 (s, 3H, OCH_3), 4.14 (m, 1H, H-9), 4.23 (m, 2H, H-8, H-9'), 4.41 (m, 1H, H-5), 4.45 (m, 1H, H-6), 5.19 (t, $J=9.5$ Hz, 1H, H-4), 5.24 (d, $J=5.0$ Hz, 1H, H-7), 5.90 (d, $J=2.5$ Hz, 1H, H-3), 6.46 (brs, 1H, NH), 7.53 (dd, $J=1.5$ Hz, 9.0 Hz, 2H, Ar), 8.26 (dd, $J=1.5$ Hz, 9.0 Hz, 2H, Ar), 8.58 (d, $J=8.5$ Hz, 1H, NH), 11.35 (s, 1H, NH); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=23.30$, 25.64, 26.63, 28.20, 28.41, 48.74 (C-4), 48.89 (C-5), 52.76 (OCH_3), 65.89 (C-9), 74.33 (C-7), 75.10 (C-6), 77.70 (C-8), 80.18, 84.37, 109.18 (C-2), 115.81, 122.47, 125.39, 126.39, 145.63 (C-2), 152.66, 152.86, 155.98, 157.50, 161.74, 162.88, 171.85; FABHRMS calcd for $C_{33}H_{45}N_5O_{15}Na$ $[M+Na]^+$ 774.2810, found 774.2848; elemental analysis calcd (%) for $C_{33}H_{45}N_5O_{15}$: C 52.73, H 6.03, N 9.32, found: C 52.66, H 6.06, N 9.15.

Conjugation of 9 to resin to give 14: PL-PEGA resin (120 mg, 0.4 mmol g^{-1}, 150–300 μm) was swollen in DMF overnight, Fmoc-Gly-OH/Boc-Gly-OH (480 μL, 0.3 M, 9:1, mol/mol), HOBt (144 μL,

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1.0 M) and DIC (22 μL) were then added to the resin. The resin was shaken for 2 h and washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). Orthogonally protected PL-PEGA resin (40 mg) was treated with 20% piperidine in DMF for 30 min, then washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). Compound **9** (25 mg, 48 μmol), HOBt (48 μL, 1.0 M), and DIC (8 μL) were added to the resin. The resin was shaken for 3 h and washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). The resin was treated with TBAF (1.0 mL, 0.1 M) and shaken overnight. After the resin had been washed, as described before, TFA in DCM (1 mL, 50%) was added, and the mixture was shaken for 30 min. After it had been washed as described, the resin (20 mg) was coupled with substrate **4** (2 mg) by using HOBt (5 μL, 1.0 M), DIC (1 μL), and Bu_3N (5 μL) to give **14**.

Conjugation of 13 to resin to give 15: Orthogonally protected PL-PEGA (40 mg) resin was treated with 20% piperidine in DMF for 30 min, then washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). Compound **13** (36 mg, 48 μmol), DMAP (12 mg, 96 μmol), and pyridine (1 mL) were added to the resin. The resin was shaken for 40 h and washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). The resin was treated with aq. NaOH (1.0 mL, 0.1 M) and shaken for 2 h. After the resin had been washed as described before, TFA in DCM (1 mL, 50%) was added, and the mixture was shaken for 30 min. After more washing, the resin (20 mg) was coupled with substrate **4** (2 mg) by using HOBt (5 μL, 1.0 M), DIC (1 μL), and Bu_3N (5 μL) to give **15**.

Preparation of acetylated blank resin 16: Orthogonally protected PL-PEGA resin (40 mg) was treated with 20% piperidine in DMF for 30 min, then washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). Ac_2O (0.5 mL) and pyridine (0.5 mL) were added to the resin. The resin was shaken overnight, and washed with DMF (5×3 mL), MeOH (5×3 mL), CH_2Cl_2 (5×3 mL), and DMF (5×3 mL). Resin (20 mg) was coupled with substrate **4** (2 mg) by using HOBt (5 μL, 1.0 M), DIC (1 μL), and Bu_3N (5 μL) to give **16**.

Neuraminidase inhibition assays: Inhibitors in solution: Compound **5** was used as a substrate in a reaction buffer containing 2-(*N*-morpholino)-ethanesulfonic acid (MES; 32.5 mM), pH 6.5, with $CaCl_2$ (4 mM). The neuraminidase (1 mU) was preincubated with various inhibitors (**a**–**d**, 0.5 mM) in MES buffer (150 μL, pH 6.5) at room temperature for 30 min. Substrate **5** (20 μL) was then added to each well. The fluorescence intensities were measured after 20 min incubation with a PerkinElmer fluorescence plate reader with an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Inhibitors and substrate in solution. Compounds **a**–**d** at different concentrations were first incubated with neuraminidases (*Clostridium perfringens* 2 mU, *Salmonella typhimurium* 10 mU, and *Vibrio cholerae* 2 mU) in MES buffer (32.5 mM, 4 mM $CaCl_2$, pH 6.5) for 30 min. Then, the substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (final concentration 10 μM) was added to each well. After incubation for 0.5–2 h (*Clostridium perfringens* 0.5 h, *Salmonella typhimurium* 2 h, and *Vibrio cholerae* 0.5 h), aqueous NaOH (20 μL, 0.034 M) was added to each well to terminate the enzymatic reaction. The fluorescence intensity of each solution was recorded with a fluorescence plate reader, and the substrate blanks were subtracted from the sample reading. The IC_{50} was calculated by plotting the percent inhibition of neuraminidase activity versus the inhibitor concentration.

Inhibitors and substrate on solid support: Resins **14**–**16** (20 μL) were each directly incubated with neuraminidase (1 mU) in MES buffer (100 μL, pH 6.5) at room temperature. After 10 min, the fluorescence intensities were measured at an excitation wavelength of 355 nm, emission wavelength of 460 nm.

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