Activity Probes

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Design of a Mechanism-Based Probe for Neuraminidase To Capture Influenza Viruses**

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Influenza viruses, which cause upper respiratory tract problems in humans, have long been a major threat to public health.[1] It is estimated that 10–20% of the general popula-

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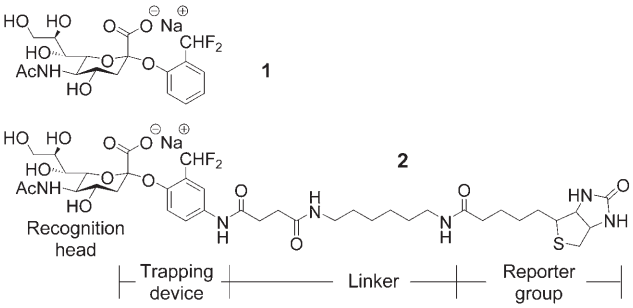
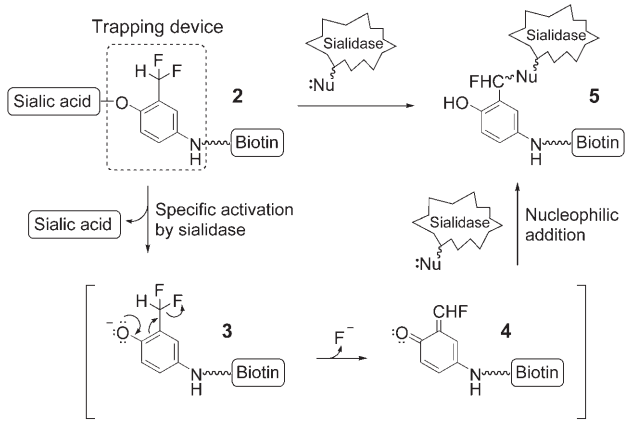
tion are affected in seasonal epidemics. Some devastating outbreaks recorded in history even claimed millions of lives worldwide.[2] Influenza viruses are typically spherical with a diameter of about 100 nm.[3] The pathogenic properties of the viruses have been extensively investigated.[4] Significant advances in therapeutic treatments and in the detection of the viruses have been made in recent years as a result of this information. Among the limited number of proteins that are encoded by the viral RNA segments, two surface glycoproteins, namely, hemagglutinin (HA) and neuraminidase (NA), have often been the focus of research. These two surface glycoproteins play important roles in the infection process; HA is responsible for the binding of viruses to the host cells, whereas NA is involved in the budding process.[5] Although the surface antigens of the viruses mutate frequently to avert attacks from the immune system of the hosts, the critical catalytic activity of NA has to be maintained for successful infection and propagation. This special feature makes it an excellent candidate for research.[6]

NA (sialidase, *N*-acylneuraminosyl glycohydrolase, EC 3.2.1.18) is an *exo*-glycosidase that hydrolyzes the linkage of sialic acid residues, which are mostly found as terminal constituents of glycoconjugates. X-ray crystallographic information about the active site of NA has revealed important residues involved in the recognition and binding of sialic acid.[7] This has assisted in the development of several reversible inhibitors of NA, such as zanamivir and oseltamivir, which have been approved for the treatment of influenza.[8] Recently, McKimm-Breschkin et al. have demonstrated that ligands derived from biotin-conjugated zanamivir were able to bind influenza virion on microtiter plates; this could in turn serve as a basis for a diagnostic method.[9] Besides influenza viruses, many NA-containing microorganisms are pathogenic and it has been proposed that this enzyme plays an important role in the pathogenicity of these infections.[10] We therefore envision that the development of activity probes that selectively form a covalent linkage with NA would be of great value, as demonstrated by the application for capturing the virus particles in this report.

Chemical probes that are able to form covalent linkages with hydrolase subfamilies have proven to be a powerful tool in modern proteomics studies.[11] We have previously developed activity probes for hydrolases such as phosphatases and β-glucosidase.[12] The concept for the design of these activity probes originated from suicide substrates, or mechanism-based inhibitors, of enzymes.[13] This approach is unique as the probes themselves are also the substrates of the corresponding hydrolases. The probes can be selectively activated once the recognition head is cleaved by the targeting hydrolase, thereby leading to covalent modifications of the hydrolase.[14] More importantly and closely related to this report, the mechanism-based approach has been successfully applied in the screening and selection of biocatalysts from phage-displayed libraries, such as in the selection of mutant β-lactamases and in the search for catalytic antibodies with β-galactosidase activity.[15] Earlier studies have shown that compound **1** was a mechanism-based inhibitor of *Clostridium perfringens* NA.[16] We thus developed probe **2** as a mechanism-based probe for NA. Probe **2** carries four structural units in its design; a sialic acid recognition head, which is connected to an *ortho*-difluoromethylphenyl latent trapping device, a linker, and a biotin reporter group. The biotin reporter group

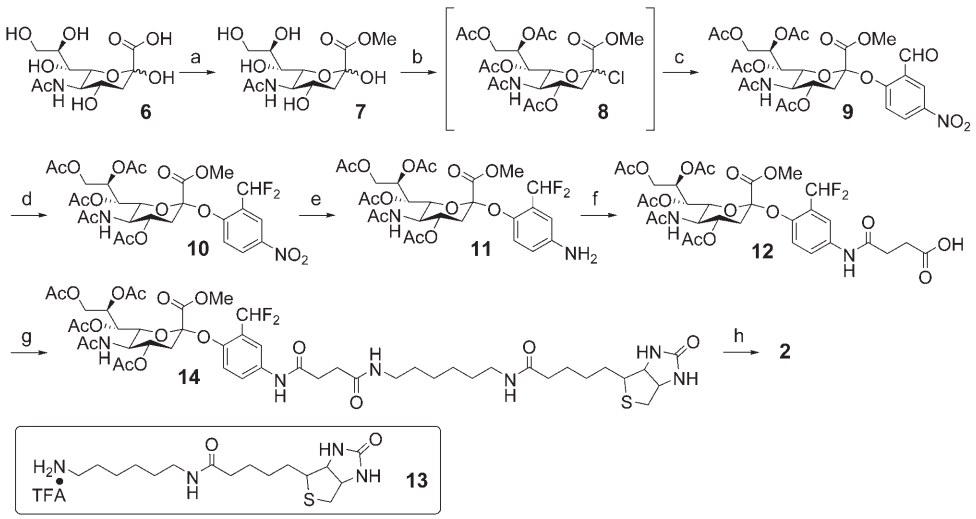


serves two functions. On one hand, it is used to visualize the labeled NA after Western blotting with streptavidin-conjugated peroxidase chemiluminescence. On the other hand, it could be used to attach the probe to the microtiter plates through avidin–biotin interactions during the virus-capturing study. When the designated glycosidic bond is cleaved by NA, the released intermediate **3** would undergo 1,4-elimination with removal of a fluoride ion to generate a reactive quinone methide, **4**. The highly reactive quinone methide intermediate **4** would alkylate nearby nucleophiles of the enzyme, thereby resulting in biotinylated adduct **5** (Scheme 1). Since the viral surface is spiked with NA, covalent attachment through NA would then lead to the capture of virus particles.



Scheme 1. Mechanism for selective activation and alkylation of neuraminidase with probe **2**.

The synthesis of probe **2** begins with a commercially available *N*-acetylneuraminic acid **6** (Scheme 2). All the synthetic procedures were combinations of efforts from previous publications.[17] In brief, fully protected glycosyl chloride **8** was prepared and used directly for coupling with 2-hydroxy-5-nitrobenzaldahyde in a $CHCl_3/H_2O$ biphasic system by using tetrabutylammonium bromide as the phase-transfer catalyst to give compound **9**. The formyl group of compound **9** could be converted into the difluoromethyl moiety of the trapping device by the DAST reagent. The structure of the difluoromethylphenyl group in compound **10**



Scheme 2. Synthesis of probe **2** for neuraminidase. Conditions: a) MeOH, IR-120 (H^+) resin, 92%; b) AcCl, AcOH; c) 2-hydroxy-5-nitrobenzaldehyde, Cs_2CO_3, Bu_4NBr, $H_2O/CHCl_3$, 67% for two steps; d) DAST, CH_2Cl_2, 47%; e) H_2, 5% Pd/C, MeOH, 95%; f) succinic anhydride, TEA, CH_2Cl_2, 94%; g) EDCI, HOBt, **13**, DIEA, DMF, 75%; h) Na_2CO_3, MeOH; aqueous Na_2CO_3, 52%. TFA = trifluoroacetic acid; TEA = triethylamine; EDCI = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide; HOBt = 1-hydroxy-1*H*-benzotriazole; DIEA = *N*,*N*-diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; DAST = (diethylamino)sulfur trifluoride.

was supported by its ^{1}H NMR spectroscopic data which showed a triplet at $\delta = 6.86$ ppm with a coupling constant of $^2J_{H,F} = 54.9$ Hz, a typical value for H–F coupling. A triplet at $\delta = 109.9$ ppm ($^1J_{C,F} = 237.2$ Hz) in the ^{13}C NMR spectrum of **10** further confirmed the presence of the CHF_2 moiety. The nitro group of compound **10** was then reduced by catalytic hydrogenation to give amine **11**. Attachment of the linker and biotin reporter group yielded the fully protected probe **14**. Final deprotection under alkaline conditions offered the desired probe **2** after LH-20 purification.

Probe **2** was first tested for its ability to biotinylate NA obtained from *Arthrobacter ureafaciens*. *Arthrobacter ureafaciens* neuraminidase (0.8 U, Sigma) was incubated in the presence or absence of probe **2** (200 μM) at 4 °C in 100 mM ammonium acetate buffer (10 μL). Bovine serum albumin (BSA) was used as a negative control. Labeled samples were applied to a 10% polyacrylamide gel, which was then subjected to sodium dodecylsulfate (SDS) PAGE. After electrophoresis, the proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked, washed, and developed by using the enhanced chemiluminescence (ECL). Western blotting protocols as recommended by the supplier (Amersham Biosciences). The result of the Western blot analysis only shows biotinylated proteins (Figure 1). In this experiment, three biotinylated bands corresponding to isoenzymes at 88, 66, and 52 KDa were observed for NA (Lane 1).[18] Probe **2** had no effect on BSA, as shown by the lack of labeled bands (Lane 2). In the absence of the probe, neither NA nor BSA was biotinylated (Lanes 3 and 4). This result firmly supports the alkylation process described in Scheme 1. The actual labeling site was not further determined in this study, because a number of biological applications utilizing the same latent trapping device have already established the covalent-bond-forming feature.[15,19] Moreover, Lee and co-workers recently used this activity-probe approach to characterize the catalytic domain of β-galactosidases from *Xanthomonas manihotis*, *Escherichia coli*, and *Bacillus circulans*, with the identification of mainly single modifications on Arg, Glu, and Glu residues, respectively.[20]

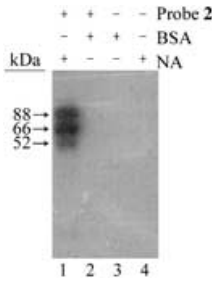


Figure 1. Western blot analysis of probe **2** labeling the *Arthrobacter ureafaciens* neuraminidase.

We also compared the inhibitory effect of probe **2** and zanamivir on the activity of NA from influenza A virus (A/WSN/33, H1N1), as well as from *Arthrobacter ureafaciens* (AU), *Clostridium perfringens* (CP), and *Vibro cholerae* (VC; Figure 2). Both compounds were tested at 3.3 mM in the NA inhibition assays with 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA, Sigma) as the substrate.[21] All samples were measured in duplicate and fluorometric determinations were performed with a fluorometer (ThermoLab systems, Sweden). The excitation wavelength was 355 nm and the emission wavelength was 460 nm. Probe **2** inhibited all four NA activities (H1N1, AU, CP, VC) with IC_{50} values of 1.7, 0.68, 0.08, and 0.53 mM, respectively. These results indicate that probe **2** interferes with the activity of NA isolated from various sources at the active site, regardless of the variations the enzymes might have. On the other hand,

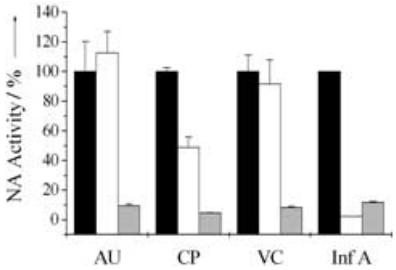


Figure 2. Inhibition study of NA activities from *A. ureafaciens* (AU), *C. perfringens* (CP), *V. cholerae* (VC), and influenza A virus (Inf A) with probe **2** (gray), zanamivir (white), and the control (black). Virus, suspended in 32.5 mM *β*-morpholinoethanesulfonic acid buffer (pH 6.5) was incubated with either probe **2** or zanamivir (3.3 mM) at room temperature for 45 min and then incubated with MUNANA at 37 °C for 30 min. The residual NA activity is expressed as the percentage of activity relative to that obtained in the absence of reagent. Assays for AU, CP, and VC were similarly carried out, except in 80 mM acetate buffer (pH 5.0).

zanamivir effectively inhibited viral NA activity with an IC_{50} value of 2–3 nM, but it displayed weak inhibitory effects on the three bacterial NA activities, a result suggesting that the original strong binding interactions could be greatly compromised by possible variations in the active site. It must be emphasized that although one of the targets in this study, the influenza virus, is the same as one in the study of McKimm-Breschkin et al.,[9] the concept and approach of the current strategy offers a versatile alternative for future applications. The labeling event in this study was a result of an activation step forming the reactive quinone methide and did not rely on the strong noncovalent binding which is a critical requirement in the previous approach, a fact which makes the current approach a more general one in targeting NA activities. It is especially worth noting that the advantage of the current approach becomes more prominent with the advent of zanamivir-resistant viruses.[22]

Having established the efficacy of probe **2** to biotinylate NA and thus influenza virus A virions, we next studied the capturing performance by utilizing the covalent-bond-forming feature. The tests were carried out by a modified ELISA method as described previously.[9] Briefly, a streptavidin-coated 96-well ELISA plate (NUNC Immobilizer) was saturated with probe **2**. BSA–biotin conjugate provided a negative control. After 1 hour of incubation, the plate was blocked with 0.1 % BSA/phosphate-buffered saline (PBS) for 1 hour and washed with PBS. Serial twofold dilutions of influenza A virus (A/WSN/33) were added and incubated for 1 hour at room temperature. After another wash, the captured viruses were detected by treatment with a polyclonal anti-FluA antibody, followed by a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate and a NeA-Blue tetramethylbenzidine substrate (TMB, Clinical Science Products, Inc.). The results indicated that probe **2** bound to the microplate wells could successfully capture influenza virus A and the intensity of the responding signal was proportional to the number of virus particles present (Figure 3). The wells loaded with BSA–biotin conjugate gave a negative response. The possibility of any noncovalent bindings could be ruled out by the modest IC_{50} value (1.7 mM) on the viral NA. More importantly, when the same procedure was applied to a mixture of influenza virus and a non-NA-containing Japanese encephalitis virus, only influenza virus was selectively captured and detected on the plate, a result strongly supporting the theory that the capture of virus particles was both probe and NA dependent. This conclusion was further supported by the experiment during which probe **2** failed to biotinylate influenza viruses that were preincubated with zanamivir, which effectively blocked the active site of NA on the viral surface. The results represent the first example of the use of a covalent-bond-forming mechanism for the capture of influenza virus particles. In addition, such covalent interactions between captured virus particles and the probe are known to be tolerant to harsh conditions,[15] thus making this methodology amenable for further manipulations.

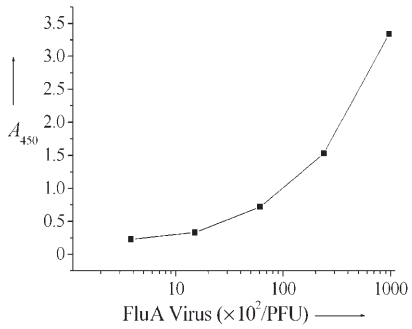


Figure 3. ELISA-based detection of influenza A (A/WSN/33) captured with probe **2** bound to streptavidin-coated microplate wells and detected with an anti-FluA antibody-HRP conjugate. PFU stands for plague-forming unit.

In summary, we have designed and synthesized a mechanism-based activity probe **2** for neuraminidase, which uses a latent quinone methide as the trapping device and forms a biotinylated adduct with *Arthrobacter ureafaciens* neuraminidase in a model study. By taking advantage of the essential role played by the NA activity in the life cycle of the influenza virus, we evaluated the interaction between probe **2** and the virus. The covalent-labeling event led to diminished NA activities. Furthermore, it serves as the basis for capturing influenza virus particles on microplate wells. This novel approach of capturing the influenza virus, which provides a stronger interaction between virus and the stationary phase, will certainly offer opportunities for developing new applications, such as rapid screening of antibodies against this group of viruses, and the development of sensitive and rapid diagnostic methods.

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