Characterization of *Vibrio cholerae* Neuraminidase by a Novel Mechanism-Based Fluorescent Labeling Reagent

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ABSTRACT: *Vibrio cholerae* neuraminidase (VCNA) plays a significant role in the pathogenesis of cholera by removing sialic acid residues from higher-order gangliosides to an unmasked GM1, the essential receptor for cholera toxin. Here we report that a novel mechanism-based fluorescent labeling reagent, 5-acetamido-2-(4-*N*-5-dimethylaminonaphthalene-1-sulfonyl-2-difluoromethylphenyl)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosonic acid (**1**), becomes a unique irreversible inhibitor of VCNA. Characterization of an inactivated VCNA by MALDI-TOF/TOFMS analysis revealed that the Asp-576 and Arg-577 residues, which are located within the ^{576}DRFF^{579} sequence, were specifically labeled with this suicide-type fluorescent substrate. Neither Asp-576 nor Arg-577 has ever been known to contribute to a specific residue in the rigid and highly conserved active site of VCNA investigated by crystallographic analysis, suggesting that a flexible β-turn structure containing this sequence may have a crucial role in the dynamic nature of substrate recognition and catalytic action by VCNA.

Sialidases (neuraminidases) make up an important class of glycoside hydrolases that catalyze the removal of the terminal sialic acid residue from various glycoconjugates (*1*). *Vibrio cholerae* neuraminidase (VCNA)[1] is a key glycoside hydrolase that elaborates the GM1 structure, the putative cholera toxin receptor, by removing sialic acids from its higher-order gangliosеds such as GD1, GT1, GQ1, etc. (*2*). Structural studies on VCNA by Taylor et al. revealed that this enzyme is composed of a central β-propeller catalytic domain flanked by two lectin-like domains (*3*). The rigid active site of VCNA appeared to be very similar to those observed in other sialidases such as *Salmonella* neuraminidase and influenza virus neuraminidase (*4*). The following features are common to these three members: (a) three arginine residues (arginine triad) which stabilize the carboxyl group of the sialic acid, (b) a glutamic acid which stabilizes one of these arginines, (c) a tyrosine residue whose hydroxyl group is close to the anomeric position of the sialic acid, (d) a glutamate with which the tyrosine has been implicated in the catalytic mechanism, (e) a hydrophobic cavity which accommodates the *N*-acetyl group of sialic acid, and includes a conserved tryptophan, and (f) a solvent-exposed aspartic acid which could act as a proton donor to the glycosidic oxygen or could be involved in stabilizing a proton-donating water molecule (*5*). This overall fold similarity defined by eight strictly conserved residues at the active site strongly suggests a similar mode of action for the entire superfamily of sialidases. However, despite extensive structural and mechanistic investigations, a number of basic questions about the mode of action by sialidases still remain. It has been suggested that sialidases have no carboxylate group favorably positioned to form a covalent sialyl−enzyme adduct during catalytic reaction, while most retaining glycosidases have been known to operate through a covalent glycosyl−enzyme intermediate involving an acid/base catalyst (*6*). The lack of structural information about the sialyl−enzyme complexes for any sialidase has made understanding both the catalytic mechanism and substrate specificity difficult.

In 2003, Withers et al. (*7*) reported the first successful trapping of the sialyl−enzyme intermediates by using 2,3-difluorosialic acid as an irreversible inactivator of *Trypanosoma cruzi* trans-sialidase. This pioneering work revealed that the Tyr-342 residue conserved in retaining-type glycoside hydrolases belonging to family GH33 plays an essential role as a catalytic nucleophile in the formation of a covalent linkage with an anomeric carbon of the sialic acid residue. In fact, it was demonstrated that other activated glycoside substrates in which a fluorine atom has been substituted adjacent to the anomeric center can also be used for identifying one of the two carboxyl groups acting as a nucleophile in the general retaining glycosidases (*8*). Our attention is now directed toward the method for searching the acid/base catalyst carboxyl group, another important amino acid residue which functions both as a general acid catalyst to assist the protonation of the glycosidic oxygen which is concomitant with bond cleavage and as a general

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[1] Abbreviations: VCNA, *V. cholerae* neuraminidase; MALDI-TOF/TOFMS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; GH, glycoside hydrolase; TLC, thin-layer chromatography; DAST, dimethylamino sulfur trifluoride; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; DANA, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid.

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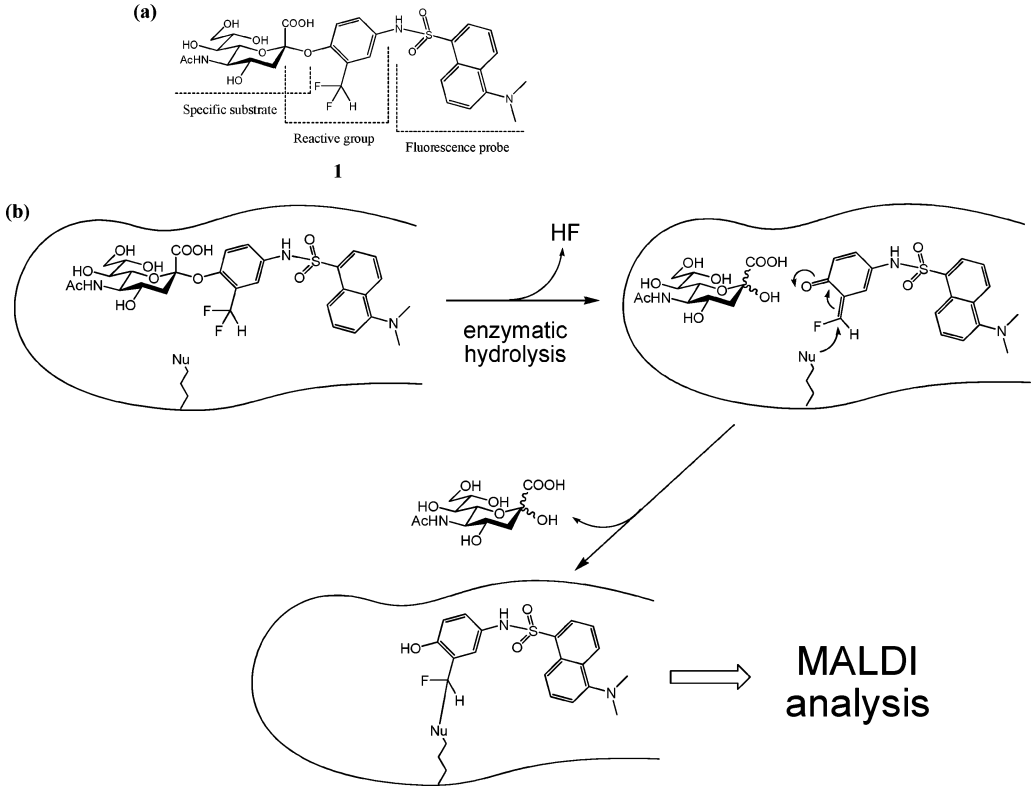


FIGURE 1: Chemical structure of the mechanism-based fluorescence labeling reagent and plausible mechanism of the specific nucleophilic labeling in an irreversible manner.

base catalyst to assist the attack of the nucleophilic water molecule to cleave the linkage of the sialyl−enzyme intermediate. In our previous report, we found that an irreversible substrate for β-galactosidases, (4-*N*-5-dimethylaminonaphthalene-1-sulfonyl-2-difluoromethylphenyl)-β-D-galactopyranoside, can be used as a specific mechanism-based fluorescent labeling agent to probe the nucleophilic amino acid residue in the active site (*9*). The proteomic analysis of inactivated *Escherichia coli* (*lacZ*) β-galactosidase using the above irreversible substrate revealed that one of the two catalytic glutamic acids, Glu-537, involved in the active site was directly labeled with the reactive aglycon generated by the hydrolysis. We thought that a similar approach with the synthetic irreversible inactivator of sialidase will allow for high-throughput identification of the candidates that act as an acid/base catalyst in the retaining sialidase activity. In this paper, we demonstrate that the mechanism-based tagging of VCNA with a fluorescent suicide substrate (Figure 1) greatly expedited an efficient isolation and an accurate sequencing of the labeled peptides by means of a tandem mass spectrometry (MALDI-TOF/TOFMS).

EXPERIMENTAL PROCEDURES

Reagent and General Methods

N-Acetylneuraminic acid was purchased from Nacalai Tesque Inc. Dimethylamino sulfur trifluoride (DAST) was purchased from Tokyo Kasei Kogyo Co. Ltd. α2,3,6,8-Neuraminidase from *V. cholerae* was purchased from Sigma. Anti-dancyl rabbit IgG was obtained from Molecular Probes. All other chemical reagents were purchased from Wako Pure Chemical Co. Ltd. All chemical reactions were performed in anhydrous solvents under a nitrogen atmosphere in the dark unless noted otherwise. TLC was performed on Merck precoated plates (20 cm × 20 cm, layer thickness of 0.25 mm, silica gel $60F_{254}$); spots were visualized by spraying a solution of a 90:5:5 (v/v/v) MeOH/*p*-anisaldehyde/concentrated sulfuric acid mixture and heating at 180 °C for ∼0.5 min, and by UV light (256 or 365 nm), when applicable. Flash column chromatography was performed on silica gel 60 (spherical type, particle size of 40−50 μm; Wako Pure Chemical Co. Ltd.) with the specified solvent systems, and the ratio of solvent systems was given on a volume basis. Organic extracts were dried over anhydrous $MgSO_4$, and solutions were concentrated under diminished pressure below 50 °C. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, with an AV-400M spectrometer (Bruker Biospin) for solutions in $CDCl_3$ unless otherwise specified. Chemical shifts are given in parts per million and referenced to internal TMS (δ_H 0.00 in $CDCl_3$), $CHCl_3$ (δ_H 7.26 in $CDCl_3$), *tert*-BuOH (δ_H 1.24 in D_2O), or $CDCl_3$ (δ_C 77.00). ^{1}H NMR assignments were made by first-order analysis of the spectra and were verified by H−H COSY, TOCSY, and HMQC experiments.

MALDI-TOF Mass Spectrometry

Samples were desalted and concentrated using 10 μL C_{18} ZipTips (Millipore) according to the manufacturer's instructions. Typically, samples were dissolved in 1 μL of 90% (v/v) acetonitrile containing trifluoroacetic acid and mixed with the same volume of a saturated solution of 2,5-dihydroxybenzoic acid in 33% acetonitrile containing 0.1% trifluoroacetic acid. The mixtures described above (1 μL) were applied to a stainless steel target MALDI plate and air-dried before being analyzed in the mass spectrometer.

All measurements were performed using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the Flexcontrol 1.2 software package (Bruker Daltonics GmbsH, Bremen, Germany). Ions generated by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated to a kinetic energy of 23.5 kV. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas. Precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell (LIFT means "lifting" the potential energy for the second acceleration of the ion source), and their masses were analyzed after the ion reflector passage. Masses were automatically annotated using XMASS 5.1.2 NT. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (*m*/*z* 1046.542), bombesin (*m*/*z* 1619.823), ACTH (*m*/*z* 2465.199), and somatostatin 28 (*m*/*z* 3147.472). The mixture of these peptides was measured on the central spot of a 3 × 3 square with external calibration. To achieve a mass accuracy of better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. Calibration of these mass spectra was performed automatically utilizing a customized macro command of the XMASS 5.1.2 NT software package. The macro command was used for the calibration of the monoisotopic singly charged peaks of the above-mentioned peptides. LIFT-TOF/TOF spectra were annotated with the BioTools 2.1 software package.

Synthesis of a Fluorescence-Labeled Suicide Substrate

*Methyl [4,7,8,9-Tetra-O-acetyl-5-acetamide-2-(4-nitrosalicylaldehyde)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid]onate (**3**).* To a solution of **2** (2.00 g, 6.46 mmol) in methanol (30 mL) was added Dowex 50W (H^+ form; 2.0 g), and the suspension was shaken violently with a voltex mixer for 1 day. The resin was filtered off from the suspension, and the filtrate was concentrated. A suspension of the residue in acetyl chloride (15 mL) was stirred vigorously for 1 day. The resulting solution was concentrated and coevaporated with toluene three times. To a suspension of the residue and 2-hydroxy-5-nitrobenzaldehyde (3.24 g, 19.4 mmol) in acetonitrile (30 mL) was added diisopropylethylamine (3.04 mL, 17.5 mmol) dropwise, and the mixture was stirred for 1 day. After solvent evaporation in vacuo, the residue was applied to a column of silica gel and eluted with an acetone/hexane mixture (4:3) to afford **3** (3.01 g, 72.7%) as a light yellow powder: ^{1}H NMR ($CDCl_3$) δ 10.42 (s, 1 H, CHO), 8.69 (d, 1 H, $J = 2.9$ Hz, Ph), 8.43 (dd, 1 H, $J = 2.9$, 9.2 Hz, Ph), 7.44 (d, 1 H, $J = 9.2$ Hz, Ph), 5.37 (ddd, 1 H, $J = 2.5$, 4.9, 9.5 Hz, H-8), 5.36 (dd, 1 H, $J = 1.6$, 9.5 Hz, H-7), 5.29 (d, 1 H, $J = 9.9$ Hz, NH), 5.05 (ddd, 1 H, $J = 4.7$, 10.3, 11.9 Hz, H-4), 4.66 (dd, 1 H, $J = 1.5$, 10.9 Hz, H-6), 4.22 (dd, 1 H, $J = 2.5$, 12.4 Hz, H-9a), 4.13 (q, 1 H, $J = 10.2$ Hz, H-5), 4.09 (dd, 1 H, $J = 5.0$, 12.4 Hz, H-9b), 3.69 (s, 3 H, Me), 2.84 (dd, 1 H, $J = 4.7$, 13.1 Hz, H-3a), 2.40 (dd, 1 H, $J = 12.1$, 13.1 Hz, H-3b), 2.20, 2.13, 2.08, 2.06, 1.95 (each s, 15 H, 5 Ac); ^{13}C NMR ($CDCl_3$) δ 187.0, 170.8, 170.6, 170.3, 170.1, 170.0, 167.6, 160.0, 143.7, 130.5, 126.3, 124.2, 119.4, 100.0, 77.2, 73.9, 68.0, 67.9, 66.9, 62.2, 53.7, 49.5, 38.6, 23.2, 21.0, 20.8, 20.7; FABHRMS calcd for $C_{27}H_{33}N_2O_{16}$ $[M + H]^+$ 641.1825, found 641.1829.

*Methyl [4,7,8,9-Tetra-O-acetyl-5-acetamide-2-(2-difluoromethyl-4-nitrophenyl)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid]onate (**4**).* To a solution of **3** (2.89 g, 4.51 mmol) in dichloromethane (30 mL) was added dimethylamino sulfer trifluoride (1.48 mL, 11.2 mmol) at 0 °C, and the mixture was stirred at room temperature for 30 min. Methanol (1.0 mL) was added to the reaction mixture at 0 °C, and the mixture was concentrated. The residue was applied to a column of silica gel and eluted with an acetone/hexane mixture (4:3) to afford the target compound **4** (2.01 g, 67.2%) as a light yellow powder: ^{1}H NMR ($CDCl_3$) δ 8.47 (d, 1 H, $J = 2.8$ Hz, Ph), 8.34 (dd, 1 H, $J = 2.8$, 9.2 Hz, Ph), 7.41 (d, 1 H, $J = 9.2$ Hz, Ph), 6.90 (t, 1 H, $J = 54.9$ Hz, CHF_2), 5.38 (ddd, 1 H, $J = 2.6$, 5.1, 9.4 Hz, H-8), 5.35 (dd, 1 H, $J = 1.6$, 9.4 Hz, H-7), 5.30 (d, 1 H, $J = 9.9$ Hz, NH), 5.03 (ddd, 1 H, $J = 4.7$, 10.3, 11.7 Hz, H-4), 4.63 (dd, 1 H, $J = 1.6$, 10.9 Hz, H-6), 4.23 (dd, 1 H, $J = 2.5$, 12.6 Hz, H-9a), 4.11 (q, 1 H, $J = 10.3$ Hz, H-5), 4.10 (dd, 1 H, $J = 5.2$, 12.6 Hz, H-9b), 3.66 (s, 3 H, Me), 2.79 (dd, 1 H, $J = 4.7$, 13.2 Hz, H-3a), 2.34 (dd, 1 H, $J = 11.9$, 13.2 Hz, H-3b), 2.20, 2.12, 2.07, 2.06, 1.94 (each s, 15 H, 5 Ac); ^{13}C NMR ($CDCl_3$) δ 170.8, 170.55, 170.3, 170.1, 170.0, 167.5, 156.5, 143.3, 127.9, 125.3, 122.6, 122.6, 122.5, 118.4, 112.4, 110.1, 107.7, 100.1, 77.2, 73.7, 68.0, 67.9, 66.9, 62.2, 53.5, 49.4, 38.5, 30.9, 23.3, 21.0, 20.8, 20.7; FABHRMS calcd for $C_{27}H_{33}F_2N_2O_{15}$ $[M + H]^+$ 663.1844, found 663.1842.

*5-Acetamido-2-(4-N-5-dimethylaminonaphthalene-1-sulfonyl-2-difluoromethylphenyl)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosonic Acid (**1**).* To a solution of **4** (100 mg, 0.151 mmol) in methanol (5.0 mL) was added 1 M sodium methoxide in methanol (50 μL) dropwise at room temperature, and the mixture was stirred for 1 h. Water and 1 M sodium methoxide in methanol (200 μL) were added to the reaction mixture dropwise, and the mixture was stirred for 4 h at room temperature and neutralized with 1% acetic acid. Palladium hydroxide on carbon (10%) was added to the reaction mixture, and the suspension was stirred vigorously under a hydrogen atmosphere for 1 day. After the catalyst was filtered, the filtrate was concentrated. To a solution of the residue in methanol (1.0 mL) were added triethylamine (42 mL, 0.30 mmol) and dansyl chloride (61 mg, 0.23 mmol). After being stirred for 20 h at room temperature, the mixture was concentrated and the residue was applied to a column of silica gel and eluted with a chloroform/methanol/water mixture (10:10:1) to afford **1** (46.6 mg, 45.2%) as a light green powder: ^{1}H NMR (D_2O) δ 8.44 (d, 1 H, $J = 8.8$ Hz, aromatic), 8.29 (d, 1 H, $J = 8.7$ Hz, aromatic), 8.15 (d, 1 H, $J = 6.9$ Hz, aromatic), 7.68 (t, 1 H, aromatic), 7.57 (t, 1 H, aromatic), 7.40 (d, 1 H, $J = 7.6$ Hz, aromatic), 7.13 (d, 1 H, $J = 8.6$ Hz, aromatic), 7.02 (brd, 1 H, aromatic), 6.97 (brs, 1 H, aromatic), 6.87 (t, 1 H, $J = 55.2$ Hz, CHF_2), 3.84−3.54 (m, 7 H, H-4, H-5, H-6, H-7, H-8, H-9a, H-9b), 2.85 (s, 6 H, NMe_2), 2.76 (dd, 1 H, $J = 4.5$, 12.6 Hz, H-3a), 2.03 (s, 3 H, NHAc), 1.78 (t, 1 H, $J = 12.0$ Hz, H-3b); FABHRMS calcd for $C_{30}H_{36}F_2N_3O_{11}S$ $[M + H]^+$ 684.2033, found 684.2032.

*Inhibitory Effect by a Suicide Substrate **1***

An ultraviolet−visible spectrometer at 410 nm was used to assess irreversible inhibitions of α2,3,6,8-neuraminidase from *V. cholerae* by a suicide substrate **1** in the presence of 4-nitrophenyl-*N*-acetylneuraminic acid as a competitive

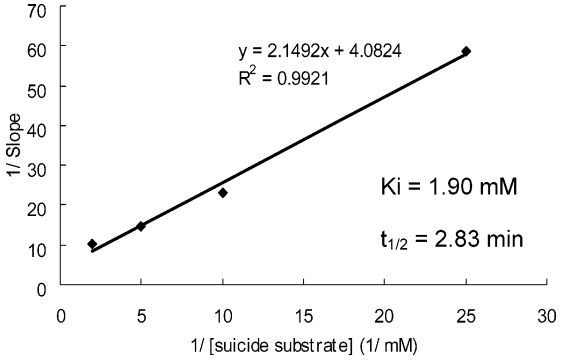


FIGURE 2: Kinetic behavior of compound **1**. To a solution of VCNA and 0−1.5 mM compound **1** was added *p*-nitrophenyl-*N*-acetylneuraminic acid (final concentration of 5 mM), and changes in the hydrolytic activity of the enzyme were determined by measuring the relative intensity at 410 nm.

substrate. The inhibition constants (K_i) and the half-life for inhibition ($t_{1/2}$) were determined as reported previously (*9*). Kinetic parameters for the enzyme were determined by incubating the enzyme in 100 mM citrate phosphate buffer (pH 5.0) containing various concentrations (0−1.0 mM) of the suicide substrate at 37 °C. Aliquots (2 μL) of the inactivation mixture were removed at different time intervals (preincubation time of 0, 15, and 30 min) and diluted into a reaction cell containing a 4-nitorophenyl-*N*-acetylneuraminic acid solution (final concentration of 5 mM, 50 μL). Residual enzyme activity was determined by monitoring the rate of release of 4-nitrophenol with an ultraviolet−visible spectrometer (410 nm) after the addition of 0.1 M glycine-NaOH buffer (pH 11) to stop the enzymatic activity. Slopes of plots of the natural algorithm of residual enzyme activity versus preincubation time yielded pseudo-first-order rate constants for inactivation at each suicide substrate concentration. Values for K_i (irreversible inhibitor constant) and the half-life of inhibition were calculated from plots of the reciprocal of the pseudo-first-order rate constants versus the reciprocal of the suicide substrate concentration. K_i and $t_{1/2}$ were calculated to be 1.90 mM and 2.83 min, respectively (Figure 2).

Mechanism-Based Fluorescence Labeling of Neuraminidase

α-2,3,6,8-Neuraminidase from *V. cholerae* (50 μg) was incubated with or without the suicide substrate **1** (5 μg, final concentration of 45 μM) at 37 °C in 200 μL of 100 mM citrate phosphate buffer (pH 5.0). The reaction mixture was subjected directly to purification with a small gel filtration column (Sephadex G-25, ϕ 8 mm × 20 mm, 1 mL) using 100 mM ammonium bicarbonate buffer (pH 8.0) to remove the excess suicide substrate. Next, the labeled enzyme was concentrated by a centrifugal evaporation system to ca. 50 μg/10 μL in 100 mM ammonium bicarbonate. To the above solution were added a 10 mM HCl solution (90 μL) and pepsin (5 μg), and the mixture was incubated overnight at 37 °C. Then, the digests were subjected to affinity purification using an anti-dansyl antibody column.

Purification of the Fluorescence-Labeled Peptide by the Anti-Dansyl Antibody Column

Anti-dansyl rabbit IgG (0.5 mL, 1 mg/mL) was dialyzed at 4 °C for 10 min against a solution of 0.1 M sodium bicarbonate containing 0.1 M NaCl using a Centricon YM-30 concentrator (Millipore) (5 × 3 mL, 10 000 rpm). Then, the anti-dansyl rabbit IgG solution was mixed with 0.5 mL of Affinity-Gel 10 (Bio-Rad) according to the manufacturer's instructions, and the mixture was packed into a 0.5 cm × 2 cm column. The Bradford method (*10*) was used to estimate that 0.98 mg of IgG was coupled with 1.0 mL of polymer support. The binding capacity (1.18 nmol/0.98 mg of IgG) of this antibody column was tested with (4-*N*-dansylphenyl)-β-D-galactopyranoside as reported previously (*9*). A typical procedure for the isolation of a fluorescence-labeled peptide fragment generated by peptidase treatment follows. A sample solution (50 μL) was allowed to pass through the anti-dansyl rabbit IgG column at room temperature with a flow rate of 0.1 mL/min. After the mixture had been washed with 3 mL of a 100 mM Hepes-NaOH solution (pH 7.5) containing 100 mM NaCl, peptides adsorbed onto the antibody column were eluted with a 10% (v/v) acetic acid/water mixture, and the fractions containing the desired materials were concentrated using centrifugal evaporation.

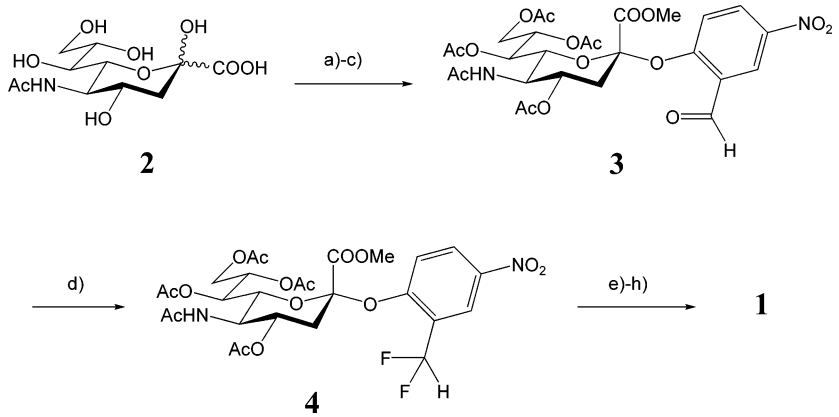
RESULTS AND DISCUSSION

Synthesis. Compound **1** was synthesized from commercially available *N*-acetylneuraminic acid (**2**) according to the procedure shown in Scheme 1. A well-known glycosyl donor, methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-2-chloro-2,3,5-trideoxy-β-D-glycero-D-galacto-2-nonulopyranosonate (*11*), was coupled with 2-hydroxy-5-nitrobenzaldehyde in the presence of diisopropylethyleneamine to afford α-glycoside **3** in 72.3% yield. Treatment of **3** with diethylamino sulfur trifluoride (DAST) furnishing intermediate **4** (67.2%) led to the desired compound **1** after de-O-acetylation, saponification of the methyl ester, reduction of the *p*-nitro group, and subsequent dansylation (four steps, 45.2%). The purity and chemical structures of compounds synthesized herein were ascertained by NMR and high-resolution mass spectrometry.

Inhibition and Specific Labeling of VCNA. According to the general method and conditions described previously by our group (*9*), Danzin et al. (*12*), and Quash et al. (*13*), we examined the effect of compound **1** on the hydrolysis of a common substrate, 4-nitrophenyl-*N*-acetylneuraminic acid, by VCNA. These results indicate that inactivation of VCNA is dependent on both the time of the preincubation and the concentration of compound **1** in the medium. Kinetic data (K_i = 1.90 mM, $t_{1/2}$ = 2.83 min) obtained by the Kitz and Wilson plot (Figure 2) (*14*, *15*) clearly suggest that compound **1** acts as a competitive and irreversible inhibitor toward VCNA.

VCNA inactivated (labeled) with an irreversible inhibitor was subjected to peptidase treatment to give rise to the complex peptide fragments observed in MALDI-TOFMS as shown in Figure 3a. It is evident that the signals resulting from the fluorescence-labeled peptide ($[M + H]^+$ = 1328.49, $[M + Na]^+$ = 1350.47, and $[M + Ka]^+$ = 1366.45) cannot be directly identified if the peptide mixture was analyzed, because of the complexity of signals of other peptides found in the upper spectra. However, the MALDI-TOFMS of the peptide readily purified by the anti-dansyl antibody column exhibited clear and simple signals due to the ions of a labeled peptide fragment as indicated in Figure 3b.

Scheme 1: Synthetic Route of the Mechanism-Based Fluorescent Labeling Reagent[a]



[a] (a) Dowex 50W (H^+ form)/ MeOH; (b) CH_3COCl; (c) 2-hydroxy-5-nitrobenzaldehyde/CH_3CN (three steps, 72.7%); (d) dimethylamino sulfur trifluoride, 0 °C (67.2%); (e) 1 M NaOMe/MeOH; (f) 1 M NaOMe/MeOH/H_2O; (g) 10% $Pd(OH)_2/C$, H_2; (h) dansyl chloride/Et_3N/MeOH (four steps, 45.2%).

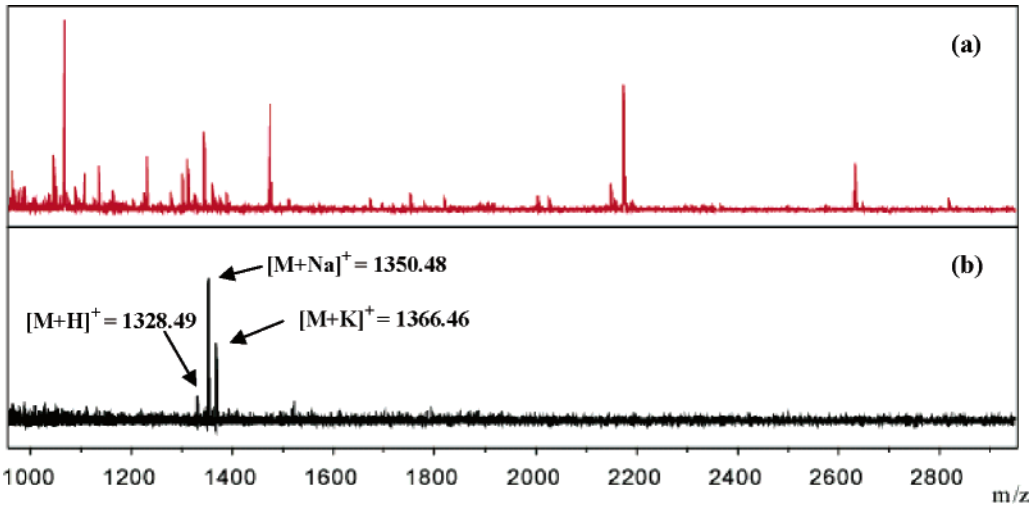


FIGURE 3: MALDI-TOF mass spectra of fluorescence-labeled peptides. Peptide fragments produced by digestion with pepsin were analyzed: (a) mixtures of peptides derived from labeled enzyme and (b) peptide isolated by using anti-dansyl antibody column chromatography.

The parent ion of *m*/*z* 1328.49 from the fluorescence-labeled peptide obtained by the peptidase treatment was subsequently applied for further LIFT-TOF/TOF fragmentation analysis to yield a spectrum having a series of b-ions (488.13, 1036.34, 1116.41, and 1310.48) and y-ions (1328.49, 841.38, 313.38, and 166.09) as shown in Figure 4. These product ions and fragmentation from two dansylated amino acids allowed us to conclude that this peptide with a calculated molecular mass ($[M + H]^+ = 1328.48$) was sequenced as ^{576}DRFF^{579}, in which Asp-576 and Arg-577 were dansylated. Surprisingly, it has been known that neither Asp-576 nor Arg-577 was assigned as a crucial amino acid residue located in the catalytic site of VCNA. Moreover, the three-dimensional structure of VCNA (Figure 5) (*3*) indicated that both Asp-576 and Arg-577 residues seemed to be located far (approximately 20 Å) from the catalytic pocket of VCNA. It has been postulated that a solvent-exposed Asp-250 located close (4.7 Å) to the anomeric carbon, one of the well-conserved residues in the rigid active site, seems to be a key amino acid residue that acts as a proton donor or a stabilizer of a proton-donating water molecule (*5*). However, we could not detect any other fluorescence-labeled peptides except the peptide sequence containing Asp-576 and Arg-577. Since a pair of residues, Asp-320 (14.3 Å) and Arg-321 (16.9 Å), can be seen between the active site and the flexible β-turn involving Asp-576 and Arg-577, the labeling of these two amino acid residues by compound **1** seems to be a quite specific and interesting result. Considering the unstable nature of the reactive intermediate of fluorinated quinone methide generated by hydrolysis, one may speculate that Asp-576 and Arg-577 could participate in the catalytic mechanism of the hydrolysis of compound **1** only when VCNA conducts a movement of the flexible β-turn structure by ~20 Å, bringing Asp-576 and Arg-577 to the active site. At present, we could not determine the mechanism of unusual dual labeling observed in the flexible β-turn, and further structural and mutagenesis studies may be required to provide a more sufficient and plausible answer to explain this phenomenon. However, Wakatsuki et al. (*16*) also demonstrated in the crystallographic studies on human cytosolic sialidase (Neu2) that the loops containing Glu-111 and the catalytic Asp-46 residues located far from the active site are disordered in the apo form but upon binding of DANA (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid) become ordered to adopt two short α-helices and come in close contact with the inhibitor and cover the catalytic site, illustrating the dynamic nature of substrate recognition and catalytic action by sialidase. Taken together, these results may suggest that

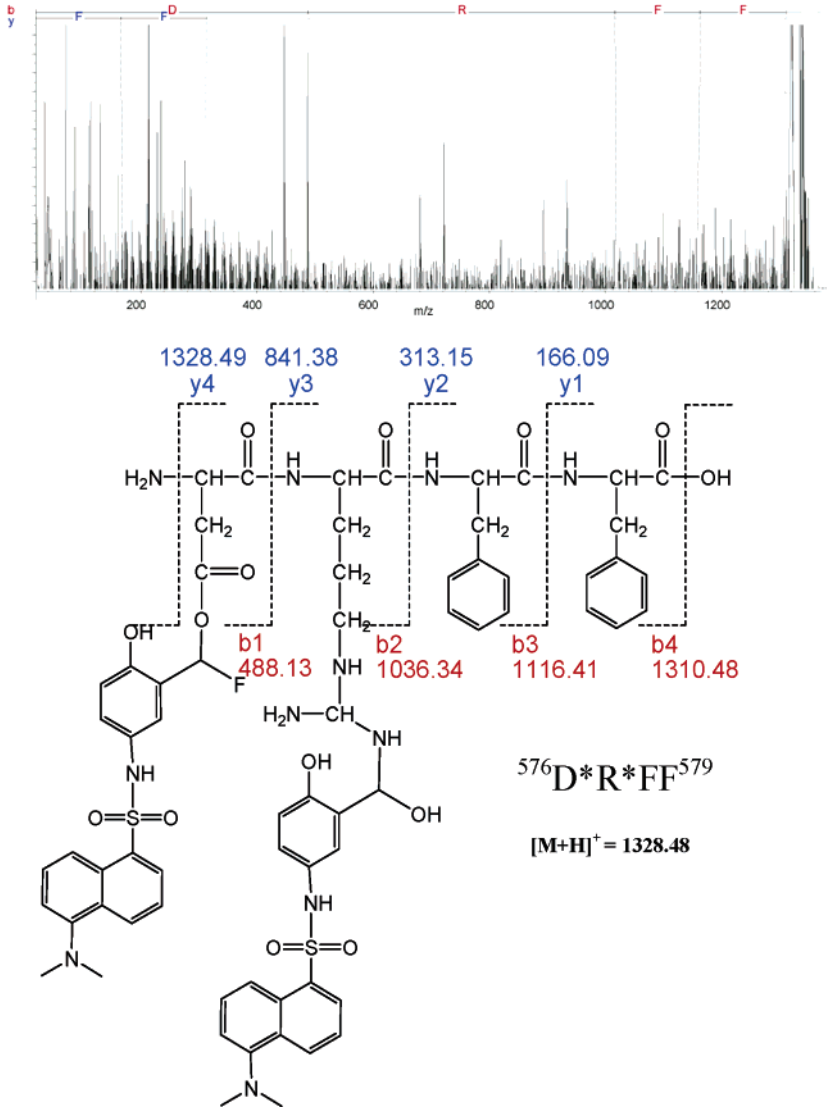


FIGURE 4: MALDI-TOF/TOF mass spectrum of the dansylated peptide fragment (*m*/*z* 1328.49) and assignment of the identified ^{576}DRFF^{579} sequence, which contains dansylated Asp-576 and Arg-577.

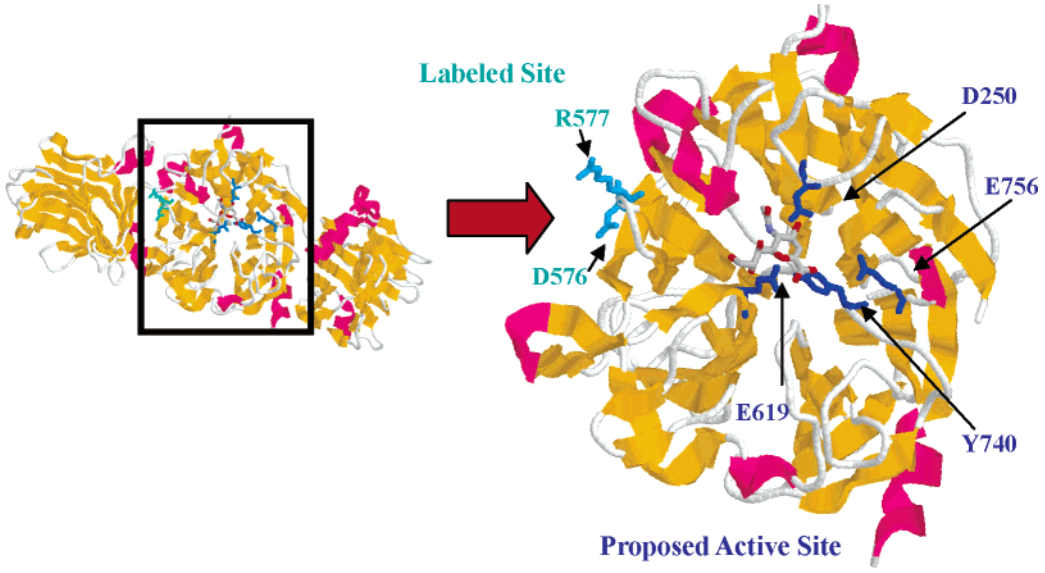


FIGURE 5: Active site and topology of fluorescence-labeled Asp-576 and Arg-577 in the three-dimensional structure of VCNA in the Protein Data Bank.

the use of the flexible loop or β-turn structure containing catalytic amino acids located far from the catalytic crevice seems to be feasible for adopting sialidase for substrates with different sizes or characteristics.

It is evident that three-dimensional structural information for enzymes at atomic resolution obtained by crystallographic analysis can often be utilized for conclusive identification of the active site amino acid residues. When the three-dimensional (3D) structure of an enzyme−inhibitor or enzyme−substrate complex is available, the residues important to catalysis, ether directly as an acid/base, as nucleophilic catalysts, or less directly through binding of the substrate in its ground state or at the transition state, will be apparent. However, static 3D information is still not useful for investigating a dynamic reaction mechanism of the enzymatic action. Even when the identities of the amino acid residues in the spatial proximity of the substrate are known, their specific functions in the catalysis frequently cannot be predicted. Therefore, verification of functional roles in catalysis of the specific residues postulated by 3D structural analysis requires further kinetic analyses of mutants preferably in conjunction with studies using specific mechanism-based labeling reagents.

In this study, we demonstrate that a novel mechanism-based fluorescence labeling agent synthesized from *N*-acetylneuraminic acid allows for the efficient isolation and characterization of labeled peptides bearing catalytic (nucleophilic) amino acids of VCNA. Characterization of an inactivated VCNA by MALDI-TOF/TOFMS analysis revealed that the Asp-576 and Arg-577 residues, which are located within the ^{576}DRFF^{579} sequence, were specifically labeled by this suicide-type fluorescent substrate. Both Asp-576 and Arg-577 have never been identified as members of the highly conserved active site of VCNA investigated by crystallographic analysis; thus, we postulated that a flexible β-turn structure containing this sequence may have a crucial role in the dynamic nature of substrate recognition and/or catalytic action by VCNA. High-throughput proteomic analysis of the mechanism-based tagged sialidases by combined use of anti-dansyl antibody column chromatography and MALDI-TOF mass spectrometry may become a facile and general method both for identifying the candidates of the catalytic amino acids and for rapidly characterizing new bacterial/viral sialidases from unknown pathogens and human sialidases as possible targets for diagnosis and therapy of colon cancer (*17*).

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