A Novel Transition-state Analogue for Lysozyme, 4-O-β-Tri-N-acetylchitotriosyl Moranoline, Provided Evidence Supporting the Covalent Glycosyl-enzyme Intermediate*

Received for publication, November 23, 2012, and in revised form, January 3, 2013. Published, JBC Papers in Press, January 9, 2013, DOI 10.1074/jbc.M112.439281

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Background: A pure and stable transition-state analogue for lysozyme has not been reported thus far.

Results: We synthesized 4-O-β-tri-N-acetyl-chitotriosyl moranoline (3), which inhibited strongly the lysozyme reaction.

Conclusion: Compound 3 was found to be a novel and stable transition-state analogue for lysozyme.

Significance: The crystal structure of lysozyme in a complex with 3 supports the covalent glycosyl-enzyme intermediate in the catalytic reaction.

4-O-β-Di-N-acetylchitobiosyl moranoline (2) and 4-O-β-tri-N-acetylchitotriosyl moranoline (3) were produced by lysozyme-mediated transglycosylation from the substrates tetra-N-acetylchitotetraose, (GlcNAc)4, and moranoline, and the binding modes of 2 and 3 to hen egg white lysozyme (HEWL) was examined by inhibition kinetics, isothermal titration calorimetry (ITC), and x-ray crystallography. Compounds 2 and 3 specifically bound to HEWL, acting as competitive inhibitors with $K_i$ values of 2.01 × 10^{-5} and 1.84 × 10^{-6} M, respectively. From ITC analysis, the binding of 3 was found to be driven by favorable enthalpy change ($\Delta H^\circ$), which is similar to those obtained for 2 and (GlcNAc)$_4$. However, the entropy loss ($-T\Delta S^\circ$) for the binding of 3 was smaller than those of 2 and (GlcNAc)$_4$. Thus the binding of 3 was found to be more favorable than those of the others. Judging from the $K_a$ value of 3 (760 nM), the compound appears to have the highest affinity among the lysozyme inhibitors identified to date. X-ray crystal structure of HEWL in a complex with 3 showed that compound 3 binds to subsites −4 to −1 and the moranoline moiety adopts an undistorted C$_1$ chair conformation almost overlapping with the −1 sugar covalently bound to Asp-52 of HEWL (Vocadlo, Davies, G. J., Laine, R., and Withers, S. G. (2001) Nature 412, 835–838). From these results, we concluded that compound 3 serves as a transition-state analogue for lysozyme providing additional evidence supporting the covalent glycosyl-enzyme intermediate in the catalytic reaction.

Lysozymes (EC 3.2.1.17) are glycosidases acting in the innate-immune system of most animals (1). The common feature of lysozymes is to exert antibacterial activity by cleaving the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine (GlcNAc) in peptidoglycan, a major bacterial cell-wall component (2). Hen egg white lysozyme (HEWL) was the first enzyme to have its three-dimensional structure determined by x-ray crystallography, revealing that the enzyme is divided into two domains by a deep substrate-binding cleft containing the catalytic site (3). The catalytic importance of the conserved residues, Glu-35 and Asp-52, was also confirmed by the crystal structure of the enzyme-ligand complex (4) and site-directed mutagenesis (5). These studies on the structure and function of HEWL offer the strongest support available to date for the theory that enzymes may catalyze reactions by binding substrates in the geometry of the transition state more strongly than in that of the ground state.

HEWL has six subsites for sugar residue binding, termed −4 to +2 (formerly A, B, C, D, E, and F (6)). The enzyme cleave a glycosidic linkage between the sugar residues at subsites −1 and +1 with the aid of the proton-donating action of Glu-35 as a general acid. The substrate analog tri-N-acetylchitotriose, (GlcNAc)$_3$, binds predominantly to subsites −4, −3, and −2 of HEWL, keeping away from subsite −1 due to the unfavorable positive free energy of the sugar residue binding at that subsite (7). This situation might have been an obstacle to understanding the catalytic mechanism of the enzyme. In the classical explanation, the lysozyme-catalyzed reaction was recognized to take place via a carbenium ion intermediate stabilized by the carboxylate of Asp-52 as a conjugate base. The intermediate state adopts a half-chair conformation with C1 carbon display-

§4 This work was supported by “Strategic Project to Support the Formation of Research Bases at Private Universities, Matching Fund Subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2011–2015, Grant 2111010356.

1 The atomic coordinates and structure factors (codes 4HPI and 4HP0) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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5 The abbreviations used are: GlcNAc, N-acetylglucosamine; HEWL, hen egg white lysozyme; (GlcNAc)$_n$, β-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of $n$; pNP-(GlcNAc)$_n$, p-nitrophenyl penta-N-acetylchitopentaoside; ITC, isothermal titration calorimetry; HRESI-MS, high-resolution electrospray ionization mass spectrometry; PDB, Protein Data Bank; BisTris, 2-(bis-2-hydroxyethylamino)-2-(hydroxymethyl)propane-1,3-diol.
ing $sp^2$ hybridization. Thus, the binding of a pyranose ring to subsite $-1$ is likely accompanied by distortion of the pyranose ring from its normal chair conformation toward the half-chair conformation (8, 9). Experimental evidence for the distortion of the $-1$ sugar was obtained by binding experiments using an unsaturated derivative from cell-wall tetrasaccharide (8) and a $\delta$-lactone derived from $(GlcNAc)_4$ (10). The affinity of the $\delta$-lactone derivative to HEWL was much higher than that of the unsaturated derivative, suggesting that the $\delta$-lactone derivative is a transition-state analogue for HEWL. However, the $\delta$-lactone derivative used is unstable and always in a mixture with the gluconic acid. Thus, the data obtained with the lactone derivative might not be accurate. It is highly desirable to obtain a pure and stable transition-state analogue for HEWL to more deeply understand the enzyme mechanism.

We recently reported a synthetic method for easily obtaining the conjugated $\delta$-lactone, 4-O-$\beta$-tri-$N$-acetlychitotriosyl-2-acetamido-2,3-dideoxydidehydroglucopyranose $\delta$-lactone (5) derived from tetra-$N$-acetlychitotetraose $(GlcNAc)_4$ by chemical modification of the reducing end residue of the tetrasaccharide (11). Compound 5 might be a candidate for the stable transition-state analogue for HEWL. Meanwhile, Vocadlo et al. (12) reported the crystal structure of HEWL covalently bound to C1 carbon of the $-1$ sugar, which exhibits a chair conformation with C1 carbon in $sp^3$ hybridization. Because 1-deoxynojirimycin (moranoline) acting as the paradigms of glycosidase inhibitors is known to bind subsite $-1$ of the corresponding enzymes (13–15), chitin oligosaccharides, $(GlcNAc)_n$ modified with moranoline at their reducing end might be another candidate for the stable transition-state analogue for HEWL.

In this study, 4-O-$\beta$-$N$-acetylglucosaminyl moranoline (1), 4-O-$\beta$-di-$N$-acetylchitobiosyl moranoline (2), and 4-O-$\beta$-tri-$N$-acetlychitotriosylmoranoline (3) were synthesized from chitin tetrasaccharide, $(GlcNAc)\beta$, with the aid of the lysozyme-catalyzed transglycosylation reaction (Scheme 1), and examined with respect to their inhibitory action toward HEWL. Isothermal titration calorimetry (ITC) and x-ray crystallography were also conducted to elucidate the binding modes of 2 and 3 to HEWL. Finally, we found that compound 3 is a novel transition-state analogue for HEWL and provides evidence supporting the covalent glycosyl-enzyme intermediate in the catalytic reaction.

**EXPERIMENTAL PROCEDURES**

*General Methods—* 4-O-$\beta$-Tri-$N$-acetylchito-triosyl-2-acetamido-2,3-dideoxydidehydroglucopyranose (4) and compound 5 (Fig. 1) were prepared by our previously described methods (11). $(GlcNAc)_n\beta$ ($n = 2, 3, 4,$ and 6) and $(GlcNAc)_n\beta$-$p$NP were kindly provided by Yaizu Suisan Kagaku Industry Co., Ltd. (Shizuoka, Japan). Moranoline was a gift from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). HEWL, which was recrystallized six times, was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Lyophilized cells of *Micrococcus lysodeikticus* were purchased from Sigma. All other reagents were of the highest quality commercially available and were used without further purification.

*Analytical Methods—* HPLC analysis was carried out to determine the amounts of products from the lysozyme-catalyzed transglycosylation reaction using an ANIDIUS column (4.6 $\times$ 250 mm, Develosil, Japan) with a JASCO Intelligent system liquid chromatograph and detection by ultraviolet light at 210 nm. The bound material was eluted with 80% CH$_3$CN at a flow rate of 1.0 ml/min at 40 °C. The HRESI-MS spectra were measured on a JMS-T100LC mass spectrometer. 500-MHz $^1$H NMR spectra and 125-MHz $^{13}$C NMR spectra were recorded using a JEOL $\lambda$-500 spectrometer. Chemical shifts were expressed in ppm relative to the methyl resonance of the external standard, sodium 3-(trimethylsilyl)propionate.

*Enzymatic Synthesis of Compounds 1, 2, and 3 by HEWL—* $(GlcNAc)_4$ (0.90 g, 1.1 mmol) and moranoline (1.24 g, 7.6 mmol) were dissolved in 50 ml of 50% DMSO, and then HEWL (1.0 g, 100,000 kU) was added to conduct the transglycosylation reaction shown in Scheme 1. The mixture was incubated for 100 h at 50 °C. The precipitate was removed by centrifugation (27,600 $\times$ g, 20 min) and the supernatant was directly applied to an ion-exchange
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column (Dowex-50W H⁺ form, 3.0 × 18 cm) equilibrated with H₂O. After washing the column with H₂O, the adsorbed portion was eluted with 0.5 M NH₄OH. The adsorbed portion was concentrated and dissolved in 10 ml of H₂O and then loaded onto a Toyopearl HW-40S column (5.5 × 60 cm). The column was developed with the 0.1 M NaCl at a flow rate of 80 ml/h and a fraction size of 7 ml/tube. The chromatogram identified four distinct peaks, F-1 to F-4 (Fig. 2B). Fractions corresponding to the individual peaks were pooled, dialyzed, concentrated, and lyophilized.

F-1 (fractions 88–92) was identified as compound 3 (22.2 mg. 2.7% based on the amount of (GlcNAc)₄ added). [α]D²⁵ + 3.4° (c 1.0, H₂O); HRESI-MS: m/z 795.31046 [M + Na⁺]⁺ (calculated for C₉₆H₅₂N₄O₉Na, 795.31234). ¹H NMR (D₂O, 500 MHz): δ 4.59 (d, 2H, J₁⁻,₂ = 9.0, J₂⁻,₃ = 9.0 Hz, H-1, H-2), 4.56 (d, 1H, J₃⁻,₄ = 9.0 Hz, H-1), 3.93 (dd, 1H, J₃⁻,₄ = 9.0, J₄⁻,₅ = 9.0 Hz, H-3), 3.37 (t, 1H, J₅⁻,₆a = 9.0 Hz, H-4), 3.10 (dd, 1H, J₅⁻,₆a = 9.0, J₆a⁻,₆b = 12 Hz, H-5), 2.88–3.85 (2H, H-6b, H-6b), 3.81–3.63 (1H, H-6b), 2.45 (t, 1H, J₃⁻,₄ = 12.5, J₄⁻,₅ = 5.0 Hz, H-1b), 2.62 (m, 1H, J₃⁻,₄ = 8.5, J₅⁻,₆b = 6.0 Hz, H-3, H-5), 2.45 (t, 1H, J₄⁻,₅ = 12.5, J₅⁻,₆b = 12.5 Hz, H-1a), 2.07 (s, 3H, CH₃CONH-), 13C NMR (D₂O, 125 MHz): δ 177.4 (CH₂CONH-), 142.4 (C-1''), 124.3 (C-4''), 117.7 (C-3''), 117.3 (C-2''), 114.5 (C-5''), 111.5 (C-3''), 69.5 (C-6''), 61.4 (C-7''), 47.3 (C-1a''), 46.0 (C-1b''), 44.4 (C-2'), 39.7 (C-3'), 31.7 (C-4'), 29.3 (C-5'), 29.0 (C-6'), 28.8 (C-7'), 27.9 (C-5''), 23.6 (C-4'), 22.2 (C-3'), 22.0 (C-6'), 19.1 (C-7'), 17.9 (C-5''), 12.5 (C-3'), 12.0 (C-6'), 11.1 (C-7'), 10.8° (C-4'), 8.5° (C-5'), 25.6° (C-4').

Enzyme Activity Assay—Lysozyme activity was determined by measuring the turbidity changes in bacterial cell suspensions of M. lysodeikticus (0.2 mg/ml) in 100 mM phosphate buffer (pH 7.0) at 25 °C as previously reported by Saint-Blancard et al. (16). The optical density (OD) of the suspension was measured at 450 nm and a decrease in OD₄₅₀ nm of 0.001 was defined as 1 unit of lysozyme activity.

Lysozyme Inhibition Assays—IC₅₀ was determined by measuring the turbidity in the presence of inhibitors, using a turbidity assay under the following conditions. The reaction mixture (0.15 ml) comprising bacterial cell suspensions of M. lysodeikticus (0.2 mg/ml) in 100 mM phosphate buffer (pH 7.0), and 0 to 1.0 mM inhibitor was preliminarily incubated at 25 °C for 5 min. Finally, the HEWL solution (2.5 μl, 50 units) in the same buffer was added. The decrease in OD₄₅₀ nm of the cell suspension was monitored for 2.5 min using a UV-visible spectrophotometer V-630 (Jasco Co., Tokyo, Japan). IC₅₀ values for the inhibitors were calculated from Dixon and Webb plots (17).

In addition, the modes of inhibition were examined for the individual compounds, 2, 3, and 5, by means of Lineweaver-Burk plots (18), which were also used for calculation of the Kᵢ values. The experimental conditions were as follows. The reaction mixture (0.03 ml) comprising 36-280 μM pNP-(GlcNAc)₃, 100 mM sodium acetate buffer (pH 5.5), and an inhibitor (0 – 0.2 mM for 2, 0 – 0.02 mM for 3, or 0 – 0.2 mM for 5) was preliminarily incubated at 40 °C for 5 min. The reaction was performed with 63 units of HEWL. Samples (4 μl) were taken at intervals (0, 5, 10, 15, 20, and 25 min) and inactivated by boiling at 100 °C for 5 min. The amounts of pNP-GlcNAc and pNP-(GlcNAc)₂ formed by the enzymatic reaction were determined by HPLC using the same system as described above except that the detection was performed by ultraviolet light at 300 nm.

ITC Experiments—The HEWL solution (45 μM) in 20 mM phosphate buffer (pH 6.0, 7.0, and 8.0) was degassed and its concentration was determined using absorbance of ultraviolet light at 280 nm. Individual (GlcNAc)₃ (n = 3 and 4) and the chitoligosaccharide derivatives (1 mM (GlcNAc)₃, 1 mM (GlcNAc)₄, 1 mM (GlcNAc)₅, 1 mM (GlcNAc)₆, 1 mM (GlcNAc)₇, and 1 mM (GlcNAc)₈) were dissolved in 20 mM phosphate buffer (pH 6.0, 7.0, and 8.0), degassed, and loaded into a syringe, whereas the HEWL solution (0.2028 ml) was loaded into the sample cell. Calorimetric titration was performed with an iTC200 system (Microcal, Northampton, MA) at 25 °C. For all titrations, 2.5 μl of a ligand was injected into the sample cell at 180-s intervals with a stirring speed of 1000 rpm. The titrations were completed after 16 injections. All experiments were performed with c values of 5 to 100 (c = nKᵢ[M]ᵢ, where [M]ᵢ is the initial protein concentration).

Analysis of Calorimetric Data—ITC data were collected automatically using the Microcal Origin version 7.0 software accompanying the iTC200 system. Prior to data fitting, all data
were corrected for heat of dilution by subtracting the heat remaining after saturation of binding sites of the enzyme. The magnitude of the heat after the saturation was similar to that obtained for the ligand titration into the buffer alone. Nonlinear least-squares fitting to the experimental data using a single-site binding model was satisfactory, providing reliable values of the equilibrium binding association constant ($K_a$), and the reaction enthalpy change ($\Delta H^\circ$) for the lysozyme-inhibitor interaction. The value of $n$ was found to be within the range from 0.9 to 1.2 for all interactions. The binding free energy change ($\Delta G^\circ$) and the entropy change ($\Delta S^\circ$) were calculated using Equation 1.

$$\Delta G^\circ = -RT\ln K_a = \Delta H^\circ - T\Delta S^\circ \quad (\text{Eq. 1})$$

Crystallography and Data Collection—Crystallization conditions for HEWL in complex with compounds 2 or 3 were screened using the sparse-matrix sampling method, by sitting drop vapor diffusion at 20 °C. Under optimized crystallization conditions, 1 μl of protein solution (17 mg/ml in water) containing 1.2 mm 2 or 3 was mixed with 1 μl of reservoir solution containing 0.2 M magnesium chloride hexahydrate, 0.1 M BisTris (pH 6.5), and 25% polyethylene glycol 3350 (PEG 3350). Crystals grew within 3 days to a size of up to 0.3 × 0.6 × 0.3 mm³. For data collection, the crystals were transferred into a cryoprotectant solution containing 0.2 M magnesium chloride hexahydrate, 0.1 M BisTris (pH 6.5), 25% PEG 3350, and 30% glycerol and then flash-cooled in a nitrogen stream at 95 K. The diffraction data were collected at beamline BL-17A of Photon Factory (Ibaraki, Japan), using an ADSC Q315 CCD detector, at a cryogenic temperature (95 K). The data were integrated and scaled with HKL2000 (19). The processing statistics are summarized in Table 1.

### Results

Enzymatic Synthesis of Compounds 1, 2, and 3 by HEWL—We have already reported the regioselective synthesis of $p$-nitrophenyl penta-N-acetylchitotetraoside, $p$NP-(GlcNAc)$_5$, through HEWL-catalyzed transglycosylation from (GlcNAc)$_5$ as a donor substrate to the C4-position of $p$-nitrophenyl N-acetylglucosaminide as an acceptor (25). Following the procedure was developed for $p$NP-(GlcNAc)$_5$, we tried to produce compounds 1, 2, and 3 from (GlcNAc)$_4$ by the use of HEWL (Scheme 1). A regioselective transglycosylation from the (GlcNAc)$_4$ donor to the moranoline acceptor successfully took place, producing a series of 1, 2, and 3. The time course of the enzymatic-transglycosylation reaction is shown in Fig. 2A, which was obtained by HPLC determination of the reaction products. The transfer reaction led to the preferential formation of 1 rather than 2 and 3. The yields of 2 and 3 did not increase even if a longer incubation was conducted. The products were easily separated from each other by successive column chromatographies of Dowex-50W and Toyopearl HW-40S. The gel-filtration profile is shown in Fig. 2B. Compounds 1, 2, and 3 were finally obtained in yields of 36.3, 5.9, and 0.0%, respectively, based on the amount of (GlcNAc)$_4$ added.
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Inhibition Analysis—The inhibitory effects of compounds 1, 2, and 3, 4-O-β-tri-N-acetylchitotriosyl-2-acetamido-2,3-dideoxydihydroglucopyranose (4), and 4-O-β-tri-N-acetylchitotriosyl-2-acetamido-2,3-dideoxydihydrogluco-δ-lactone (5) (Scheme 1 and Fig. 1) on the lytic activity of HEWL toward M. lysodeikticus cells were compared with those of (GlcNAc)$_n$ ($n = 2, 3,$ and 4), which were used as reference compounds. The results are listed in Table 2. At pH 7.0 the IC$_{50}$ value of 3 (0.57 μM) was one-fourteenth of that of (GlcNAc)$_4$ (7.7 μM), which is preferentially bound in a nonproductive manner. In fact, at saturation of the enzyme with (GlcNAc)$_n$ only 0.11% of the tetrascaraccharide demonstrates productive modes of binding (26). Compounds 2 and 5 also acted as inhibitors and the effects were approximately equivalent to that of the reference compound (GlcNAc)$_3$. Compound 3 was found to be the most effective inhibitor of lysozyme lysis. Comparison between the data for 3 and (GlcNAc)$_4$ led to an important finding that the moranoline moiety of 3 is most significantly responsible for the inhibitory action of this compound.

We then tried to determine the inhibition constants of these inhibitors. Although turbidimetry is widely used for lysozyme assays using bacterial cells, this method is less quantitative due to the heterogeneity of the bacterial cell powder and is not appropriate for kinetic measurements. Thus, a kinetic analysis in the presence of the inhibitors was conducted using a well defined low molecular weight substrate, pNP-(GlcNAc)$_5$. The use of pNP-(GlcNAc)$_5$ as the substrate provided fully reliable kinetic data, as shown in Fig. 3. The mode of inhibition of compounds 2, 3, and 5 toward HEWL was found to be competitive based on the Lineweaver-Burk plots. The $K_i$ values were graphically determined to be $2.01 \times 10^{-5}$ M for 2, $1.84 \times 10^{-6}$ M for 3, and $3.51 \times 10^{-5}$ M for 5, respectively, by the method of Dixon and Webb (17). The inhibitory activity of 3 was 10-fold higher than those of the others. We also confirmed that the three inhibitors tested were not hydrolyzed by HEWL during the individual inhibition experiments.

**TABLE 2**

| Inhibitors | M. lysodeikticus cell, IC$_{50}$ μM | Relative ratio |
|------------|------------------------------------|----------------|
| (GlcNAc)$_3$ | 470 ± 50 | 0.03 |
| (GlcNAc)$_3$ | 15.0 ± 2.6 | 1 |
| (GlcNAc)$_4$ | 7.7 ± 0.3 | 1.9 |
| 1 | 374 ± 71 | 0.04 |
| 2 | 16.7 ± 1.2 | 0.9 |
| 3 | 0.57 ± 0.03 | 26.3 |
| 4 | 271 ± 5.6 | 0.6 |
| 5 | 14.7 ± 2.3 | 1.0 |

FIGURE 2. A, time course of the product formation by HEWL-catalyzed transglycosylation. The amounts of the products were determined by HPLC using an ANIDIUUS column. Details are described in text. Unfilled circle, 1; filled circles, 2; unfilled triangles, 3, 5, purification of the moranoline derivatives by Toyopearl HW-40S column chromatography. Conditions are described under "Experimental Procedures."
FIGURE 3. Lineweaver-Burk plots showing the inhibition of HEWL by 2 (A), 3 (B), and 5 (C). The inhibition assay was described under “Experimental Procedures.” ○, with an inhibitor ([I] = 0.2 mM for 2, 0.02 mM for 3, and 0.2 mM for 5); ●, no inhibitor. All experiments were repeated three times. Error bars denote S.D. (n = 3).

FIGURE 4. Thermograms (upper panels) and binding isotherms with theoretical fits (lower panels) obtained for the binding of (GlcNAc)_3 (A), (GlcNAc)_4 (B), 2 (C), 3 (D), and 5 (E) to HEWL.
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| Ligands          | $n$     | $\Delta H^\circ$ kcal mol$^{-1}$ | $-T\Delta S^\circ$ kcal mol$^{-1}$ | $\Delta G^\circ$ kcal mol$^{-1}$ | $K_d$ μM |
|------------------|---------|----------------------------------|------------------------------------|----------------------------------|--------|
| (GlcNAc)$_3$     | 1.01 ± 0.02 | $-13.5 ± 0.34$                   | 6.6                                | $-6.9$                           | 9.3    |
| (GlcNAc)$_4$     | 0.93 ± 0.02 | $-10.7 ± 0.34$                   | 3.7                                | $-7.0$                           | 7.0    |
| 2                | 0.94 ± 0.01 | $-11.0 ± 0.17$                   | 4.4                                | $-6.6$                           | 14.2   |
| 3                | 1.12 ± 0.01 | $-11.0 ± 0.10$                   | 2.6                                | $-8.4$                           | 0.76   |
| 5                | 1.24 ± 0.03 | $-9.2 ± 0.32$                    | 2.2                                | $-6.9$                           | 8.7    |

Table 4 lists the values of thermodynamic parameters for 3 obtained at various pH values. The affinity for 3 was found to decrease at a higher pH, 8.0, due to the considerable decrease in favorable enthalpy change.

Crystal Structures of HEWL in Complex with Compounds 2 and 3—HEWLs in complex with 2 and 3 adopted similar conformations to that of unbound HEWL (PDB code 1DPX, Ref. 28), whose structure could be superimposed with a root mean square deviation value of 0.163 and 0.214 Å, respectively, over the corresponding 128 Ca-atoms of the individual complex structures. Fig. 5 shows the simulated annealing omit maps of compounds 2 and 3 bound to HEWL. In both cases, clear electron density for the individual sugar units was found at glycosylation binding site (negatively numbered subsites). The moranoline moiety was located at subsite $-1$, whereas the remaining GlcNAc residues bound to subsites $-2$, $-3$, and $-4$. It is well known that (GlcNAc)$_3$ and (GlcNAc)$_4$ bind to subsites $-2$, $-3$, and $-4$, keeping away from subsite $-1$ because of the unfavorable positive binding free energy at that subsite. Thus, the structures shown in Fig. 5 suggest that the moranoline moiety has a strong affinity toward subsite $-1$. This is the most important requirement for designing an effective inhibitor for lysozyme. However, more careful comparison between the two complex structures indicated that the moranoline moiety of 3 is more closely located to the Asp-52 side chain than that of 2.

The amino acid residues responsible for the binding of compounds 2 and 3 can be identified from Fig. 6. In the complex structure with 2 (Fig. 6A), the nitrogen atom of the moranoline moiety significantly interacts with the carboxylate of Asp-52. The hydrogen bonding network mediated by a water molecule is also formed between the nitrogen atom of the moranoline moiety, the Glu-35 side chain, the C6-OH of the moranoline moiety, and the Val-109 main chain -NH. In the other side of subsite $-1$, the C2-OH of the moranoline moiety appears to interact with the side chains of Asp-48 and Asn-59 through a water molecule. In contrast to the strong interaction of the $-2$ sugar of the HEWL-(GlcNAc)$_3$ complex (PDB code 1HEW, Ref. 29), the GlcNAc residue at subsite $-2$ was found to interact only with the Ala-107 main chain C = O. As for the $-3$ sugar, in addition to the face-to-face stacking interaction with the indole side chain of Trp-62, several hydrogen bonds are formed with Asp-101 and Asn-103.

Compound 3 appears to more strongly interact with HEWL (Fig. 6B). In fact, an additional interaction of the moranoline moiety was found between the Asn-46 side chain and the C2-OH of the moranoline moiety. The $-2$ GlcNAc residue forms several interactions with Asn-59, Trp-62, and Trp-63, in addition to that with Ala-107. The Asp-48 side chain also has a water-mediated interaction with the C6-OH of the $-2$ sugar. As in the case of HEWL in complex with 2, the $-3$ sugar has a face-to-face stacking interaction with the indole side chain of Trp-62 and a hydrogen bond with the side chain of Asp-101, but the interaction with the Asn-103 side chain is missing. Two hydrogen bonds are formed between the Asp-101 side chain and the $-4$ sugar. The great difference in the binding affinities between 2 and 3 may be due to the slight shift in the location of 3 from that of 2 in the substrate-binding cleft of HEWL (Fig. 5).
The moranoline molecule, which has been recognized to interact with the catalytic base (or the nucleophile) of the targets, glycoside hydrolases (13), may mimic the transition state of enzymatic hydrolysis. To mimic the transition state of the lysozyme-catalyzed reaction, we designed and tried to produce 4-O-β-N-acetylglucosaminyl moranoline (1), 4-O-β-di-N-acetylchitobiosyl moranoline (2), and 4-O-β-tri-N-acetylchitotriosyl moranoline (3), in which the reducing end GlcNAc residues of (GlcNAc)$_2$, (GlcNAc)$_3$, and (GlcNAc)$_4$ are substituted with the moranoline, respectively. HEWL successfully catalyzed a regioselective transglycosylation from the (GlcNAc)$_4$ donor to the moranoline acceptor, producing compounds 1, 2, and 3. However, the enzyme produced 1 in preference to 2 and 3 in the entire course of the reaction (Fig. 2A). Compound 3 was obtained in only a low yield. A major fraction of (GlcNAc)$_4$ prefers to bind nonproductively to the lysozyme-binding cleft occupying subsites −4, −3, and −2, with the additional GlcNAc extending beyond the substrate-binding cleft at the nonreducing end (26). Thus, the second (GlcNAc)$_4$ molecule binds to the vacant subsites, −1, +1, and +2, resulting in hydrolysis into the nonreducing end GlcNAc and the remaining (GlcNAc)$_3$ (30). After releasing the (GlcNAc)$_3$ fragment, the nonreducing end GlcNAc is transferred to the moranoline molecule, producing a higher amount of 1. Compounds 2 and 3 might have been produced from the productive HEWL-(GlcNAc)$_4$ complexes, in which (GlcNAc)$_4$ occupies subsites −2 to +2 and subsites −3 to +1, respectively, via the bond cleavage and the moranoline transfer reaction. Fractions of the productive HEWL-(GlcNAc)$_4$ complex are much lower than that of the nonproductive complex occupying subsites −4, −3, and −2 (26), resulting in the lower yields of 2 and 3.

The inhibitory activities of 1 and 2 were comparable with those of (GlcNAc)$_2$ and (GlcNAc)$_3$, respectively (Table 2), indicating that the moranoline moiety itself does not enhance the binding ability to lysozyme. It is well known that the moranoline molecule alone strongly binds to subsite 1 of glucosidases (13–15). However, it appears that the moranoline does not bind to the lysozyme by itself. The δ-lactone derivative (5) containing $sp^2$ hybridization and adopting a stable half-chair conformation also exhibited a similar inhibitory activity to that of (GlcNAc)$_3$. The inhibitory activity of 4 was even lower than those of (GlcNAc)$_3$ and 5. However, only compound 3 was found to strongly inhibit the lysozyme activity. The stable binding of (GlcNAc)$_3$ to subsites −4, −3, and −2 (sites A, B, and C) of HEWL and a suitable substitution on the reducing end GlcNAc appear to be essential for the activity.
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location of the moranoline moiety might have cooperatively enhanced the binding ability of 3, explaining the highest inhibitory activity of the compound. Kinetic determination afforded $K_i$ values of $2.01 \times 10^{-5}$, $1.84 \times 10^{-6}$, and $3.51 \times 10^{-5}$ M, and by ITC the binding free energy changes were determined to be $-6.6$, $-8.4$, and $-6.9$ kcal/mol, for 2, 3, and 5, respectively (Table 3). The experimental data for the IC$_{50}$ values, $K_i$ values, and binding free energy changes were consistent with each other. Judging from the $K_a$ value of 3 (0.76 µM), the compound appears to have the highest affinity among the lysozyme inhibitors identified to date. The pH dependence of $\Delta G^*$ for 3 (Table 4) might be derived from the change in the protonation state of the moranoline moiety, of which the $pK_a$ value was reported to be 6.3–6.7 (13, 31). The protonation state of Glu-35 ($pK_a = 6.7$, Ref. 32), which strongly interacts with the moranoline moiety of 3 (Fig. 6B), might have partly affected the binding ability of the compound.

X-ray crystallography of the structures of HEWL in complex with 2 and 3 revealed significant differences in binding mode.

FIGURE 7. A, stereo view of the superimposition of the structures of 2 (orange) and 3 (cyan) bound to HEWL. B, stereo view of the superimposition of the structures of 3 (cyan) and (GlcNAc)$_4$ (green) bound to HEWL (PDB code 1HEW). Important amino acid residues in HEWL are also indicated by stick model colored in yellow.

FIGURE 8. Stereo view of the superimposition of the structures of 3 bound to HEWL (cyan) and NAG2FGlcF (magenta) covalently bound to Asp-52 of HEWL. The distances between the atoms linked by broken lines are also listed in the figure. Important amino acid residues in HEWL are also indicated by stick model colored in yellow.
almost overlap at the ring C4, C5, C6, and N(O) positions. The partial overlap of the moranoline moiety of the bound compound 3 with the −1 sugar of the covalently bound NAG2FGlCF appears to demonstrate the covalent glycosyl-HEWL intermediate. Evidence of a covalent intermediate using a wild type enzyme was obtained for an invertebrate lysozyme from *Tapes japonica* (37), which is highly similar to HEWL in their three-dimensional structure and in the catalytic mechanism. Taken together, we concluded that compound 3 is a stable and pure transition-state analogue with sufficient inhibitory activity for wild-type HEWL, providing additional evidence for a covalent glycosyl intermediate.

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