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# Selective and slow-binding inhibition of shikonin derivatives isolated from *Lithospermum erythrorhizon* on glycosyl hydrolase 33 and 34 sialidases

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ABSTRACT

Sialidases are enzymes that catalyze the hydrolysis of sialic acid residues from various glycoconjugates, which are widely found in a number of viral and microbial pathogens. In this study, we investigated the biological evaluation of isolated six shikonins (**1**–**6**) and three shikonofurans (**7**–**9**) from *Lithospermum erythrorhizon*. The nine isolated compounds **1–9** showed strong and selective inhibition of glycosyl hydrolase (GH) 33 and -34 sialidases activities. In GH33 bacterial-sialidase inhibition assay, the inhibitory activities against GH33 siadliase of all shikonofuran derivatives (**7**–**9**) were greater than shikonin derivatives (**1**–**6**). Shikonofuran E (**8**) exhibited the most potent inhibitory activity toward GH33 sialidases ($IC_{50}$ = 0.24 μM). Moreover, our detailed kinetic analysis of these species unveiled that they are all competitive and simple reversible slow-binding inhibitors. Otherwise, they showed different inhibitory capacities and kinetic modes to GH34 viral-sialidase activity. All the naphthoquinone derivatives (**1**–**6**) were of almost equal efficiency with $IC_{50}$ value of 40 μM and shikonofurans (**7**–**9**) did not show the significant inhibitory effect to GH34 sialidase. Kinetic analyses indicated that naphthoquinones acted via a noncompetitive mechanism.



## 1. Introduction

Glycosyl hydrolases (GH) are key enzymes of carbohydrate metabolism found in the three major kingdoms of archaebacteria, eubacteria, and eukaryotes. Currently, carbohydrate active enzymes are classified into 115 separate families of glycoside hydrolases based on the amino acid sequence similarities (CAZy: http://www.cazy.org).[1] Among these hydrolases, exo-sialidases (EC 3.2.1.18, also called neuraminidase) are enzymes that catalyze the hydrolysis of sialic acid residues from various glycoconjugates,[2] which are widely found in higher eukaryotes and in a number of viral and microbial pathogens.[3–5] Exo-sialidases have been classified into three different sequence-based families, GH33, -34, and -83.[1] Glycoside hydrolase family 33 (GH-33) includes most bacterial and simple eukaryotic sialidases and trans-sialidases, GH-34 includes viral sialidases and GH-83 includes the hemagglutinin-neuraminidases. Despite their low sequence similarity, however, all sialidases share a similar catalytic domain with a six bladed β-propeller topology,[5] observed in viral,[6–8] bacterial,[9] and eukaryotic homologs.[10] This overall fold similarity and common catalytic machinery, defined by eight strictly conserved residues at the reaction center, strongly argue in favor of a common evolutionary origin and a similar mode of action for the entire superfamily of sialidases.[11]

GH33 and -34 have been receiving increasingly greater attention recently owing to its inhibitors developing, such as Oseltamivir, DANA, and transition-state analogue Neu5Ac2en.[12,13] Previously, our group has succeeded in isolating and characterizing the naturally occurring sialidase inhibitors, which are xanthones, flavonoids, pterocarpans, and phlorotannins from *Cudrania tricuspidata*, *Sophora flavescens*, *Rhodiola rosea*, *Glycyrrhiza uralensis*, *Angelica keiskei*, and *Ecklonia cava*.[14–19] Especially, xanthones derivatives have been shown to display nanomolar inhibitory activity against GH33 with competitive inhibition modes, manifested slow-binding inhibition.

As part of our continuous research, a medicinal plant *Lithospermum erythrorhizon*, which is rich in naphthoquinones (shikonin derivatives), was taken as our target material to provide unique sialidase inhibitors. *L. erythrorhizon* belong to the Boraginaceae family and it is widely planted in Korea, Japan, and China, and it is used as a dye for staining fabrics and food colorants. This plant has been classified as edible by the KFDA (Korea Food & Drug Administration). Shikonin and its derivatives have been reported

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**Nomenclature**

| | |
|---|---|
| $IC_{50}$ | the inhibitor concentration leading to 50% activity loss |
| $K_i$ | inhibition constant |
| $K_i^{app}$ | apparent $K_i$ |
| $K$ | rate constant |
| $V_{max}$ | maximum velocity |
| $K_m$ | Michaelis-Menten constant |
| $k_{obs}$ | apparent first-order rate constant for the transition from $v_i$ to $v_s$ |
| $v_i$ | initial velocity |
| $v_s$ | steady-state rate |
| wavelength | intensity at ex: 365 nm, em: 450 nm |

to have many medicinal properties such as antibacterial, wound healing, anti-inflammatory, antithrombiotic, and antitumor effects.[20] In a clinical trial, a shikonin-containing mixture has been reported to reduce lung cancer growth at an effective rate of 63.3%, and administration of this mixture increased the body weight and appetite of patients. No harmful effects on peripheral system, heart, kidney and liver have been observed after shikonin treatment.[21]

In this study, we conducted a biological evaluation of six isolated shikonins and three shikonofurans from *L. erythrorhizon*, investigating the inhibition selectivity of nine constituents, which exerted strong and selective inhibition of GH 33 and -34 activities. On the basis of their inhibition properties, we characterize the kinetic profile for binding between shikonins and purified sialidase and HPLC quantitative analysis.

## 2. Results and discussion

In the preliminary study, we confirmed the sialidase (GH33) inhibitory activity of an isopropyl alcohol extract of *L. erythrorhizon* exhibited 83% inhibition at 30 μg/mL. The crude extract of *L. erythrorhizon* was directly analyzed by HPLC chromatography as shown in Figure 1A. As can be seen, more than nine principal naphthoguinone peaks were detected by PDA at 254 nm. For the choice of best extract conditions, we analyzed extracts of *L. erythrorhizon* prepared using varying solvents (*n*-hexane, chloroform, ethyl acetate, acetonitrile, and isopropyl alcohol) by HPLC (Fig. 1B). *n*-Hexane, a common solvent for the extraction of secondary metabolites from *L. erythrorhizon*, showed the best profile. We then used repeated silica gel, RP-18 gel, and Sephadex (LH-20) chromatography to isolate bioactive compounds from *n*-hexane extract by bioactivity-guided fractionation. The isolated compounds (**1**–**9**) were identified as known species shikonin (**1**), acetylshikonin (**2**), β,β-dimethylacrylshikonin (**3**), β-hydroxyisovalerylshikonin (**4**), isobutylshikonin (**5**), deoxyshikonin (**6**), shikonofuran D (**7**), shikonofuran E (**8**), and shikonofuran C (**9**) (Fig. 2). Their chemical structures were elucidated on the basis of comprehensive spectral analysis of MS (EIMS and HREIMS) and NMR ($^1H$ and $^{13}C$) data, and also comparing with the data published previously.[20,22,23]

To investigate whether the nine compounds (**1**–**9**) that exhibited potent inhibitory activity on the GH33 and 34 sialidases, with or without treatment with test compounds, were measured by fluorogenic methods. Unless otherwise stated, all compounds were first tested at a single maximum concentration of 200 μM, after which $IC_{50}$ determinations were using twofold serial dilutions starting from 200 μM, following an in-house protocol.

Firstly, each isolated constituents (**1**–**9**) of *L. erythrorhizon* were tested in an in vitro GH33 sialidase inhibition assay. As *Clostridium perfringens* type A is the causative agent of human gas gangrene, or clostiridial myonecrosis, and human food poisoning.[24] It produces many secreted hydrolytic enzymes and toxins, including α-toxin and perfringolysin O. *C. perfingens* strains can encode up to three sialidases, *nanH*, *nanI*, and *nanJ*. Among the gene, NanI contains a signal peptide, and is secreted from the cell. NanI is thus readily isolated form cell-free supernantants.[25] NanI may also play a role in nutrition, releasing sialic acid from higher-order gangliosides for subsequent transport into the cell.[26] As a result of its location, NanI may also interact with the extracellular environment of the host tissue during infection.

All the isolated (**1**–**9**) showed a dose-dependent inhibitory effect against the bacterial sialidase activity (Fig. 3A). However, the activity was slightly affected by subtle changes in structure. As shown in Table 1, four acyl group substituted compounds (**2**–**5**) showed stronger activity than the parental compounds (**1** and **6**). Among the acyl substituted shikonins acethylshikonin (**2**), β,β-dimethyl acrylshikonin (**3**), β-hydroxyisovalerylshikonin (**4**), and isobutylshikonin (**5**) exhibited inhibitory effects on sialidase, with $IC_{50}$ values of 2.5, 1.9, 3.3, and 1.8 μM, respectively, whereas shikonin (**1**) and deoxyshikonin (**6**) showed moderate activity in the GH33 silaidase with $IC_{50}$ values 53.8 and 27.5 μM, respectively. Taken as an ensemble, the following general features of the SAR can be deduced from these data. The presence of aryl, acetyl, valeryl, and isobutyl group at C-1′ were more active than hydroxyl and deoxyshikonin. A similar pattern was observed for compounds **2**–**5**. Perhaps most pointedly, acylation of the C-1′ hydroxyl group enhanced inhibition potency significantly. Likewise, shikonofurans **7**–**9** showed similar pattern. This implies hydrophobicity is important for inhibition. Interestingly, shikonofuran, furyl-hydroquinone derivatives were found to be more active than naphthoquinone, shikonin derivatives with $IC_{50}$ values ranged from 0.2 to 1.0 μM. Shikonofuran E was a highly potent nanomolar GH33 sialidase inhibitor with an approximately 10-fold more potent inhibition ($IC_{50}$ = 0.24 μM) than naphthoquinone scaffold. This interesting result shows that free hydroquinone may be correlated in the sialidase amino acid with hydrogen bond.

To investigate the enzyme activity as a function of time exposed to the inhibitor, we measured the residual enzyme activity of preincubated enzyme with inhibitors over a number of exposure times. All isolated compounds showed a time-dependent inhibitory effect on GH33 sialidase activity. In our previous work, it was proven that many secondary metabolites showed a time-dependent inhibitory effect. As shown in Figure 3B, isolated compounds from *L. erythronrhizon* exposed by 10 min preincubation times have more inhibition than those with no incubation. As a decrease in residual activity was seen as a function of preincubation time, GH33 sialidase inhibitors emerged to be a slow-binding inhibitor at low concentrations. Slow-binding inhibition mechanisms can be investigated by preincubation of the enzyme with inhibitor followed by measurement of initial velocities for substrate hydrolysis as a function of preincubation time. Increasing preincubation time with compounds (**1**–**9**) led to a decrease in both the initial velocity ($v_i$) and the steady-state rate ($v_s$). Representatively, Figure 3C showed typical progress curves for slow binding behavior, when the hydrolysis of substrate by sialidase in the presence of compound **9** concentrations. The $k_{obs}$ values for the inhibition of sialidase at concentrations of compound **9** were determined by fitting the data to the slow-binding equation (Eq. (3)–(5)). $K_{obs}$ was then plotted as a function of inhibitor concentrations. The inhibition was shown to be time dependent by

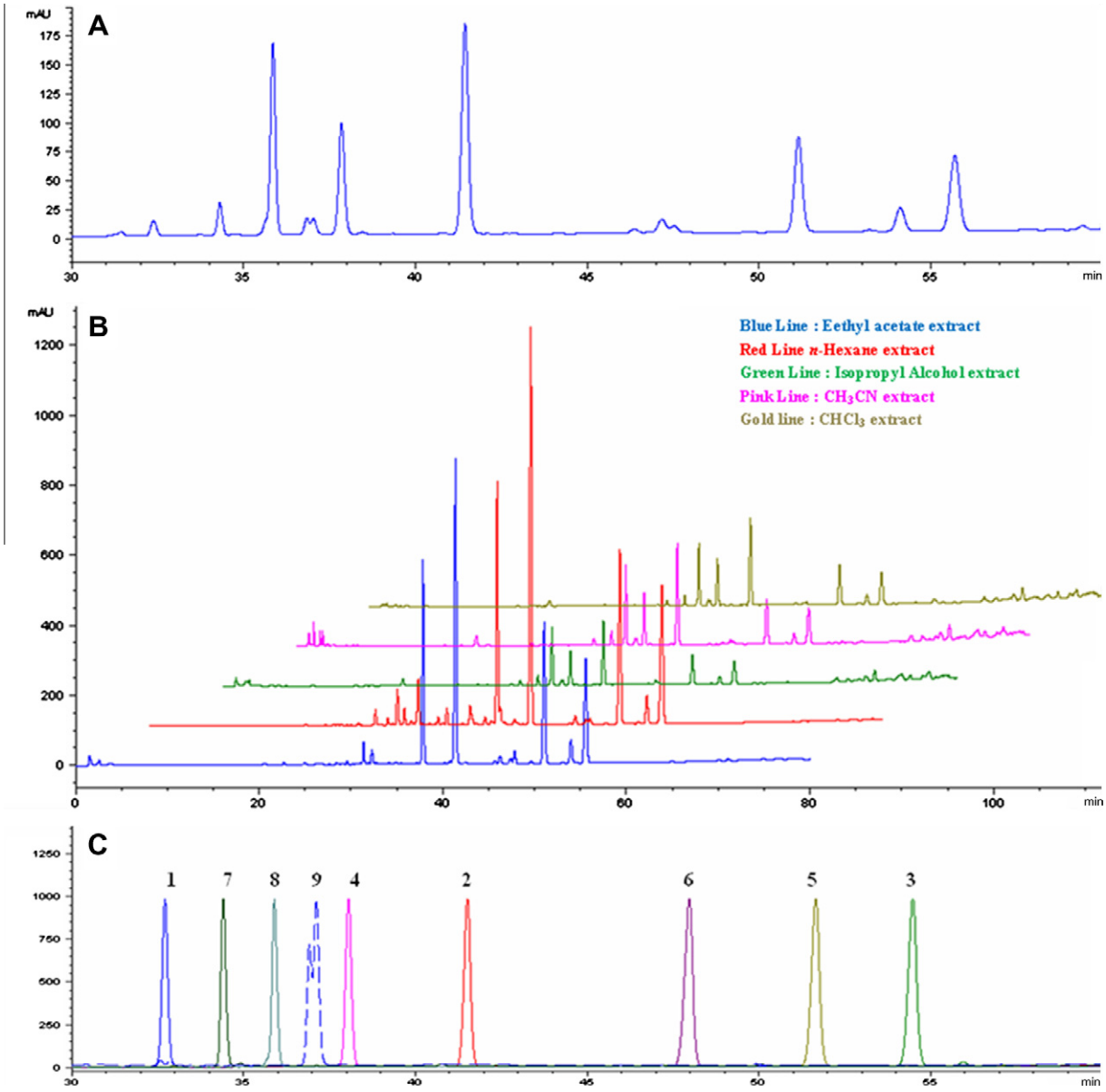



**Figure 1.** (A) HPLC chromatogram of an isopropyl crude extract of *Lithospermum erythrorhizon* at 254 nm. (B) Chromatographic profiles at 254 nm for varying solvents. (C) Chromatogram of isolated compounds standard mixture.

the fact that $k_{obs}$ exhibited a liner dependence on the inhibitor concentration as depicted in Figure 3D. Thus, these results prove that all of isolated constituents (**1**–**9**) from *L. erythronrhizon* inhibit GH33 sialidase by the simple reversible slow-binding model according to the data yielded by the analysis by Eqs. 3 and 4 as listed in Table 2.

Most slow-binding enzyme inhibitors act as competitive inhibitors, binding at the enzyme active site,[27] although it is possible for them to interact with the enzyme by competitive, noncompetitive, or uncompetitive inhibition patterns. For further study of the inhibition mode, we used the kinetic plots (Lineweaver–Burk or Dixon plots). The kinetic and inhibition constants ($K_i$) obtained are listed in Table 1. The inhibition type of GH33 sialidase by compounds **5** and **6**–**9** are illustrated in Figure 4, representatively. As a result of this kinetic analysis, we found that *L. erythronrhizon*-derived GH33 inhibitors have competitive inhibition modes. Because the Lineweaver–Burk plots of $1/V$ versus $1/[S]$ result in a family of straight lines with the same $y$-axis intercept as illustrated, respectively, for the nine sialidase inhibitor.

In a separate experiment, shikonin and shikonofuran derivatives were tested for the inhibition of GH34 influenza virus sialidase. The influenza virus sialidase (also called neuraminidase) is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles. Specifically, sialidase cleaves the α-ketosidic bond that links a terminal sialidic acid residue to the adjacent oligosaccharide moiety. Sialidase is, therefore, essential for the movement of the virus to and from sites of infection in the respiratory tract.

The biological activity of isolated compounds **1**–**9** were assessed against sialidase from recombinant influenza virus A (rvH1N1). All isolated compounds apart from shikonofurans **7**–**9** showed a dose-dependent inhibitory effect toward influenza virus sialidase activity (Fig. 5A), whereas compounds emerged not to be a time-dependent inhibition profiles. As shown in Table 1, acyl group substitutued naphthoqinones (**2**–**5**) have similar inhibitory activity with $IC_{50}$ value range of 40 μM. Among the isolates, shikonin (**1**, $IC_{50}$ = 30.1 μM) showed the most potent inhibitory activity in GH34 inhibition assay and twofold active than corresponding

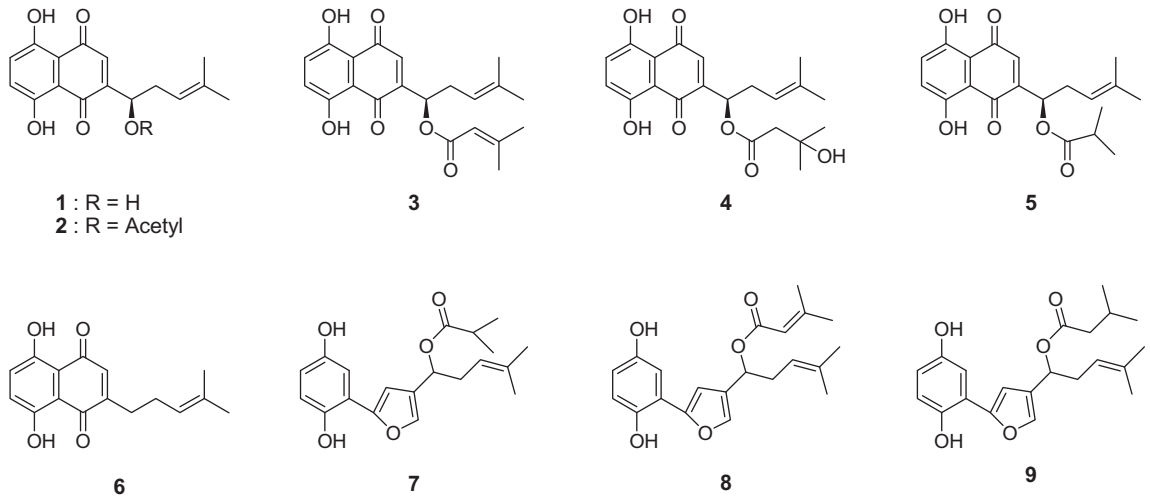



**Figure 2.** Chemical structures of isolated compounds **1**–**9** from the root of *L. erythrorhizon*.

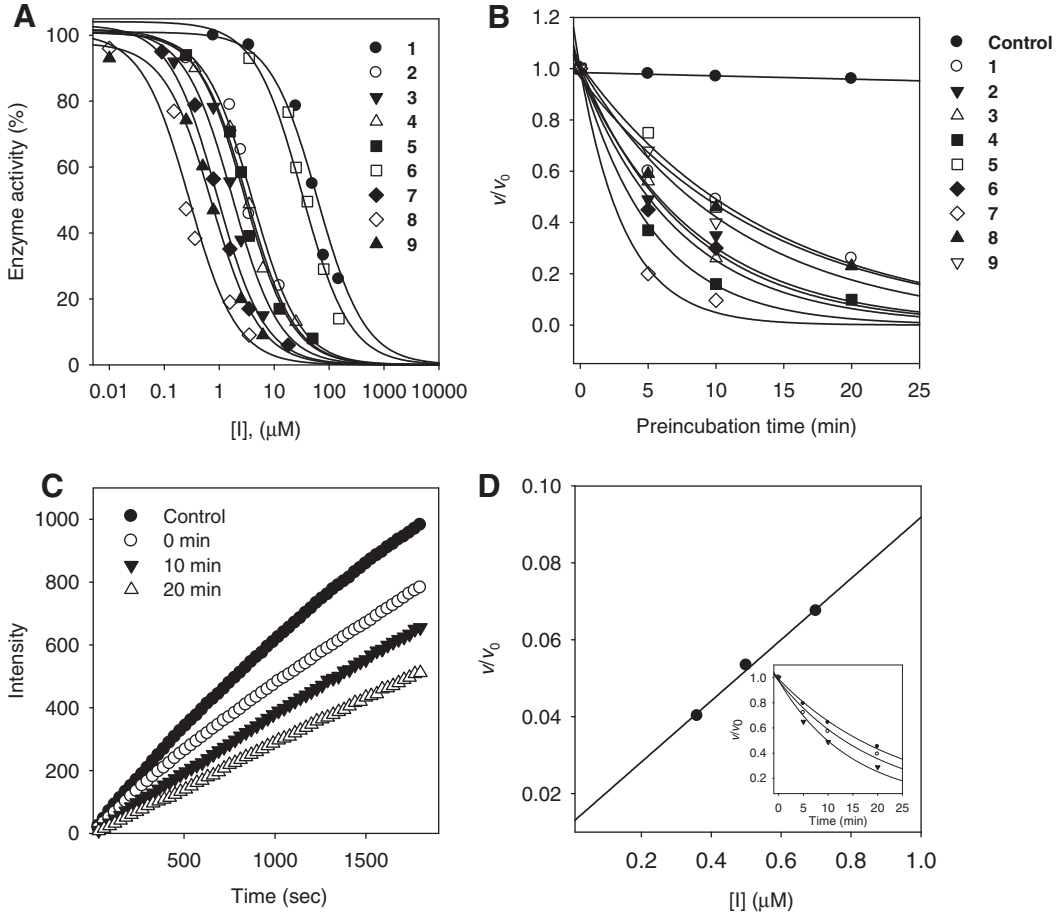



**Figure 3.** (A) Effects of compounds on the activity of GH33 bacterial sialidase fro hydrolysis of substrate. (B) Inhibition as a function of preincubation time for compounds **1**–**9** at $IC_{50}$. (C) Slow-binding inhibition of compound **9** at time course. (D) Plot of $k_{obs}$ as a function of compound **9** concentrations for a slow-binding inhibition fitted by Eqs. (4) and (5) (Inset) preincubation time dependence of the fractional velocity of an enzyme-catalyzed reaction in the presence of varying concentrations of a compound **9**.

deoxyshikonin ($IC_{50}$ = 63.4 μM). Interestingly, shikonofuran derivatives (**7**–**9**) exerted no significant inhibition of GH34 sialidase activity even at 200 μM. These findings suggest that the position of attachment of a hydroxy, acetyl, acryl, valeryl, and isobutyl does not affect their inhibitory potency, and that the naphthoquinone moiety is more active than furyl-hydroquinone moiety in influenza virus sialidase inhibition.

We subsequently progressed to analyze the mechanism of binding of inhibitors of influenza virus sialidase using double-reciprocal plots, Influenza virus sialidase inhibitors **1**–**6** possessed noncompetitive inhibition modes because increasing substrate concentrations resulted in a family of lines with different slopes, but a common *x*-axis intercept from kinetic studies (Fig. 5B for compound **1**, representatively).

**Table 1**
Sialiases inhibitory activities of isolated shikonin derivatives (**1**–**9**).

| Compound | Family | | | |
|---|---|---|---|---|
| | GH33 (*Clostridium perfringens*) | | GH34 (A/Bervig_Mission/1/18) | |
| | $IC_{50}$[a] (μM) | Inhibition type ($K_i$, μM) | $IC_{50}$[a] (μM) | Inhibition type |
| **1** | 53.8 ± 3.5 | Competitive (21.4 ± 2.1) | 34.1 ± 2.3 | Noncompetitive |
| **2** | 2.5 ± 0.1 | Competitive (1.1 ± 0.1) | 41.4 ± 0.7 | Noncompetitive |
| **3** | 1.9 ± 0.2 | Competitive (0.6 ± 0.1) | 47.3 ± 1.4 | Noncompetitive |
| **4** | 3.4 ± 0.6 | Competitive (0.8 ± 0.1) | 40.5 ± 0.5 | Noncompetitive |
| **5** | 2.9 ± 0.2 | Competitive (0.9 ± 0.1) | 40.5 ± 0.3 | Noncompetitive |
| **6** | 27.5 ± 3.3 | Competitive (15.9 ± 2.6) | 63.4 ± 1.2 | Noncompetitive |
| **7** | 1.0 ± 0.1 | Competitive (0.3 ± 0.1) | >100 | NT[b] |
| **8** | 0.2 ± 0.1 | Competitive (0.1 ± 0.0) | >100 | NT |
| **9** | 0.6 ± 0.1 | Competitive (0.2 ± 0.1) | >100 | NT |

[a] All compounds were examined in a set of duplicated experiment; $IC_{50}$ values of compounds represent the concentration that caused 50% enzyme activity loss.
[b] NT = not tested.

**Table 2**
Kinetic parameters for time-dependent inhibition of GH33 sialidase by compounds **1**–**9**

| Compounds | $k_3$ ($\mu M^{-1}\ s^{-1}$) | $k_4$ ($s^{-1}$) | $K_i^{app}$ (μM) |
|---|---|---|---|
| **1** | 0.00113 | 0.00665 | 58.8 |
| **2** | 0.02134 | 0.02119 | 1.01 |
| **3** | 0.0218 | 0.03833 | 1.75 |
| **4** | 0.001714 | 0.0385 | 2.24 |
| **5** | 0.0202 | 0.005264 | 0.26 |
| **6** | 0.0029 | 0.03464 | 11.9 |
| **7** | 0.06185 | 0.01299 | 0.21 |
| **8** | 0.4426 | 0.01299 | 0.05 |
| **9** | 0.0813 | 0.0122 | 0.15 |

Furthermore, we examined effects of isolated compounds (**1**–**9**) on the other glycosyl hydrolases such as α,β-glucosidase and rhamosidase. Glycosidases are well known targets in the design and development of antiviral, antibacterial, anticancer, and antidiabetic agents. This wide range of glucosidase inhibitors exemplifies the pervasive importance of glycosylation/deconjugation of sugars in such varied processes as cell signaling/recognition, cell cycle regulation, and metabolism. The inhibitory profiles of compounds **1**–**9** against glycosidases are shown in Table 3. As shown in Table 3, only three shikonofurans (**7**–**9**) exhibited a significant degree of inhibition ($IC_{50}$ = 20.8–37.8 μM) for α-glucosidase activity, while naphthoquinones (**1**–**6**) did not show any glycosidase inhibitory activities. These shikonofurans (**7**–**9**) are similar in potency to sugar-derived α-glucosidase inhibitors, such as voglibose ($IC_{50}$ = 23.4 μM), which is currently used for therapeutic purposes.[28]

On the basis of the biological data presented above, we determined the amount of isolated bioactive compounds (**1**–**9**) by HPLC. The standard curves for nine active components (**1**–**9**) were $y = 5304.19x + 15.1051$ ($R^2 = 0.9998$, **1**), $y = 7240.59x + 64.3901$ ($R^2 = 0.9996$, **2**), $y = 8753.65x + 70.2512$ ($R^2 = 0.9999$, **3**), $y = 6175.39x + 42.9128$ ($R^2 = 0.9998$, **4**), $y = 7595.39x + 77.5300$ ($R^2 = 0.9998$, **5**), $y = 10642.72x + 21.9682$ ($R^2 = 0.9998$, **6**), $y = 5953.71x + 10.7149$ ($R^2 = 0.9999$, **7**), $y = 11295.27x + 19.0482$ ($R^2 = 0.9994$, **8**), and $y = 3336.73x + 9.4452$ ($R^2 = 0.9998$, **9**), respectively. As shown in Table 4, the clearly resolved peaks were obtained for isolated compounds **1**–**9** after extraction with six solvents (*n*-hexane, chloroform, ethyl acetate, ethanol, isopropyl alcohol, and acetonitrile, respectively). The *n*-hexane extract was found to contain the highest amount among these nine compounds. All the isolated compounds **1**–**9** were present at the following concentrations in the indicated extracts. Quantification using a calibration curve with the respective isolated compound revealed that acetylshikonin (**2**) is the main shikonin in the *n*-hexane extract with a concentration of 218.69 ± 3.5 g/Kg for extract.

## 3. Conclusion

In conclusion, we focused on plenty of shikonin derivatives of edible plant, *L. erythrorhizon*. An analysis of *L. erythronrhizon* hexane extracts identified nine shikonin derivatives: shikonin, acetylshikonin, β,β-dimethylacrylshikonin, β-hydroxyisovalerylshikonin, isobutylshikonin, deoxyshikonin, shikonofuran D, shikonofuran E, and shikonofuran C. All the isolated compounds **1**–**9** showed markedly inhibitory activities to the bacterial source (*Clostridium perfringens*, GH33) and the viral source (Influenza virus A/Bervig_Mission/1/18, GH34) sialidases. Especially, nine components exerted significant inhibition of GH33 sialidase inhibitory activity. The inhibitory activities against GH33 siadliase of all shikonofuran derivatives (**7**–**9**) were greater than naphthoquinone derivatives (**1**–**6**). In additon, our detailed kinetic analysis of these species has unveiled that they are all competitive and simple reversible slow-binding inhibitors. Interestingly, they showed different inhibitory capacities and kinetic modes to GH34 sialidase activity. All the naphthoquinone derivatives (**1**–**6**) were of almost equal efficiency with $IC_{50}$ values and shikonofurans (**7**–**9**) did not show a significant inhibitory effect to GH34 sialidase. Kinetic analyses indicated that naphthoquinones acted via a noncompetitive mechanism. The properties of isolated components (**1**–**9**), although unclear about the types of sialidase activity, may have free/rigid hydroquinone moiety effects on sialidase binding sites. These observations led us to suggest that further research on these important lead compounds may show them to be a new alternative to anti-bacterial and influenza treatments.

## 4. Materials and methods

### 4.1. General apparatus and chemicals

Melting points were measured on a Thomas Scientific Capillary Melting Point Apparatus (Electronthermal 9300, UK) and are uncorrected. $^1H$ and $^{13}C$ NMR data were obtained on JNM-ECA 500 (Jeol, Japan) spectrometer in $CDCl_3$ and tetramethylsilane (TMS) as internal standard. Optical rotation values were measured by a Perkin–Elmer 343 polarimeter and $[\alpha]_D$-values are given in units of $10^{-1}$ deg $cm^2$ $g^{-1}$. ESI Mass spectra were scanned using ESI in negative mode. All the reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chromatographic separations were carried out by Thin-layer Chromatography (TLC) (E. Merck Co., Darmastdt, Germany), using commercially available glass plate pre-coated with silica gel and visualized under UV at 254 and 366 nm. Column chromatography was carried out using 230–400 mesh silica gel (kieselgel 60, Merck, Germany). Sephadex LH-20 (GE Healthcare) was used for column chromatography.

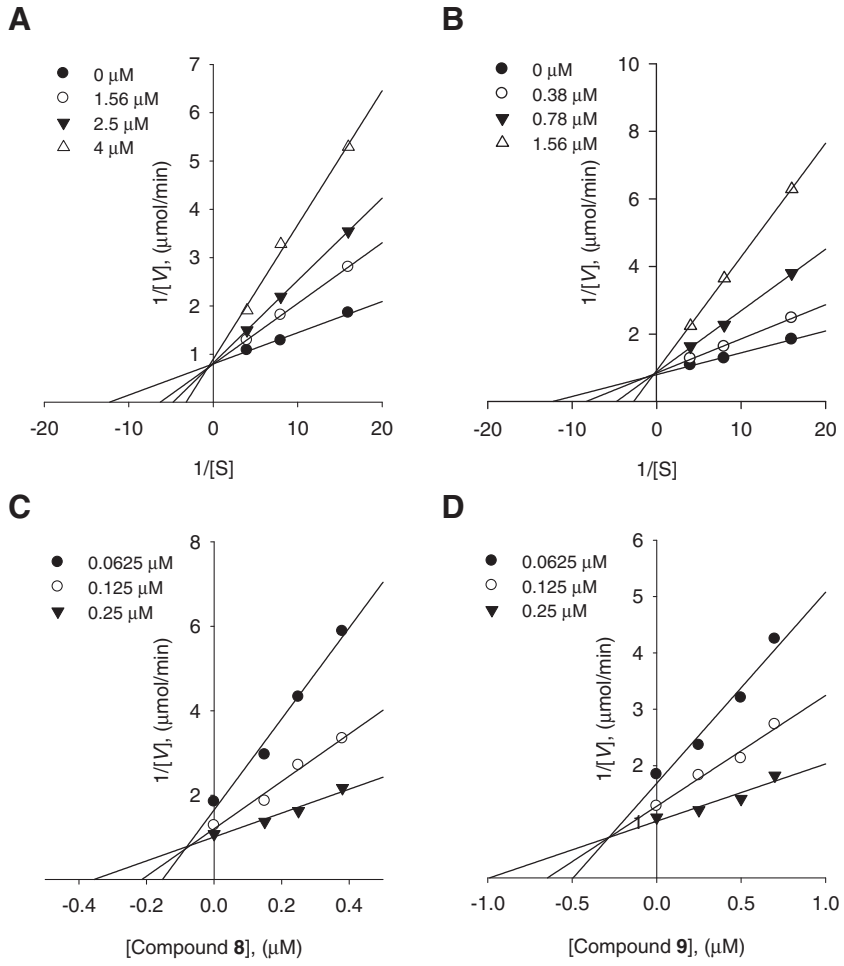



**Figure 4.** Lineweaver–Burk plots for GH33 sialidase inhibition by compounds **5** (A) and **7** (B). Dixon plots for inhibition of compounds **8** (C) and **9** (D) on GH33 sialidase for the hydrolysis of substrate.

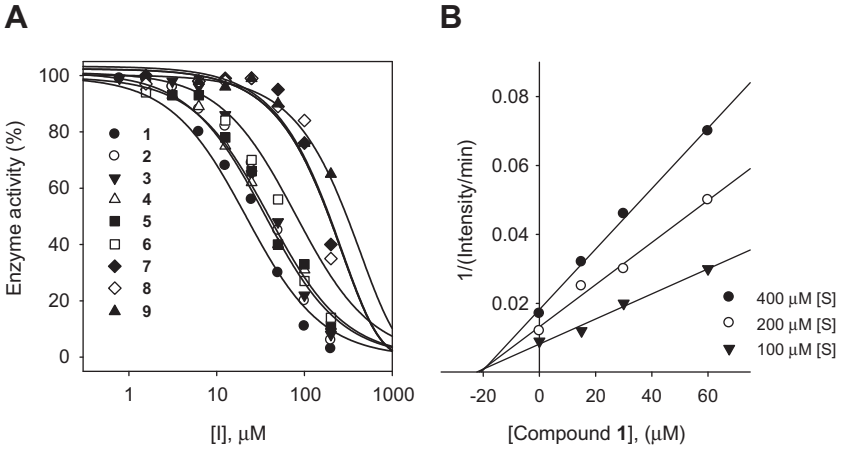



**Figure 5.** (A) Effects of compounds **1–9** on GH34 influenza A virus sialidase for the hydrolysis of substrate. (B) Dixon plots for GH34 sialidase inhibition by compound **1**.

### 4.2. HPLC apparatus and chromatographic conditions

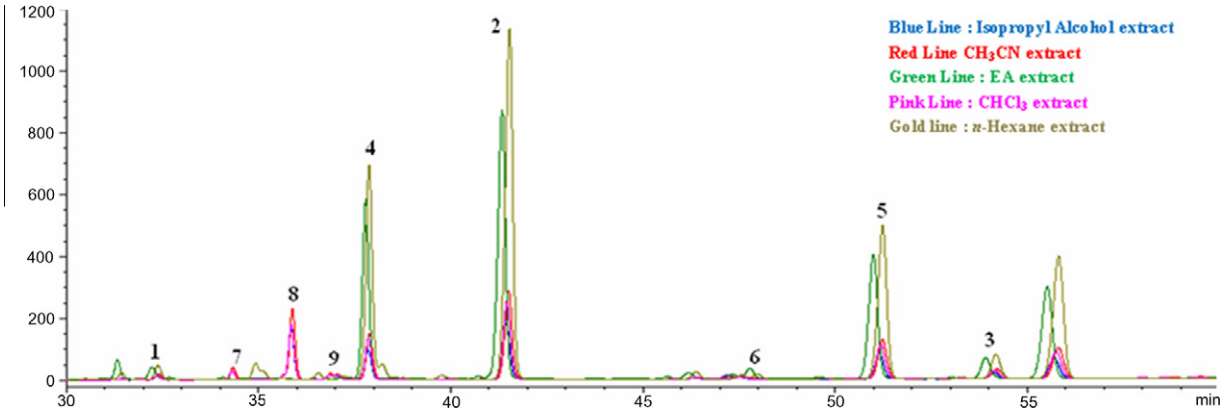
The pulverized roots of *L. erythrorhizon* (50 g) were extracted with various solvent (500 mL) at 30 °C. The extracts used for HPLC analysis were passed through 0.45-μm filters (Millipore, MSI, Westboro, MA). Chromatographic separation was achieved using an Agilent 1200 liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary HPLC pump, a degasser, autosampler and UV detector (VWD). The mobile phase for HPLC consisted of solvent A, 0.1% trifluoroacetic acid in water, and solvent B, 0.1% trifluoroacetic acid in acetonitrile. The solvent gradient was as follows (relative to solvent A): 0 min, 30% B; 10 min, 45% B; 20 min, 55% B; 30 min, 70% B; 40 min, 85% B; 50 min, 100% B; 70 min, 100% B. The flow rate was 1.0 mL/min and the injection was volume 10 μL. The eluent was detected at 254 nm and all HPLC analyses were performed at 30 °C.

**Table 3**
Glycosidases inhibitory activity of shikonins **1**–**6** and shikonofurans **7**–**9**

| Compounds | Glycosidases | | |
|---|---|---|---|
| | α-Glucosidase ($IC_{50}$, μM)[a] | β-Glucosidase ($IC_{50}$, μM) | Rhamnosidase ($IC_{50}$, μM) |
| **1** | NA[b] | NA | NA |
| **2** | 157.8 ± 10.4 | NA | NA |
| **3** | 140.9 ± 8.7 | NA | NA |
| **4** | NA | NA | NA |
| **5** | 144.2 ± 3.9 | NA | NA |
| **6** | NA | NA | NA |
| **7** | 37.8 ± 2.1 | NA | NA |
| **8** | 26.6 ± 3.0 | NA | NA |
| **9** | 20.8 ± 1.7 | NA | NA |

[a] All compounds were examined in a set of duplicated experiment; $IC_{50}$ values of compounds represent the concentration that caused 50% enzyme activity loss.
[b] NA = no activity at 200 μM.

**Table 4**
Quantitative analysis of isolated compounds **1**–**9** in Kg of *Lithospermum erythrorhizon* extracts




| Compounds | Content (g/Kg, extract) | | | | | |
|---|---|---|---|---|---|---|
| | EtOH | Isopropyl alcohol | $CH_3CN$ | Ethyl acetate | $CHCl_3$ | *n*-Hexane |
| **1** | – | 2.76 ± 0.04 | 1.49 ± 0.04 | 1.55 ± 0.03 | 3.20 ± 0.04 | 0.11 ± 0.02 |
| **2** | 0.23 | 33.92 ± 7.1 | 56.99 ± 4.9 | 166.14 ± 12.5 | 46.5 ± 2.8 | 218.69 ± 3.5 |
| **3** | 0.11 | 3.64 ± 0.12 | 10.61 ± 2.1 | 12.68 ± 3.1 | 4.57 ± 1.2 | 14.89 ± 1.25 |
| **4** | – | 18.48 ± 3.4 | 31.40 ± 3.8 | 113.54 ± 6.8 | 25.31 ± 3.5 | 135.58 ± 1.5 |
| **5** | 0.43 | 16.69 ± 6.8 | 32.71 ± 3.2 | 85.99 ± 5.9 | 22.9 ± 3.12 | 107.58 ± 1.2 |
| **6** | 0.62 | 0.74 ± 0.2 | 4.09 ± 0.9 | 4.89 ± 1.2 | 1.01 ± 0.26 | 2.19 ± 0.15 |
| **7** | 0.38 | 4.74 ± 1.2 | 8.38 ± 1.8 | 1.90 ± 0.1 | 5.06 ± 1.0 | 0.22 ± 0.01 |
| **8** | – | 15.85 ± 3.1 | 23.55 ± 5.0 | 0.35 ± 0.08 | 17.09 ± 2.5 | 0.67 ± 0.08 |
| **9** | – | 7.86 ± 1.5 | 10.5 ± 0.8 | 1.63 ± 0.37 | 7.63 ± 1.1 | 5.28 ± 1.25 |

### 4.3. Extraction and isolation

Dried roots of *L. erythrorhizon* (2.0 kg) were extracted with *n*-hexane for one week at room temperature. The *n*-hexane extract was concentrated on a rotary evaporator, and the dried extract (15.8 g) was chromatographed on silica gel using mixtures of *n*-hexane–EtOAc of increasing polarity (100:0→30:70), yielding nine fractions. Fraction 2 (1.6 g) was divided into four sub-fractions, 2-1, 2-2, 2-3, 2-4, by column chromatography on silicagel eluted with *n*-hexane–EtOAc (70:30, v/v). Sub-fraction 2-3 (0.8 g) was purified on silicagel chromatography to give compound **2** (300 mg). Compound **3** (22 mg) and compound **5** (32 mg) were isolated from sub-fraction 2-2 using preparative-HPLC ($CH_3CN/H_2O$, 80/20, v/v). Fraction 3 (480 mg) was further purified by silicagel chromatography eluting with *n*-hexane–EtOAc (70:30, v/v) to give compound **1** (10 mg) and compound **6** (12 mg). Fraction 5 (1.9 g) was separated by chromatography on a Sephadex LH-20 column and preparative-HPLC to yield compound **7** (22 mg), compound **8** (12 mg), and compound **9** (18 mg).

Shikonin (**1**): red brown powder; $[\alpha]_D^{20}$ = +140 (*c* 0.1, $CHCl_3$); mp: 145–146 °C; ESI-MS *m*/*z* = 287[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) δ 12.59 (s, 1H, –OH), 12.49 (s, 1H, –OH), 7.19 (d, *J* = 3.8 Hz, 2H), 7.16 (d, *J* = 1.3 Hz, 1H), 5.19 (t, *J* = 7.6 Hz, 1H), 4.91 (s, 1H), 2.62 (m, 1H), 2.35 (m, 2H), 1.75 (s, 3H), 1.65 (s, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 180.8, 180.0, 165.6, 165.0, 151.5, 137.6, 132.5, 132.4, 132.0, 118.5, 112.0, 111.6, 68.4, 35.8, 26.0, 18.2.

Acetylshikonin (**2**): red powder; $[\alpha]_D^{20}$ = +456 (*c* 0.1, $CHCl_3$); mp: 105–106 °C; ESI-MS *m*/*z* = 329[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) δ 12.56 (s, 1H, –OH), 12.40 (s, 1H, –OH), 7.16 (s, 2H), 6.97 (d, *J* = 1.2 Hz, 1H), 6.0 (ddd, *J* = 1.2, 4.6, 7.2 Hz, 1H), 5.11 (t, *J* = 7.26 Hz, 1H), 2.58 (m, 1H), 2.46 (m, 1H), 2.12 (s, 3H), 1.68 (s, 3H), 1.56 (s, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 178.3, 176.8, 169.8, 167.5, 167.0, 148.3, 136.2, 132.9, 132.8, 131.5, 117.7, 111.9, 111.6, 69.6, 32.9, 25.8, 21.0, 18.0.

β,β-Dimethylacrylshikonin (**3**): dark red powder; $[\alpha]_D^{20}$ = + 304 (*c* 0.5, $CHCl_3$); mp: 112−113 °C; ESI-MS *m*/*z* = 369[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 12.59 (s, 1H, –OH), 12.43 (s, 1H, –OH), 7.18 (s, 2H), 6.97 (s, 1H), 6.01 (ddd, *J* = 1.3, 4.6, 7.1 Hz, 1H), 5.77 (t, *J* = 1.3 Hz, 1H), 5.14 (t, *J* = 7.1 Hz, 1H), 2.62 (m, 1H), 2.48 (m, 1H), 2.15 (s, 3H), 1.93 (s, 3H), 1.70 (s, 3H), 1.57 (s, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 179.1, 166.9, 166.4, 165.4, 159.0, 149.1, 135.9, 132.7, 132.5, 131.7, 118.1, 115.4, 112.0, 111.7, 68.7, 33.0, 27.7, 25.8, 20.5, 18.04.

β-Hydroxyisovalerylshikonin (**4**): dark red powder; $[\alpha]_D^{20}$ = +102 (*c* 0.5, $CHCl_3$); mp: 89−90 °C; ESI-MS *m*/*z* = 387[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 12.59 (s, 1H, –OH), 12.40 (s, 1H, –OH), 7.17 (s, 2H), 7.02 (d, *J* = 1.26 Hz, 1H), 6.08 (ddd, *J* = 1.2, 4.6, 7.6 Hz, 1H), 5.11 (t, *J* = 7.6 Hz, 1H), 3.25 (s, 1H, –OH), 2.61 (m, 1H), 2.58 (d, *J* = 2.9 Hz, 2H), 2.49(m, 1H), 1.69 (s, 3H), 1.58 (s, 3H), 1.30 (s, 3H), 1.29 (s, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 177.0, 175.4, 171.8, 168.9, 168.3, 147.6, 136.5, 133.5, 133.3, 131.4, 117.7, 111.9, 111.7, 69.9, 69.2, 46.5, 33.0, 29.3, 29.2, 25.8, 18.0.

Isobutylshikonin (**5**): dark red powder; $[\alpha]_D^{20}$ = +135 (*c* 0.5, $CHCl_3$); mp: 89−90 °C; ESI-MS *m*/*z* = 357[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 12.58 (s, 1H, –OH), 12.42 (s, 1H, –OH), 7.18 (s, 2H), 6.96 (d, *J* = 1.3 Hz, 1H), 6.01 (ddd, *J* = 1.3, 4.6, 7.4 Hz, 1H), 5.11 (t, *J* = 7.1 Hz, 1H), 2.62 (m, 2H), 2.47 (m, 1H), 1.70 (s, 3H), 1.57 (s, 3H), 1.21 (d, *J* = 5.0 Hz, 3H), 1.19 (d, *J* = 5.0 Hz, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 178.3, 176.8, 175.7, 167.3, 166.8, 148.5, 136.0, 132.8, 132.7, 131.3, 117.8, 111.8, 111.6, 69.0, 34.0, 32.9, 25.7, 18.9, 18.4, 17.9.

Deoxyshikonin (**6**): dark red powder; mp: 93−94 °C; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 12.61 (s, 1H, –OH), 12.45 (s, 1H, –OH), 7.18 (d, *J* = 1.3 Hz, 2H), 6.82 (d, *J* = 1.3 Hz, 1H), 5.14 (t, *J* = 7.1 Hz, 1H), 2.62 (t, *J* = 7.6 Hz, 2H), 2.29 (q, *J* = 7.6 Hz, 2H), 1.69 (s, 3H), 1.59 (s, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 183.2, 183.1, 162.9, 162.2, 151.5, 134.6, 133.7, 132.0, 130.9, 122.4, 112.0, 111.9, 29.8, 26.6, 25.7, 17.9.

Shikonofuran D (**7**): pale yellow oil; $[\alpha]_D^{20}$ = +56 (*c* 0.1, $CHCl_3$) ESI-MS *m*/*z* = 343[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.43 (s, 1H), 7.02 (d, *J* = 3.4 Hz, 1H), 6.80 (d, *J* = 8.8 Hz, 1H), 6.69–6.66 (m, 2H), 5.76 (t, *J* = 6.7 Hz, 1H), 5.08 (t, *J* = 7.14 Hz, 1H), 2.62–2.48 (m, 3H), 1.68 (s, 3H), 1.61 (s, 3H), 1.17 (d, *J* = 6.7 Hz, 3H), 1.15 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 176.8, 152.4, 149.3, 146.6, 138.8, 135.2, 127.2, 118.6, 118.1, 117.0, 116.6, 112.1, 106.7, 68.2, 34.3, 33.6, 25.8, 19.0, 18.9, 18.1.

Shikonofuran E (**8**): pale yellow oil; $[\alpha]_D^{20}$ = −69 (*c* 0.5, $CHCl_3$); ESI-MS *m*/*z* = 355[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.43 (s, 1H), 6.97 (d, *J* = 2.9 Hz, 1H), 6.79 (d, *J* = 8.4 Hz, 1H), 6.69 (s, 1H), 6.67 (dd, *J* = 2.9, 8.8 Hz, 1H), 5.78 (t, *J* = 6.7 Hz, 1H), 5.70 (t, *J* = 1.3 Hz, 1H), 5.09 (t, *J* = 7.1 Hz, 1H), 2.61 (m, 1H), 2.52 (m, 1H), 2.16 (d, *J* = 1.3 Hz, 3H), 1.89 (d, *J* = 1.3 Hz, 3H), 1.68 (s, 3H), 1.60 (s, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 166.3, 157.7, 152.4, 149.3, 146.5, 139.0, 135.2, 127.3, 118.7, 118.1, 117.0, 116.5, 116.1, 112.1, 106.8, 67.7, 33.7, 27.6, 25.9, 20.4, 18.1.

Shikonofuran C (**9**): pale yellow oil; $[\alpha]_D^{20}$ = +64 (*c* 0.1, $CHCl_3$); ESI-MS *m*/*z* = 357[M−1]$^-$; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.43 (d, *J* = 3.2 Hz, 1H), 7.00 (d, *J* = 2.0 Hz, 1H), 6.81 (d, *J* = 8.4 Hz, 1H), 6.69 (m, 2H), 5.76 (t, *J* = 6.9 Hz, 1H), 5.08 (m, 1H), 2.61 (m, 1H), 2.51 (m, 1H), 2.20 (m, 2H), 2.09 (m, 1H), 1.68 (s, 3H), 1.62 (s, 3H), 0.94 (d, *J* = 1.6, 6.8 Hz, 6H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 172.8, 152.4, 149.2, 146.6, 139.0, 135.2, 127.2, 118.6, 118.1, 116.5, 111.2, 106.8, 106.7, 68.2, 43.8, 33.6, 25.8, 22.4, 18.0, 16.7.

### 4.4. Expression and purification of truncated sialidase gene (*NanI*) of *Clostriduim perfringens* (ATCC 10543)

The truncated sialidase gene encoding the C-terminal catalytic domain (amino acids Val243-Lys694) was PCR-amplified using genomic DNA of *Clostridium perfringens* ATCC 10543 and two primers designed according to the *nanI* sequence of *C. perfringens* ATCC 10543: sense primer (5′–GGATCCCCGTAGAGGGAGC TG–3′ having underlined *Bam*HI site) and antisense primer [5′–CTCGAGTTATTTATTAGC TCCACTC–3′ having underlined *Xho*I site]. The amplified fragments were digested with *Bam*HI and *Xho*I and inserted into pET23d vector (Novagen, Madison, WI, USA). *Escherichia coli* BL21 (DE3) codon plus RIL (Invitrogen, Carlsbad, CA, USA) transformants harboring the expression vector was grown in Luria-Bertani medium (Becton and Dickinson; 1,000 mL) containing 50 μg/mL of ampicillin at 37 °C up to an absorbance of 0.5 at 600 nm. Expression was induced by 0.2 mM IPTG and it was continued at 18 °C for 8 h.

Wet cells collected by centrifugation at 4,000*g* for 5 min were suspended in 20 mM sodium phosphate buffer (buffer A, pH 7.0). The suspended cells were sonicated and centrifuged at 10,000*g* for 20 min, and the supernatant was used as a crude enzyme solution. Purification of the truncated nanI was carried out with Q Sepharose Fast Flow (GE Healthcare, Piscataway, NJ, USA) column equilibrated with buffer A. The column was washed with buffer A, the absorbed proteins were loaded onto a phenyl Sepharose equilibrated with buffer A containing 1.0 M ammonium sulfate. After washing the column with the same buffer, the absorbed proteins were eluted with a linear gradient (1–0 M) of ammonium sulfate in buffer A. The active fractions were dialyzed against buffer A, concentrated using a CentriPrep YM-10 (Millipore, Billerica, MA, USA). Approximately 14.5 mg of purified protein was obtained from 1000 mL of the culture medium. Protein concentration was determined by Bradford's method[29] with bovine serum albumin as standard. SDS–PAGE was performed with precast 4–12% Bis-Tris gels in an Xcell Sure Lock Mini-Cell (Invitrogen), according to the manufacturer's procedure.

### 4.5. Glycosyl hydrolase family 33 sialidase inhibition assay

The GH33 sialidase inhibitory assay was performed as previously reported.[14,15] Briefly, 15 μL GH33 sialidase solution (0.1 U/mL) was pre-mixed with 15 μL of sample solution at different concentrations in 510 μL of 50 mM sodium acetate buffer (pH 5.0) in cuvette. Then, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (SIGMA, M8639) 0.125 mM in buffer (pH 5.0) as a substrate added (60 μL) to the mixture to start the reaction at 37 °C. 4-Methylumbelliferone was immediately quantified by fluorometric determination with a SpectraMax M$^{2e}$ Multimode Reader (Molecular Devices, USA). The excitation wavelength was 365 nm and the emission wavelength was 450 nm. For the determination of enzyme activity (fitting experimental data to the logistic curve by (Eq. 1), used time-drive protocol with initial velocity was recorded over a range of concentrations and the data were analyzed using a nonlinear regression program [Sigma Plot (SPCC Inc., Chicago, IL)].

$$\text{Activity}(\%) = 100[1/(1 + ([\text{I}]/\text{IC}_{50}))] \quad (1)$$

### 4.6. Glycosyl hydrolase family 34 sialidase (Influenza A virus, A/Bervig_Mission/1/18, rvH1N1) inhibition assay

Recombinant influenza A virus H1N1 (rvH1N1) sialidase was from R&D systems, Inc. (4858-NM).[16] The inhibitory effect on GH34 sialidase was measured using fluorometric method developed in our previous work.[16,17] All samples were dissolved in MeOH at 5 mM and diluted. 50 μL substrate, 800 μM 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate solution, was mixed with 80 μL of 50 mM Tris buffer (pH 7.5) at

room temperature. 20 μL of sample solution and 50 μL of sialidase (0.05 pg/mL) were added to a well in a plate. The mixture was recorded at excitation and emission wavelengths of 365 nm and 445 nm with a FLx 800 (BioTeck Instrument Inc., USA).

$$\text{Activity}(\%) = [(S - S_0)/(C - C_0)] \times 100 \quad (2)$$

where $C$ is the fluorescence of the control (enzyme, buffer, and substrate) after 20 min of incubation, $C_0$ is the fluorescence of the control at zero time, $S$ is the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation, and $S_0$ is the fluorescence of the tested samples at zero time. To allow for the quenching effect of the samples, the sample solution was added to the reaction mixture $C$, and any reductions in fluorescence were assessed.

### 4.7. Progress curves determination and time-dependent assay

For further investigation of the inhibition mechanism, we measured initial velocities of substrate hydrolysis as a function of preincubation time of the sialidase with the inhibitor, probing the time-dependence of inhibition of the hydrolysis of substrate by these inhibitors. The data were analyzed using the a nonlinear regression program [Sigma Plot (SPCC Inc., Chicago, IL)] to give the individual parameters for each curve; $v_i$ (initial velocity), $v_s$ (steady-state velocity), $k_{obs}$ (apparent first-order rate constant for the transition from $v_i$ to $v_s$), $I$ (intensity at ex: 365 nm, em: 450 nm), and $K_i^{app}$ (apparent $K_i$) according to the following equations:

$$I = v_s t + (v_i - v_s)[1 - \exp(-k_{obs}t)]/k_{obs} \quad (3)$$

$$v/v_0 = \exp(-k_{obs}t) \quad (4)$$

$$k_{obs} = k_4(1 + [I]/K_i^{app}) \quad (5)$$

### 4.8. Statistical analysis

All the measurements were made triplicate. The results were subject to analysis of variance using Sigma plot to analyze the difference. Differences were considered significant at $p < 0.05$.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.011.

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