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Influenza Virus Neuraminidase Inhibitory Activity of Phlorotannins from the Edible Brown Alga *Ecklonia cava*

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Supporting Information

ABSTRACT: Influenza A virus infections continue to pose a major threat to humans and several animal species. Neuraminidase (NA) is one of the most promising targets for the development of drugs against influenza viruses because of its critical role in the viral life cycle. During the course of a search for NA inhibitors from edible natural sources, we found that the ethyl acetate layer of ethanol extracts of *Ecklonia cava* showed extremely high NA-inhibitory activity (72.1% inhibition at 30 μg/mL). Bioactivity-guided fractionation of the ethyl acetate layer yielded five phlorotannins, identified as phloroglucinol (**1**), eckol (**2**), 7-phloroeckol (**3**), phlorofucofuroeckol (**4**), and dieckol (**5**). The inhibitory activities of these compounds (**1**−**5**) against NAs from group-1 (A/Bervig_Mission/1/18 [H1N1], A/PR/8/34 [H1N1]) and group-2 (A/Hong Kong/8/68 [H3N2], A/Chicken/Korea/MS96/96 [H9N2]) influenza A were evaluated to determine potencies and kinetic behavior. Analyses using various in vitro influenza A virus NA assays showed that all five phlorotannin derivatives were selective NA inhibitors. Of the phlorotannins, phlorofucofuroeckol (**4**) exhibited the most potent inhibitory activities toward group-1 NAs (IC_{50} values, 4.5 and 14.7 μM), whereas dieckol (**5**) potently inhibited group-2 NAs. Kinetic analyses indicated that compounds **1**−**5** were all noncompetitive. Notably, these noncompetitive inhibitors synergized with oseltamivir to enhance the NA-inhibitory effects of oseltamivir.

KEYWORDS: Influenza virus, neuraminidase, *Ecklonia cava*, phlorotannin

INTRODUCTION

The influenza virus is a highly infective agent that causes acute pulmonary disease. Influenza viruses are negative strand RNA viruses with a segmented genome that belongs to the *Orthomyxoviridae* family, which includes influenza viruses A, B, and C.[1,2] Type A viruses account for all human pandemics in the last century, namely, the 1918 H1N1 "Spanish," 1957 H2N2 "Asian," and 1968 H3N2 "Hong Kong" influenza viruses.[3,4] In June 2009, the World Health Organization (WHO) declared a new strain of swine-origin H1N1, raising the level of influenza pandemic alert from phase three to phase six.[5] Although vaccination is considered that first line of defense, current strategies for vaccine design and manufacturing may not be sufficient to combat an influenza pandemic. A current target for the design of new drugs against the influenza virus is the enzyme neuraminidase (NA), a glycoprotein found on the surface of the virus particle. The function of NA is to cleave sialic acid residues from the surface of progeny virus particles[6] and the infected cell.[7] In the absence of NA activity, viruses form aggregates, severely limiting the spread of infection. If the activity of NA can be inhibited, virus spread can be essentially stopped. Thus, its key role in the pathogenicity of many respiratory viruses makes NA an attractive target for anti-influenza virus strategies. There is considerable interest in the development of NA inhibitors from edible natural plants, as these can be readily applied to neutraceutics for the prevention/treatment of virus infection.

We recently reported that polyphenols isolated from the plants *Rhodiola rosea*,[8] *Glycyrrhiza uralensis*,[9] *Sophora flavescens*,[10] and *Cudrania tricuspidata*[11] inhibit NA and display anti-influenza virus activities. We have established a screening system for identifying new, natural product-derived influenza virus NA inhibitors and understanding the structure−activity relationships of constituents from native plants. During the course of searching for anti-influenza virus inhibitor from edible natural sources, we found that the ethanol extract of *Eckolnia cava* possessed NA-inhibitory activity.

The brown alga *Eckolina cava* (Laminariaceae) is abundant in the subtidal regions of Jeju Island in Korea. *E. cava* is used to produce food ingredients, animal feed, fertilizers, and folk medicine in gynecopathy.[12] Previous studies have presented a wealth of evidence showing that *E. cava* exhibits antioxidative,[13,14] anticancer,[12] antiallergic,[15] and anti-inflammatory[16,17] activities, and inhibits melanin formation in mouse B16F10 melanoma cells.[18] Phlorotannin components, which are oligomeric polyphenols of phloroglucinol units, are responsible for the pharmacological activities of *E. cava*. Phlorotannins, such as eckol (a closed-chain trimer of phloroglucinol), phlorofucofuroeckol (a pentamer), and dieckol (a hexamer) were identified in *Ecklonia* species.

As part of an effort to develop a safe and effective anti-influenza virus natural product, we here applied an activity-guided fractionation of the edible brown alga *E. cava* that resulted in the isolation of five known phlorotannins (**1**−**5**). The inhibitory effects and

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mechanisms of action of these phlorotannins were tested against influenza virus NAs (A/Bervig_Mission/1/18 [H1N1], A/PR/8/34 [H1N1], A/Hong Kong/8/68 [H3N2], and A/Chicken/Korea/MS96/96 [H9N2]). These compounds were further evaluated according to their activity against influenza A virus NAs divided phylogenetically into two distinct subtypes: group-1 (N1, N4, N5, and N8) and group-2 (N2, N3, N6, N7, and N9).[19] We also assessed the ability of individual isolated inhibitors (**2**−**5**) to synergize with oseltamivir.

MATERIALS AND METHODS

General Apparatus and Chemicals. Melting points were measured on a Thomas Scientific Capillary Melting Point apparatus (Electronthermal 9300, UK) and are uncorrected. ^{1}H- and ^{13}C NMR along with 2D-NMR data were obtained on JNM-ECA 500 and 600 (Jeol, Japan) spectrometers in methanol-d_3 and tetramethylsilane (TMS) as internal standards. ESI mass spectra were scanned using ESI in negative or positive mode. All of 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 plates precoated 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). RP-18 (ODS-A, 12 μm, S-150 Å, YMC) and Sephadex LH-20 (Amersham Biosciences) were used for column chromatography.

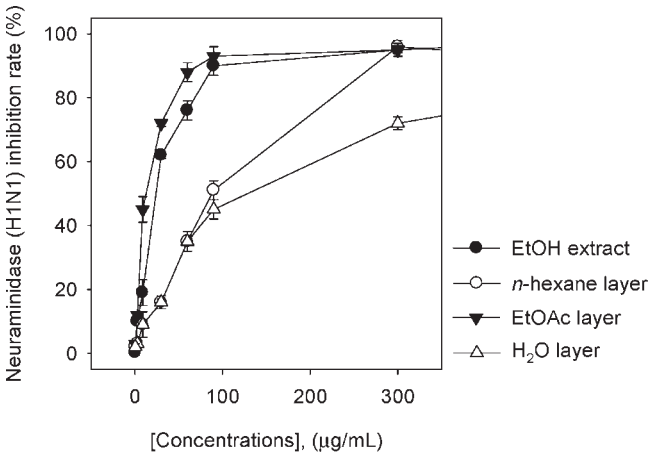

Figure 1. Influenza virus NA inhibitory activities of *E. cava* EtOH extract and its different organic solvent soluble layers.

Extraction and Isolation. Dried powder of *Ecklonia cava* (2.0 kg) were extracted with EtOH (20 L) for one week at room temperature. The EtOH extract was concentrated on a rotary evaporator, and the dried extract (157 g) was suspended in H_2O and partitioned with *n*-hexane (25.4 g) and ethyl acetate (60.5 g). The ethyl acetate soluble fraction (60 g) was chromatographed on silica gel using mixtures of $CHCl_3$/MeOH of increasing polarity (100:0→20:80), yielding six fractions. Fraction 2 (3.0 g) was divided into four subfractions, 2−1, 2−2, 2−3, and 2−4, by column chromatography on silica gel eluted with $CHCl_3$/MeOH of increasing polarity (100:0→50:50). Subfraction 2−1 (0.23 g) was separated through chromatography on a Sephadex LH-20 column to yield compound **1** (30 mg). Compound **3** (18 mg) was isolated by subfraction 2−3 using preparative-HPLC (CH_3CN/H_2O, 60/40, v/v). Fraction 4 (3.5 g) was divided into five subfractions, 4−1, 4−2, 4−3, 4−4, 4−5, by column chromatography on silica gel eluted with $CHCl_3$/MeOH of increasing polarity (100:0→30:70). Subfraction 4−2 (0.5 g) was further purified by silica gel chromatography eluted with $CHCl_3$/MeOH (70:30, v/v) and RP-C18 chromatography to yield compound **2** (18 mg). Subfraction 4−3 (0.48 g) was separated through chromatography on a RP-C18 chromatography column and preparative-HPLC to yield compound **4** (23 mg) and compound **5** (13 mg).

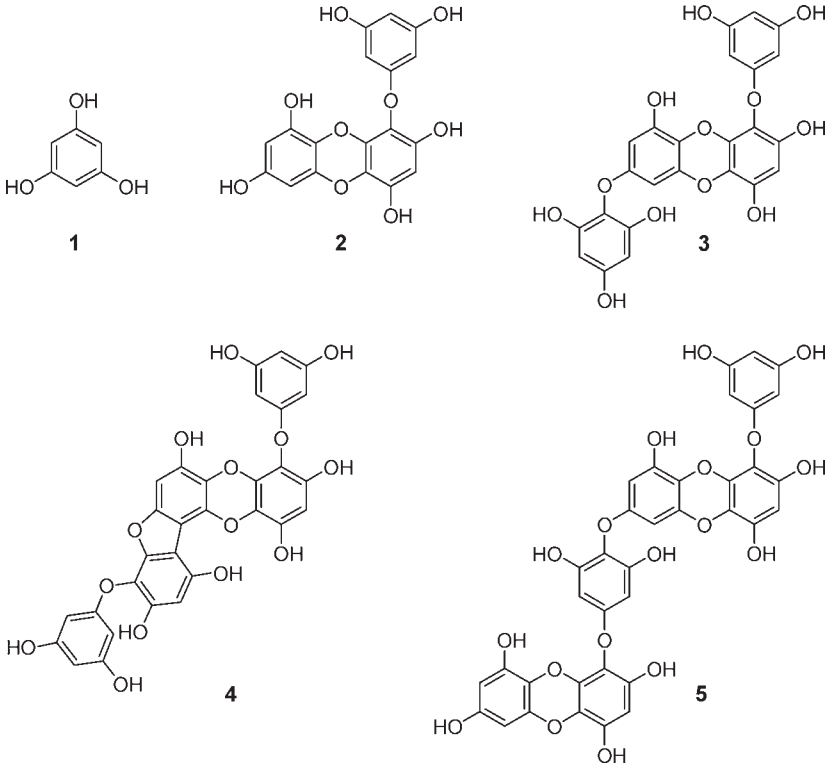

Figure 2. Structures of compounds isolated from the edible brown alga *E. cava*.

Table 1. Influenza Virus NA (rvH1N1) Inhibitory Activities of Isolated Phlorotannin Derivatives (1−5)

compounds	IC_{50} (μM)[a]	inhibition type (K_i, μM)
1	>200	NT[b]
2	89.5 ± 9.0	noncompetitive (52.0 ± 1.7)
3	44.2 ± 1.7	noncompetitive (36.5 ± 3.0)
4	4.5 ± 2.2	noncompetitive (7.2 ± 0.1)
5	20.2 ± 3.2	noncompetitive (16.0 ± 3.5)

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

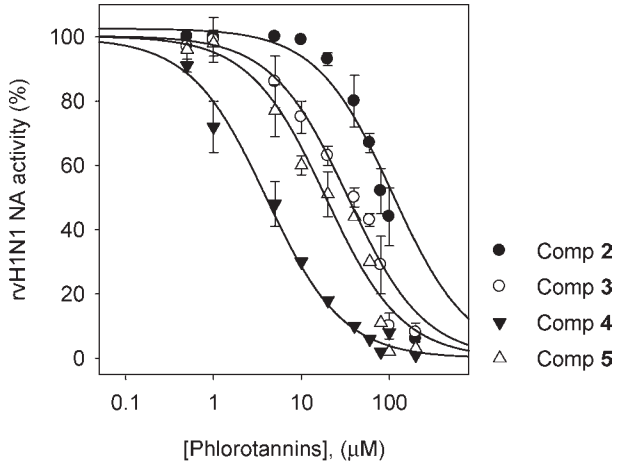

Figure 3. Effects of compounds **2**−**5** on the activity of influenza virus NA (H1N1).

Compound ***1***. White powder; mp 218−219 °C; purity 98% (HPLC analysis conditions, 70% aqueous acetonitrile, 1 mL/min, λ = 210 nm, and R_t = 3.16 min); ^{1}H NMR (300 MHz, methanol-d_4) δ 5.80 (s, 3H); ^{13}C NMR (75 MHz, methanol-d_3) δ 158.7, 94.0.

Compound ***2***. Light brown powder; mp 246−247 °C; purity 98% (HPLC analysis conditions, 80% aqueous acetonitrile, 1 mL/min, λ = 210 nm, and R_t = 7.59 min); ESI-MS m/z = 371 $[M - H]^-$; ^{1}H NMR (600 MHz, methanol-d_3) δ 6.14 (s, 1H), 5.95 (s, 2H), 5.93(s, 3H); ^{13}C NMR (150 MHz, methanol-d_3) δ 160.6, 158.8, 153.2, 145.9, 145.7, 142.9, 142.0, 137.2, 124.2, 123.5, 123.2, 98.5, 96.3, 94.4, 94.0.

Compound ***3***. Brown powder; mp 276−277 °C; purity 93% (HPLC analysis conditions, 70% aqueous acetonitrile, 1 mL/min, λ = 254 nm, and R_t = 10.5 min); ESI-MS m/z = 495 $[M - H]^-$; ^{1}H NMR (600 MHz, methanol-d_3) δ 6.14 (s,1H), 6.07 (s, 2H), 5.96 (m, 4H), 5.85 (s, 1H); ^{13}C NMR (150 MHz, methanol-d_3) δ 160.6, 160.5, 159.0, 158.8, 155.1, 153.2, 150.8, 147.4, 145.8, 145.0, 141.7, 137.3, 125.5, 124.0, 123.4, 123.0, 98.6, 96.6, 96.5, 96.3, 94.8, 94.4, 94.2, 93.9.

Compound ***4***. Light brown powder; mp 292 °C (decomp); purity 95% (HPLC analysis conditions, 70% aqueous acetonitrile, 1 mL/min, λ = 210 nm, and R_t = 6.02 min); ESI-MS m/z = 603 $[M + H]^+$; ^{1}H NMR (500 MHz, methanol-d_3) δ 6.62 (s, 1H), 6.39 (s, 1H), 6.25 (s, 1H), 5.95 (d, J = 2.0 Hz, 2H), 5.92 (m, 1H), 5.90 (m, 1H), 5.87 (d, J = 2.0 Hz, 2H); ^{13}C NMR (125 MHz, methanol-d_3) δ 160.6, 160.5, 160.4, 158.8, 151.8, 150.4, 149.8, 146.9, 147.0, 146.9, 144.6, 142.6, 137.0, 134.0, 126.7, 123.7, 123.4, 121.0, 104.0, 103.9, 98.6, 98.0, 96.4, 96.3, 94.9, 94.1, 94.0.

Compound ***5***. Light brown powder; mp 278 °C (decomp); purity 94% (HPLC analysis conditions, 70% aqueous acetonitrile, 1 mL/min, λ = 210 nm, and R_t = 10.13 min); ESI-MS m/z = 743 $[M + H]^+$; ^{1}H NMR (500 MHz, methanol-d_3) δ 6.13 (s, 1H), 6.11 (s, 1H), 6.07 (s, 2H), 6.05 (d, J = 2.9 Hz, 1H), 6.03 (d, J = 2.9 Hz, 1H), 5.96 (d, J = 2.6 Hz, 1H), 5.93 (d, J = 2.9 Hz, 1H) 5.90 (m, 1H); ^{13}C NMR (125 MHz, methanol-d_3) δ 160.4, 158.7, 156.5, 154.7, 153.2, 151.1, 146.0, 145.9, 145.8, 145.6, 143.0, 142.8, 142.1, 142.0, 137.3, 137.1, 125.1, 124.9, 124.3, 124.2, 123.5, 123.2, 123.1, 98.5, 98.3, 98.1, 98.0, 96.3, 94.8, 94.5, 94.4, 94.0.

Table 2. IC_{50} Values (μM) of Isolated Phlorotannin Derivatives (1−5) from *E. cava* on Various Strains of Influenza Virus Neuraminidases[a]

compounds	A/PR/8/34 (H1N1)	A/Hong Kong/8/68 (H3N2)	A/Chicken/Korea/MS96/96 (H9N2)
1	>200 μM	>200 μM	>200 μM
2	>200 μM	>200 μM	152.1 ± 15.7
3	41.2 ± 6.9	37.4 ± 7.1	32.2 ± 9.9
4	14.7 ± 0.8	20.7 ± 1.2	22.7 ± 5.7
5	20.5 ± 2.0	16.6 ± 3.2	13.2 ± 4.2

[a] All compounds were examined in a set of triplicate experiments. IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss.

HPLC Apparatus and Chromatographic Conditions. Dried powder of *Ecklonia cava* (2 g) were extracted with various solvents (20 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 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary HPLC pump, a degasser, and an 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 column was maintained at 30 °C, the injection volume was 10 μL, and the flow rate was 1.0 mL/min. The eluent was detected at 210 and 254 nm.

Viruses and Cells. In this study, the influenza strains A/PR/8/34 (H1N1) (ATCC VR-1469), A/Hong Kong/8/68 (H3N2) (ATCC VR-544), and A/Chichen/Korea/MS96/96 (H9N2) were propagated in MDCK cells (ATCC CCL-3; Manassas, VA, USA) in the presence of 10 μg/mL trypsin (1:250; GIBCO Invitrogen Corporation, California). Then, in order to increase the signal-to-background ratio, the viruses were concentrated by ultracentrifugation at 25,000 rpm for 4 h at 4 °C. The pellets were subsequently suspended in 2 mL of NA-*Star* buffer (Applied Biosystems, Foster City, CA).

Chemiluminescent NA Inhibition Assay. NA inhibition was determined with the commercially available NA-*Star* kit (Tropix, Applied Biosystems, Darmstadt, Germany) that utilizes a 1,2-dioxetane sialic acid derivative for the substrate. The assay was distributed by Applied Biosystems, with opaque white 96-well plates, according to the manufacturer's protocol as described previously.[20] At least three independent experiments were performed for each virus. The mean NA activity of the six untreated virus controls was set to 100%, and the concentration required to reduce NA enzyme activity of virus controls by 50% was calculated.

Recombinant Influenza Virus NA (A/Bervig_Mission/1/18, rvH1N1) Inhibition Assay. The inhibitory effect on rvH1N1 NA was measured using the fluorometric method developed by our previous work.[8,9] All samples were dissolved in MeOH at 5 mM and diluted. Fifty microliters of 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. Twenty microliters of the 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

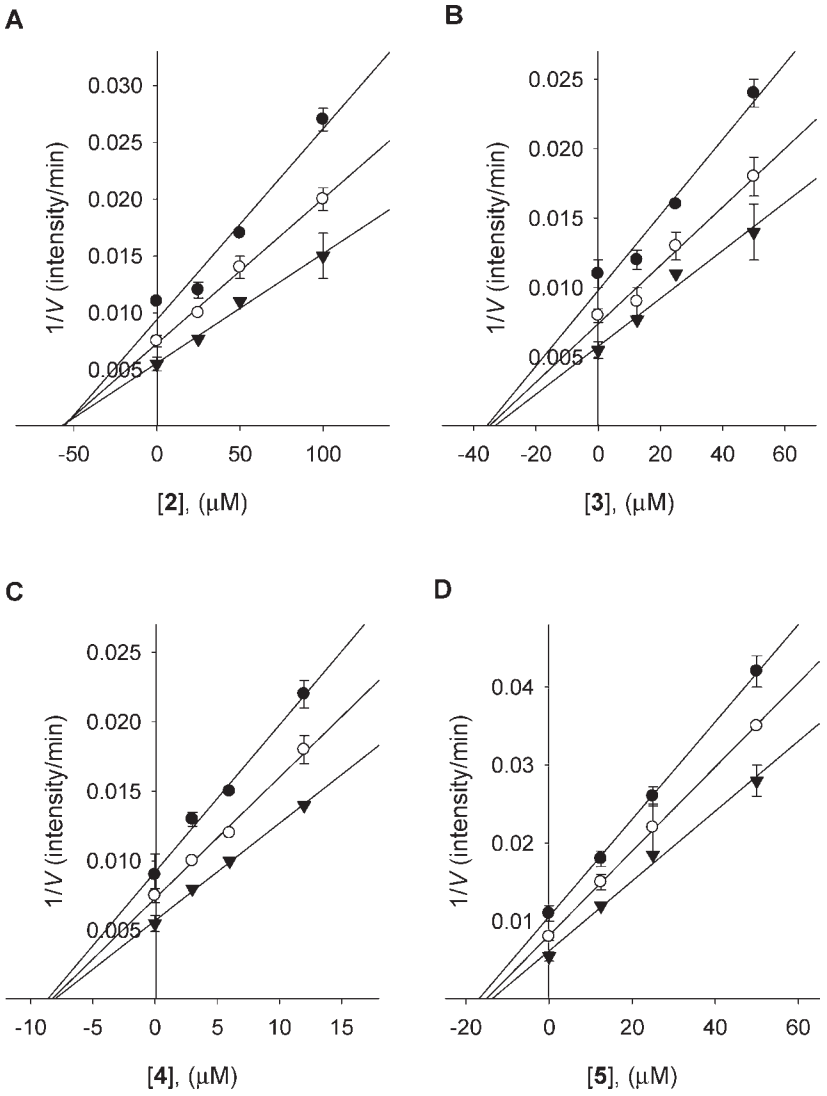

Figure 4. Graphical determination of the type of inhibition for compounds **2**−**5**. A−D are Dixon plots for the inhibition of compounds **2**−**5**, respectively, on the hydrolysis activity of viral NA (H1N1) in the presence of different concentrations of substrates (50 μM, ●; 100 μM, ○; and 200 μM, ▼).

wavelengths of 365 and 445 nm, respectively, with a FLx 800 (BioTeck Instrument Inc., USA).

RESULTS AND DISCUSSION

Natural sources, mainly plants, have provided products for food preservation and fulfilled the primary healthcare needs of every known culture. In this study, we assessed the neuraminidase inhibitory activity of an ethanol extract of *Ecklonia cava*, along with its solvent soluble fractions (*n*-hexane, ethyl acetate [EtOAc], and H_2O). The choice of extraction conditions is a critical step in natural bioactive compounds research. The ethanol extracts (420 g) of the dried brown alga *E. cava* (3.5 kg), which exhibited 33.6% inhibition at 30 μg/mL, were solvent-fractionated to obtain *n*-hexane (54 g), EtOAc (160 g), and H_2O (188 g) layers, and the inhibitory activity of each layer against influenza virus NA (rvH1N1, A/Bervig_Mission/1/18) was evaluated (Figure 1). As shown in Figure 1, the EtOAc layer exerted 72.1% inhibition at 30 μg/mL, a level of influenza virus NA-inhibitory activity greater than that of other fractions. The high potency of the EtOAc layer encouraged us to identify the compounds responsible for this effect.

To isolate the compounds by NA-guided fractionation, we subjected the EtOAc layer of the ethanol extract of *E. cava* to a succession of chromatographic procedures, including silica gel, Sephadex LH-20, and octadecyl-functionalized silica gel to yield five phlorotannins (**1**−**5**) as active principles apart from phloroglucinol (**1**). Through analysis of spectroscopic data (^{1}H-, ^{13}C NMR, and MS) and comparison with previous studies,[21−23] the isolated compounds were identified as the known species phloroglucinol (**1**), eckol (**2**), 7-phloroeckol (**3**), phlorofucofuroeckol (**4**), and dieckol (**5**) (Figure 2).

The inhibitory potencies and capacities of these phlorotannins toward influenza virus NA (rvH1N1, A/Bervig_Mission/1/18) were investigated. Influenza virus NA-inhibitory activity was assayed by following the hydrolysis of the fluorogenic substrate-Mu-Neu5Ac in the presence or absence of test compounds, as described in our previous studies.[8,9] Fifty percent inhibitory (IC_{50}) values were determined from a 10-point concentration curve by

Table 3. Synergetic Effect of Isolated NA Noncompetitive Inhibitors (2−5) with Oseltamivir

compound (μM)	neuraminidase activity (%)				compound (μM)	neuraminidase activity (%)			
	H1N1[a]	H1N1[b]	H3N2[b]	H9N2[b]		H1N1[a]	H1N1[b]	H3N2[b]	H9N2[b]
oseltamivir (5 nM)	46.7	65.1	59.2	62.1	oseltamivir (5 nM)	46.7	65.1	59.2	62.1
oseltamivir (5 nM) + **2** (100)	21.5	23.3	24.4	21.7	oseltamivir (5 nM) + **4** (100)	2.8	9.2	11.8	5.9
oseltamivir (5 nM) + **2** (50)	34.4	37.6	39.2	37.4	oseltamivir (5 nM) + **4** (50)	10.5	24.4	24.5	12.5
oseltamivir (5 nM) + **2** (25)	46.2	45.1	45.7	48.3	oseltamivir (5 nM) + **4** (25)	12.0	35.4	36.3	20.3
oseltamivir (5 nM) + **2** (12.5)	48.0	49.3	52.9	55.1	oseltamivir (5 nM) + **4** (12.5)	30.1	44.0	39.7	32.1
oseltamivir (5 nM) + **2** (6.0)	45.3	56.2	57.2	60.4	oseltamivir (5 nM) + **4** (6.0)	35.9	52.1	47.0	43.7
oseltamivir (5 nM) + **2** (3.0)	46.9	65.3	59.0	63.2	oseltamivir (5 nM) + **4** (3.0)	41.2	55.3	51.8	49.7
oseltamivir (5 nM) + **3** (100)	18.0	6.0	11.4	7.4	oseltamivir (5 nM) + **5** (100)	13.7	1.4	4.6	2.1
oseltamivir (5 nM) + **3** (50)	34.2	17.2	24.3	16.0	oseltamivir (5 nM) + **5** (50)	22.4	5.6	14.2	7.3
oseltamivir (5 nM) + **3** (25)	40.8	32.0	38.0	27.6	oseltamivir (5 nM) + **5** (25)	33.2	16.7	29.0	17.7
oseltamivir (5 nM) + **3** (12.5)	45.1	41.7	47.9	39.2	oseltamivir (5 nM) + **5** (12.5)	40.8	30.7	41.4	31.4
oseltamivir (5 nM) + **3** (6.0)	46.0	51.2	49.9	50.5	oseltamivir (5 nM) + **5** (6.0)	45.1	45.0	51.8	42.8
oseltamivir (5 nM) + **3** (3.0)	45.7	57.5	54.5	59.2	oseltamivir (5 nM) + **5** (3.0)	47.7	51.6	57.3	52.5

[a] Determination of A/Bervig_Mission/1/18 (rvH1N1) NA inhibitory activities of phlorotannins (**2**−**5**) by using an enzymatic assay. [b] Determination of A/PR/8/34 (H1N1), A/Hong Kong/8/68 (H3N2), and A/Chicken/Korea/MS96/96 (H9N2) virus NA inhibitory activities of phlorotannins (**2**−**5**) by using the NA-*Star* assay kit.

a hyperbola curve-fitting routine using a two-parameter logistic equation. As shown in Table 1 and Figure 3, all isolated phlorotannins examined, apart from phloroglucinol (**1**), exhibited a dose-dependent inhibitory effect on influenza virus NA. As the concentrations of the inhibitors were increased, the residual NA activity decreased dramatically (Figure 3). Among the isolated compounds, eckol (**2**), 7-phloroeckol (**3**), phlorofucofuroeckol (**4**), and dieckol (**5**) exhibited inhibitory effects on NA, with IC_{50} values of 89.5, 44.2, 4.5, and 20.2 μM, respectively (Table 1). Monomeric phloroglucinol (**1**) did not show a significant inhibitory effect (30% at 200 μM). From this, it may be inferred that the number of phloroglucinol moieties, and thus the number of hydroxyl groups, on the phlorotannin backbone contributes to significant and specific NA-inhibitory activity. In particular, phlorofucofuroeckol (compound **4**, IC_{50} = 4.5 ± 2.2 μM) exhibited inhibitory effects that were 5-, 10-, and 20-fold greater than those of compounds **5**, **3**, and **2**, respectively. Although the structure−activity relationships of phlorotannins were not thoroughly investigated, these results suggest that oligomerization and the existence of a cyclopentan ring (**4**) might be important for the in vitro NA-inhibitory activity of these compounds. Moreover, this inhibition potential appears to be caused by the hydrogen bond and the steric hindrance of the hydroxyl and aryl groups in phlorotannins with NA amino acids.

Next, the effects of isolated compounds (**1**−**5**) on the NAs from various influenza virus strains, namely, A/PR/8/34 (H1N1), A/Hong Kong/8/68 (H3N2), and A/Chicken/Korea/MS96/96 (H9N2), were further evaluated using the chemiluminescence NA-*Star* assay kit. In this assay, a decrease in chemiluminescence indicates NA-mediated inhibition of the release of 1,2-dioxetane by enzymatic hydrolysis of NA-*Star*. As shown in Table 2, the NA-inhibitory activities of phlorotannin derivatives were slightly affected by subtle changes in virus strains. Compound **4**, the most potent inhibitor of the NA from A/Bervig_Mission/1/18 (rvH1N1), was similarly potent against the group-1 NA from A/PR/8/34 (H1N1; IC_{50} = 14.7 μM). However, this inhibitor exhibited the second-lowest activity against group-2 NAs. In the case of group-3 NA, dieckol (**5**) exhibited the most potent, dose-dependent inhibitory activity of all tested phlorotannin derivatives, exhibiting IC_{50} values of 13.2 μM and 16.6 μM for A/Chicken/Korea/MS96/96 (H9N2) and A/Hong Kong/8/68 (H3N2), respectively. Phloroglucinol (**1**) did not show significant inhibitory effects against group-1 or -2 NAs (IC_{50} values > 200 μM). Although not all isolated phlorotannins exhibited significant NA-inhibitory effects, the data clearly indicate the potential of these compounds as inhibitors of influenza virus NA.

We next studied the inhibition mechanisms of the isolated NA inhibitors. In this assay, we used rvH1N1 NA because this commercially available enzyme is suitable for use in an in-house manufactured NA-inhibitory assay. For all inhibitors, enzyme activity decreased rapidly with increasing inhibitor concentration, indicating reversible inhibition. Double-reciprocal Lineweaver−Burk and Dixon plots, which are the most straightforward means of diagnosing inhibitor modality, were used to determine the inhibition mode. As illustrated in Figure 4, inhibition kinetics analyzed by Dixon plots showed that compounds **2**−**5** are noncompetitive inhibitors because increasing substrate concentrations resulted in a family of lines with different slopes, but a common *x*-axis intercept. Lineweaver−Burk plots confirmed that NA inhibitors exhibited noncompetitive inhibition characteristics against influenza virus NA (see Supporting Information, S12).

Because the inhibitory phlorotannins (**2**−**5**) act via a noncompetitive mechanism, it is possible that cotreatment with the competitive inhibitor oseltamivir might exert synergistic inhibitory effects on NA activity. To address this possibility, we tested the NA-inhibitory activity of combinations of individual isolated phlorotannins with oseltamivir. As shown in Table 3 and Figure 5, NA activity in the presence of oseltamivir was further diminished by coincubation with the noncompetitive phlorotannin NA-inhibitors. Notably, all phlorotannins decrease the IC_{50} value of oseltamivir, even at concentrations below those required to produce NA-inhibitory activity when used alone. These observations suggest that oseltamivir and phlorotannin derivatives **2**−**5** might act through different inhibitory mechanisms and synergistically inhibit NA activity by binding to different sites of the free enzyme and/or product-bound enzyme.

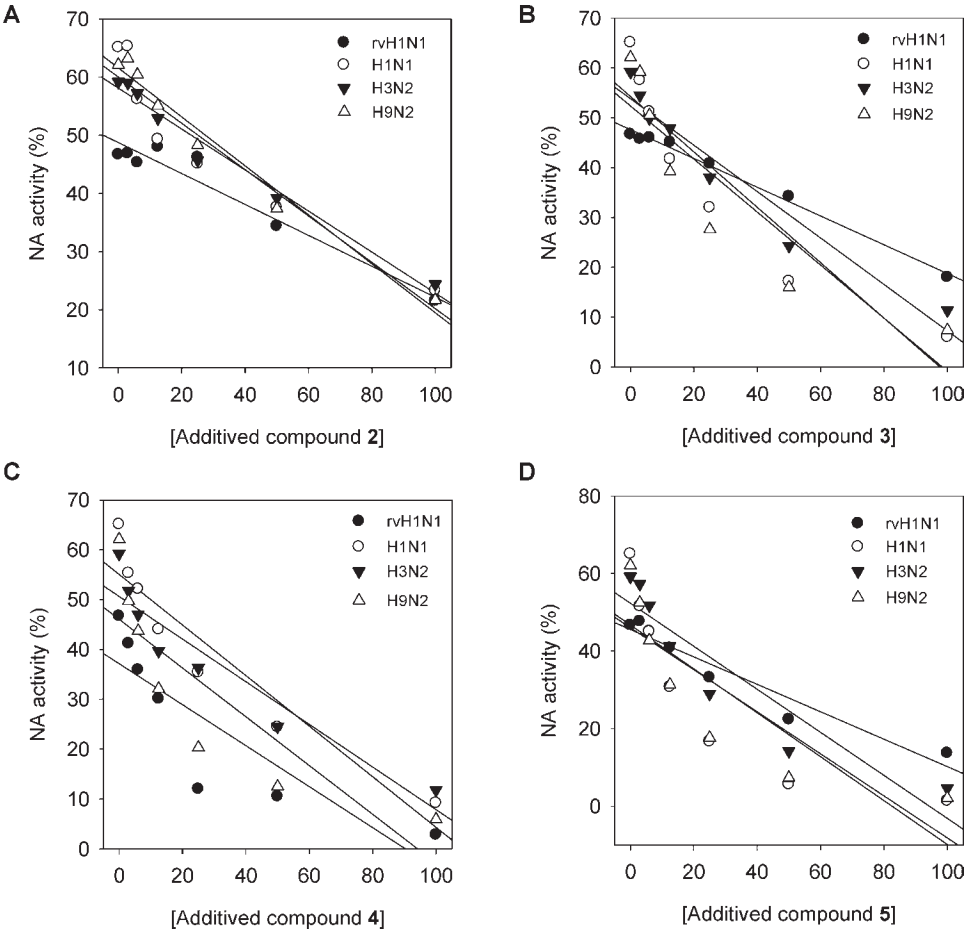

Figure 5. Isobologram for inhibitory effects by oseltamivir (5 nM) in the presence of different concentrations (0, 3.0, 6.0, 12.5, 25.0, 50.0, and 100 μM, respectively) of phlorotannins (**2**−**5**) on NAs.

Recently, marine brown algae have been documented as an important source of natural bioactive components, including polyphenols and phlorotannins with unique linkages (ether and/or phenyl).[24,25] In the present study, we focused on the polyphenol-rich brown alga, *E. cava*. An analysis of *E. cava* extracts identified five phlorotannin derivatives: phloroglucinol, eckol, 7-phloroeckol, phlorofucofuroeckol, and dieckol. In vitro NA activity assays using group-1 (A/Bervig_Mission/1/18 and A/PR/8/34, both H1N1) and group-2 (A/Hong Kong/8/68 [H3N2] and A/Chicken/Korea/MS96/96 [H9N2]) influenza viruses further showed that phlorotannin derivatives, especially phlorofucofuroeckol and dieckol, were selective NA inhibitors. Kinetic analyses indicated that all isolated derivatives acted via a noncompetitive mechanism. The ability to synergize with oseltamivir suggests that these noncompetitive inhibitors have potential therapeutic value against viral infections. These observations further highlight the possibility that the anti-influenza properties of phlorotannins derived from *E. cava* might be harnessed for use in functional food, animal feed, and pharmaceutical industries.

■ ASSOCIATED CONTENT

Supporting Information. Spectral data (NMR, LC chromatogram, and LC/MS) of phlorotanins (**2**−**5**) and Lineweaver−Burk plots of phlorotanins (**2**−**5**). This material is available free of charge via the Internet at http://pubs.acs.org.

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