Homoisoflavonoids from *Caesalpinia sappan* Displaying Viral Neuraminidases Inhibition

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In this study, twelve neuraminidase (NA) inhibitory compounds 1–12 were isolated from heartwood of *Caesalpinia sappan* on the basis of their biological activities against three types of viral NAs. Of isolated homoisoflavonoids, sappanone A (2) showed the most potent NAs inhibitory activities with IC_{50} values of 0.7 μM [H1N1], 1.1 μM [H3N2], and 1.0 μM [H9N2], respectively, whereas saturated homoisoflavonoid (3) did not show significantly inhibition. This result revealed that α,β-unsaturated carbonyl group in A-ring was the key requirements for viral NAs inhibitory activity. In our enzyme kinetic study, all NA inhibitors screened were found to be reversible noncompetitive types.

Key words *Caesalpinia sappan*; homoisoflavonoid; α,β-unsaturated carbonyl; neuraminidase

Influenza virus neuraminidase (NA, EC 3.2.1.18) has been proven as a valid therapeutic target for antiviral drugs due to its essential role in the viral replication cycle.[1–3] NA is thought to enhance viral mobility *via* hydrolysis of the α-(2,3)- or α-(2,6)-glycosidic linkage between a terminal sialic acid (Neu5Ac) residue and its adjacent carbohydrate moiety on the host receptor.[4–6] These molecules with terminal Neu5Ac are also the target receptors for viral HA (hemagglutinin),[7] the major surface glycoprotein on the viral particle. NA destroys these HA receptors allowing progeny virus particles budding from infected cell surfaces to be released.[8,9] Zanamivir (Relenza) and oseltamivir (Tamiflu), which are highly polar cyclic species designed to mimic the native substrate of neuraminidase, sialic acid, have clearly established NA inhibitors as a drug target. Although these agents are effective for the treatment and prophylaxis of influenza virus infection, drug-resistant strains have already emerged in some patients who received oseltamivir treatment.[10] To solve these problems, many researchers have sought to develop NA inhibitors from natural plants.[11,12] This is because NA inhibitors from natural plants as these can be readily applied to neutraceutics for the prevention of virus infection. For instance, our previously reported that polyphenols derived from *Rhodiola rosea*[13] and *Glycyrrhiza uralensis*[14] exhibit potent NA-inhibitory activity and Du and co-workers[15] suggested the order of potency for NA inhibition in flavonoids. A recent study reported that sappanchalcone (**1**) and brazilin (**12**) derived from *Caesalpinia sappan* were shown to inhibit three influenza virus NAs (A/PR/8/34 [H1N1], A/Guangdong/243/72 [H3N2], and B/Jiangsu/2003).[16] Also, Heller and Tamm[17] have proven a biogenetic pathway from chalcone (**1**) to brazilin (**12**) *via* several homoisoflavonoid intermediates **2**–**11**. However, NA inhibitory effects of key intermediates, homoisoflavonoids **2**–**11**, have not been demonstrated.

MATERIALS AND METHODS

General Melting points were measured on a Thomas Scientific Capillary Melting Point apparatus (Electronthermal 9300, U.K.) and are uncorrected. Optical rotations were measured on Perkin-Elmer 343 polarimeter. ^{1}H- and ^{13}C-NMR along with 2D-NMR data were obtained on JNM-EX 400 (Jeol, Japan) spectrometers in acetone-d_6 and tetramethylsilane (TMS) as internal standards. Electrospray ionization (ESI) mass spectra were scanned using ESI in negative or positive mode with bruker esquire 6000 mass spectrometer. Chromatographic separation was achieved using an Agilent 1200 liquid chromatography (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a binary HPLC pump, a degasser, and an autosampler and UV detector (VWD). All of the reagent grade chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). 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.

Plant Material The dried heartwood of *Caesalpinia sappan* L. (Leguminosae) was purchased at medicinal store of Jeongeup city.

Extraction and Isolations The dried heartwood of *Caesalpinia sappan* L. (Leguminosae) (4.8 kg) was extracted with 95% EtOH (20 L) for 2 weeks at room temperature. The residue was filtered and evaporated under reduced pressure. The dried extract (400 g) was suspended with H_2O (4 L) and partitioned with the organic solvents of the different polarities (*n*-hexane and ethyl acetate, respectively). The ethyl acetate layer (180 g) was subjected to silicagel column chromatography using a gradient system of CH_3Cl_3–CH_3OH (100 : 0 to 1 : 4) to give seven fractions (E1–E7). Fraction (E4, 40 g) was chromatographed on silicagel column chromatography with $CHCl_3$–CH_3OH (100 : 0 to 1 : 1) to give ten sub-fractions (E41–E410). Sub-fraction (E43, 8 g) was subjected to Sephadex LH-20 chromatography using a methanol to yield five fractions (E431–E435). Sappanone A (**2**, 12 mg) and

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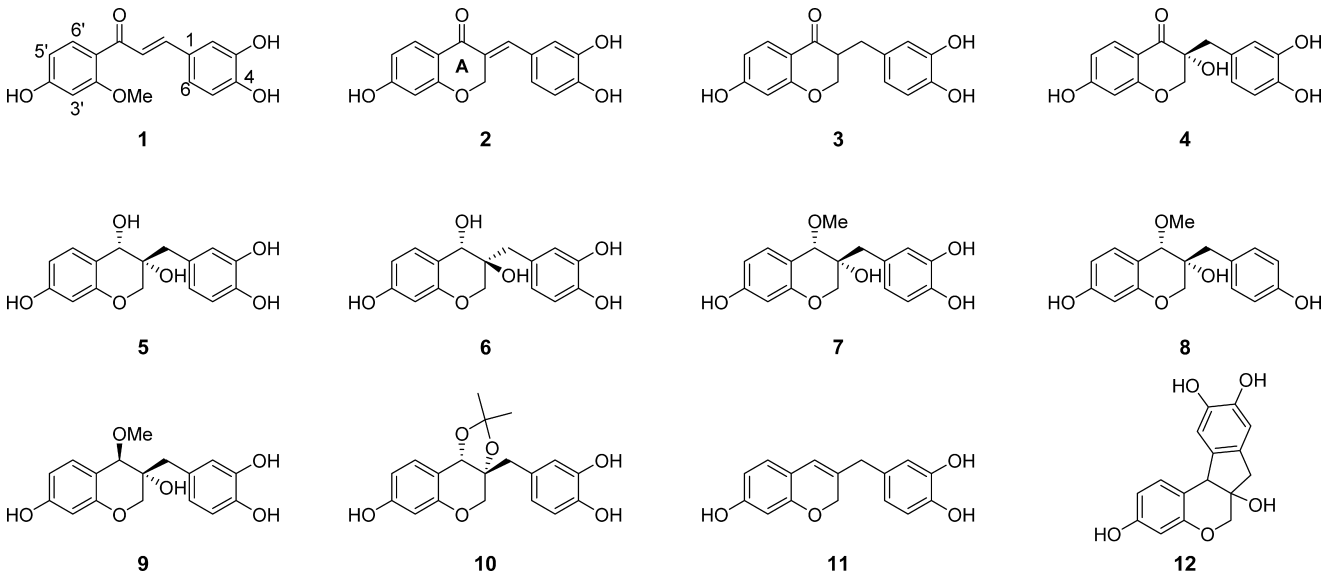

Fig. 1. Chemical Structures of Homoisoflavonoid Derivatives **1**–**12** from *C. sappan*

3′-deoxy-4-*O*-methylsappanol (**8**, 14 mg) were isolated from sub-fraction (E431, 500 mg) using RP-C18 column chromatography (CH_3OH–H_2O, 40 : 60, v/v). Sub-fraction (E434, 300 mg) was separated through chromatography on a Sephadex LH-20 column to give 4-*O*-methylsappanol (**7**, 70 mg). Sub-fraction (E44, 20 g) was divided into five sub-fractions (E441–E445) by RP-C18 column chromatography eluted with CH_3OH–H_2O (1 : 9 to 4 : 1, v/v). 4-*O*-methylepisappanol (**9**, 40 mg) was isolated from sub-fraction (E442, 120 mg) using preparative-LC (CH_3CN–H_2O, 80 : 20, v/v). Sub-fraction (E443, 6.5 g) was further purified by preparative-LC (CH_3CN/H_2O, 70/30, v/v) to give sappanone B (**4**, 10 mg) and 4-(7-hydroxy-2,2-dimethyl-9*βH*-1,3,5-trioxa-cyclopenta[*α*]naphthalene-3-lymethyl)-benzene-1,2-diol (**10**, 6 mg). Sappanol (**5**, 14 mg), episappanol (**6**, 8 mg), 3-deoxysappanone B (**3**, 7 mg), and 7,3′,4′-trihydroxy-3-benzyl-2*H*-chromene (**11**, 9 mg) were obtained by sub-fraction (E432, 1.4 g) using preparative-LC (CH_3CN–H_2O, 60 : 40, v/v). Fraction (E5, 11 g) was subjected to silicagel column chromatography ($CHCl_3$–CH_3OH, 100 : 0 to 1 : 4, v/v) to give five fractions (E51–E55). Sub-fraction (E54, 3.2 g) was separated through RP-C18 column chromatography to give sappanchalcone (**1**, 200 mg) and brazilin (**12**, 150 mg).

Synthesis of 1a For selective 1,4-reduction of *α*,*β*-carbonyl compound **1**, palladium on activated chalcoal (5 mg, 5% Pd basis) was added mehanol solution (7 mL) containing compound **1** (20 mg, 0.07 mmol) at room temperature and the reaction mixture was stirred at room temperature for 4 h at H_2 atmosphere. On completion of the reaction, the suspended material was removed by filtration and filtrate was evaporated, and then the crude product was subjected to purification through silica gel column chromatography (*n*-hexane : ethyl acetate=1 : 1) to afford **1a** in 40% yields.

Compound **1a**: White powder; ^{1}H-NMR (500 MHz, methanol-d_4) δ: 7.61 (d, J=8.59 Hz, 1H, H-6′), 6.63 (d, J=8.02 Hz, H-5), 6.61 (d, J=2.0 Hz, H-3′), 6.49 (dd, J=2.0, 8.02 Hz, H-6), 6.45 (d, J=2.0 Hz, H-2), 6.39 (dd, J=2.29, 8.59 Hz, H-5′), 3.85 (s, 3H, –OCH_3); 3.14 (t, J=7.73 Hz, 2H, H-7), 2.75 (t, J=7.73 Hz, 2H, H-8); ^{13}C-NMR (125 MHz, methanol-d_4) δ: 200.9 (C=O), 163.6 (C-4′), 161.6 (C-2′), 144.8 (C-3), 143.0 (C-4), 133.3 (C-1), 132.4 (C-6′), 119.2 (C-6), 119.0 (C-1′), 115.1 (C-2), 115.0 (C-5), 107.6 (C-5′), 98.5 (C-3′), 54.6(–OCH_3), 45.4 (C-7), 30.2 (C-8).

Viruses and Cells Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC CCL-3; Manassas, VA, U.S.A.) and grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. 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 (H9N2) were propagated in MDCK cells (ATTCC CCL-3; Manassas, VA, U.S.A.) in the presence of 10 μg/mL trypsin (1 : 250; GIBCO Invitrogen Corporation, CA, U.S.A.) at 37°C and 5% CO_2. The propagated influenza viruses were harvested at ultracentrifugation at 25000 rpm for 4 h at 4°C. Supernatant, which infected MDCK cells with virus, was suspended in NA-Star buffer (Applied Biosystem, Foster City, CA, U.S.A.).

Chemiluminescent Neuraminidase Inhibition Assay The enzyme assay inhibitory activities were performed using the commercial available NA-star kit and determined NA inhibition as previously reported with modifications.[18,19] Briefly, The NA activity was measured in 96-well plates containing 25 μL of all the compounds which were dissolved in dimethyl sulfoxide (DMSO) and diluted to the corresponding concentrations and virus supernatant in NA-Star buffer (26 mM 2-*N*-morpholinoethanesulfonic acid [pH 6.0] containing 4 mM $CaCl_2$), respectively. After the plates were preincubated for 15 min without shaking at the room temperature, 10 μL of 10 mM NA-Star 1,2-dioxetane chemiluminescent substrate diluted 1000 folds in NA-Star buffer was added to each well. The mixture without shaking was preincubated for 15 min at room temperature and 60 μL of NA-Star accelerator was added to a well in a plate. The intensity of luminescence was measured in a Centro XS^3 LB 960 (Berthold Technology GmbH and Co., KG, Germany). Each of three subtype virus performed three independent experiments and The IC_{50} was determined to measure a half values of reducing NA activities. The data were analyzed using Sigmaplot 10.0 (SPCC Inc.,

Table 1. Inhibitory Effects of Tested Compounds **1**–**12** on Neuraminidase Activities

Compound	A/PR/8/34 [H1N1] IC_{50} (μM)	A/Hong Kong/8/68 [H3N2] IC_{50} (μM)	A/Chicken/Korea/MS96/96 [H9N2] IC_{50} (μM)
1	15.9	18.1	17.0
1a	34.6	43.2	32.9
2	0.7	1.1	1.0
3	134.5	134.6	115.2
4	138.0	99.4	112.6
5	197.1	195.6	167.7
6	204.4	192.9	192.0
7	94.5	134.7	99.6
8	192.2	181.5	175.2
9	63.2	63.2	42.8
10	154.7	159.4	170.9
11	34.6	39.5	50.5
12	0.2	0.3	0.4
Oseltamivir	5.8 nM	5.6 nM	1.2 nM

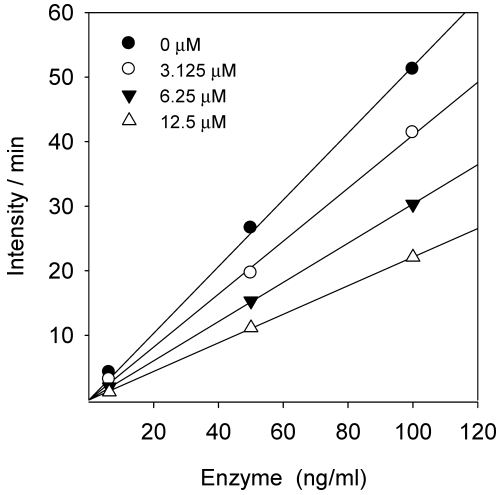

Fig. 2. Relationship of the Hydrolytic Activity of NA with Enzyme Concentrations at Different Concentrations of Compound **2**, Representatively

Chicago, IL, U.S.A.).

$$\% \text{ inhibition} = 100[1/(1+([I]/IC_{50}))]$$

$[I]$=concentration of inhibitor (μM);
IC_{50}=half-maximal inhibitory concentration (μM)

RESULTS AND DISCUSSION

In this study, we investigated the inhibitory effect and structure requirements of a series of homoisoflavonoids (3-benzylidene-4-chromanones) derived from the dried heartwood of *C. sappan*. Aside from sappanchalcone (**1**) and brazilin (**12**), the known constituents of NA inhibitors from *C. sappan*, we have isolated sappanone A (**2**), sappanone B (**3**), 3-deoxysappanone B (**4**), sappanol (**5**), episappanol (**6**), 4-*O*-methylsappanol (**7**), 3′-deoxy-4-*O*-methylsappanol (**8**), 4-*O*-methylepisappanol (**9**), 4-(7-hydroxy-2,2-dimetyl-9β*H*-1,3,5-trioxa-cyclopenta[α]naphtalen-3α-methyl)-benzen-1,2-diol (**10**), and 7,3′,4′-trihydroxy-3-benzyl-2*H*-chromene (**11**) (Fig. 1). The chemical structures of **2**–**11** were elucidated by their spectroscopic data.[20–26]

The biological activities of **1**–**12** were assessed against NAs on the surface of influenza viruses (A/PR/8/34 [H1N1], A/Hong Kong/8/68 [H3N2], and A/Chicken/Korea/MS96/96 [H9N2])[18,19] and confirmed by the positive control with oseltamivir (IC_{50}s=5.8 nM [H1N1], 5.6 nM [H3N2], and 1.2 nM [H9N2]). The biological data for compounds **1**–**12** has been shown in Table 1.

Of tested homoisoflavonoids **2**–**11**, compound **2** having an α,β-unsaturated carbonyl group in A-ring was the most effective inhibitor with an IC_{50} values of 0.7 μM for H1N1, 1.1 μM for H3N2, and 1.0 μM for H9N2, respectively. Compound **2** shows more potent inhibitions compared with these homoisoflavonoids obtained from *C. sappan*. On the other hand, 3-deoxysappanone B (**3**), which did not contain the α,β-unsaturated double bond, was more 100-fold less active (IC_{50}s=134.5, 134.6, 115.2 μM). Sappanone B (**4**) substituted with hydroxyl group at C-3 position was of similar potency with compound **3**. In addition, 2*H*-chromene homoisoflavonoid (**11**), which did not contain the carbonyl group in which is present in the corresponding homoisoflavonoid (**2**), showed 4-fold more effective (IC_{50}s=34.6, 39.5, 50.5 μM) than **3** and **4**. Taken together, it seems that an α,β-unsaturated carbonyl group increases the potency of the inhibitor. Similar trends can also be seen in unsaturated chalcone, sappanchalcone (**1**) and corresponding hydrogenated chalcone **1a** which were obtained using H_2 and Pd/C.

Sappanchalcone (**1**) inhibited NAs with IC_{50} values of 15.9, 18.1, and 17.0 μM, whereas the saturated chalcone **1a** exhibited 2-fold less inhibitory potencies, with IC_{50} values of 34.6, 43.2, and 32.9 μM, respectively. Thus, above these findings suggest that the α,β-unsaturated carbonyl group may play a pivotal role in NA inhibition, by interacting with the NA nucleophiles.

Next, non-carobonyl homoisoflavonoid derivatives (**5**–**10**) activities were significantly affected by *R*/*S* configuration of substituent at C-4 in A-ring. The IC_{50} values of these homoisoflavonoids (**5**–**10**) for NAs inhibition were determined to range between 42.8 and 204.4 μM (Table 1). Comparison of *R*-configuration **9** with *S*-configuration **5**–**8** revealed that the activities of *R*-configuration homoisoflavonoid (IC_{50}s=63.2 [H1N1 and H3N2] and 42.8 μM [H9N2]) was stronger than those of isomers (**5**; 197.1, 195.6, 167.7 μM, **6**; 204.4, 192.9, 192.0 μM, **7**; 94.5, 134.7, 99.6 μM, **8**; 192.2, 181.5, 175.2 μM, respectively). Homoisoflavonoid **10** protected with 2,2-dimethoxy propane also was shown low activity (>100 μM), as shown in Table 1.

All NA inhibitors manifested the same relationship between NA activity and concentration. The inhibition of NA by sappanone A (**2**) is illustrated in Fig. 2, representatively. Plots of the initial velocity *versus* NA concentrations in the presence of different concentrations of **2** gave a family of straight lines, all of which passed through the origin. Increasing the inhibitor concentration resulted in the lowering of the slope of the line, indicating that these compounds were reversible inhibitors. Subsequently, the selected viral NAs inhibitors were analyzed in further detail. To get the enzyme kinetic models, NAs inhibitors were examined by linear plots of $1/V$ (intensity/min) against $[I]$. Kinetic models analyzed the intersection at some distance above *x*-axis in Dixon plot, which indicate that inhibitor **2** is noncompetitive inhibitors of NAs, as shown

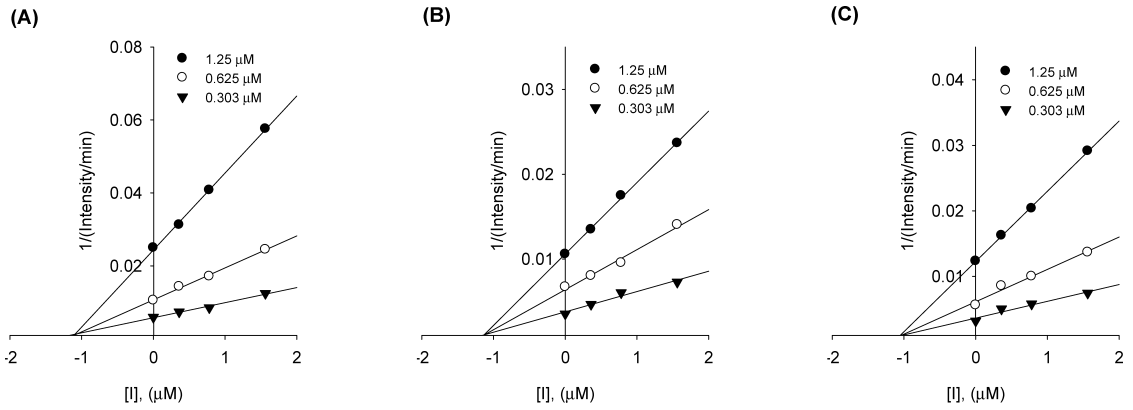

Fig. 3. Dixon Plots for the Inhibition of Hydrolase Activities of NA by Representative Compound **2**

For sappanone A (**2**) kinetic plots (A; H1N1, B; H3N2, and C; H9N2, respectively).

in Fig. 3, representatively.

In summary, we isolated twelve bioactive constituents **1**–**12** from the dried heartwood of *C. sappan* and evaluated their activities against three types of viral NAs to study structure–activity relationship (SAR) and to select lead compounds **2** and **12** with high potency against three viral NAs. On the basis of above result findings, an α,β-unsaturated carbonyl group in A-ring is critical factors for NA inhibition activity of homoisoflavonoid. We are hopeful that these preliminary data may be able to open new avenues for research targeted toward reducing the threat of influenza pandemic/epidemic.

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