Inhibition of HIV-1 Reverse Transcriptase and Protease by Phlorotannins from the Brown Alga *Ecklonia cava*

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The bioassay-directed isolation of a marine brown alga, *Ecklonia cava*, afforded four phlorotannin derivatives, eckol (1), 8,8′-bieckol (2), 8,4‴-dieckol (3), and phlorofucofuroeckol A (4). Among these compounds, 2 and 3 exhibited an inhibitory effect on human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) and protease. Specifically, they inhibited the RT more potently than the protease. The inhibitory activity of compound 2 (IC_{50}, 0.51 μM) against HIV-1 RT was comparable to that of nevirapine (IC_{50}, 0.28 μM), a reference compound. An enzyme kinetic assay showed that this compound inhibited the RNA-dependent DNA synthesis activity of HIV-1 RT noncompetitively against dUTP/dTTP with a K_i value of 0.78 μM. With respect to the homopolymeric template/primer, $(rA)_n(dT)_{15}$, 8,8′-bieckol (2) displayed an uncompetitive type of inhibition (K_i, 0.23 μM).

Key words *Ecklonia cava*; 8,8′-bieckol; 8,4‴-dieckol; HIV-1 reverse transcriptase; noncompetitive inhibition; protease

The reverse transcriptase (RT) of human immunodeficiency virus type-1 (HIV-1) converts the single-stranded (+) viral RNA genome into a double-stranded proviral DNA prior to its integration into the host genomic DNA. The RT is a multifunctional enzyme with three recognized enzymatic activities of RNA-dependent DNA polymerase (RDDP), DNA-dependent DNA polymerase (DDDP), and ribonuclease H activities. HIV-1 protease is an aspartic protease required for the proteolytic processing of the large *Gag* and *Gag–Pol* viral polyprotein precursors into the mature virion structural proteins, as well as the virion enzymes. These two enzymes, HIV-1 RT and protease, have key roles in HIV replication and inhibition of these enzymes along with HIV-1 integrase has been a major target of acquired immunodeficiency syndrome (AIDS) therapy.[1)]

In addition to the well-known nucleoside RT inhibitors (NRTIs), zidovudine (AZT), didanosine (ddC), zalcitabine (ddT), stavudine (d4T), lamivudine (3TC), and abacavir (ABC), non-nucleoside RT inhibitors (NNRTIs), such as nevirapine, delavirdine, and efavirenz have been formally approved to treat HIV infection. On the other hand, saquinavir, ritonavir, and indinavir are the three main HIV protease inhibitors available today. A number of inhibitors interacting with RT have been isolated from plants (baicalin, avarol, avarone, and psychotrine) and marine resources (illimaquinone, peyssonol, and KM043).[2—4)] In the case of HIV-1 protease, natural products, such as mangostin, ursolic, and maslinic acid, have been reported to show inhibitory activity against this enzyme.[5)] We previously reported the results of screening inhibitory activities of 47 types of Korean seaweed on HIV-1 RT and found that the EtOAc fraction of *Ecklonia cava* strongly inhibited the RDDP activity of this enzyme.[6)] In this study, we report the isolation of four phlorotannin compounds containing dibenzo[1,4]dioxin elements in common from *E. cava* and the kinetic study of HIV-1 RT by 8,8′-bieckol (**2**) that showed the most potent inhibitory effect on this enzyme.

MATERIALS AND METHODS

Materials The thalli of *E. cava* KJELLMAN were collected from the coasts of Korea including Sungsanpo, Wando, and Namhaedo from January 1999 to June 2000. After cleaning the surface of the thalli with water to remove visible epiphytes and dirt, samples were dried at 60 °C for 12 h in an oven and then ground in a coffee grinder. This seaweed was identified by Prof. S. M. Boo of the Department of Biology, Chungnam National University, Korea, and Prof. Y. S. Oh of the Department of Aquaculture, Gyeongsang National University, Korea. A voucher specimen (SSI-06) was deposited in the Herbarium of the Department of Biological Sciences, Sungkyunkwan University.

Extraction and Isolation The dried thalli (1 kg) of *E. cava* were extracted three times with 100% MeOH and evaporated *in vacuo*. The MeOH extract (180 g) was dissolved in water and partitioned with *n*-hexane. After the H_2O layer was further partitioned with ethyl acetate, the organic solvent fraction was concentrated *in vacuo* and divided into 10 fractions (f. 1—f. 10) on silica gel column chromatography (Merck, 230—400 mesh, 300 g) using $CHCl_3$–EtOAc–MeOH mixtures of increasing polarity (50 : 2 : 1 (f. 1), 25 : 5 : 1 (f. 2, f. 3), 10 : 5 : 1 (f. 4—f. 6), 5 : 5 : 1 (f. 7, f. 8), 100% MeOH (f. 9, f. 10); 1 l each). Compound **1** (13.6 mg) was purified from f. 4 by HPLC (YMC ODS, 10×250 mm, MeOH–H_2O, 30 : 70, t_R: 8.0 min). Compounds **2** (204.0 mg) and **3** (97.0 mg) were obtained by recrystallization of f. 5 in MeOH/H_2O and compound **4** (102.0 mg) was purified from f. 6 using reverse-phase silica gel column chromatography (MeOH).

HIV-1 Retroviral Transcriptase (RT) Assay Non-radioactive HIV-1 reverse transcriptase activities were evaluated with an HIV-1 RT nonradioactive assay kit (Roche Diagnostics GmbH, Germany) according to the method described previously.[6)] Briefly, 20 μl of the reaction mixture containing a homogenous template/primer hybrid, $(rA)_n(dT)_{15}$ and a

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triphosphate substrate, dUTP/dTTP, was added to the wells of a streptavidine-coated microtiter plate that contained 20 μl of test sample solution and 4 ng of the HIV-1 RT in 20 μl of lysis buffer. Final concentrations of the template/primer hybrid and triphosphate substrate (dUTP/dTTP) were 750 $mA_{260\,nm}$/ml and 10 μM, respectively. The reaction was carried out at 37 °C for 1 h and followed by the addition of each 200 μl solution of anti-digoxigenin-peroxide, and ABTS [2,2′-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt] substrate for coloring reaction. The absorbance of each well was recorded at 405 nm with the reference wavelength at 490 nm. Nevirapine (Viramune, Boehringer-Ingelheim Pharma KG, Germany) was used as a reference compound.

Enzyme kinetic assay was carried out as outlined above except for the concentration of enzyme solution (1 ng of the HIV-1 RT), incubation time (30, 52, 80, 105, 130 min), and various concentrations of either the template/primer (1500, 750, 187.5 $mA_{260\,nm}$/ml) or the triphosphate substrate (20, 15, 10, 5, 2.5 μM) in the presence of the inhibitor, 8,8′-bieckol (**2**).

HIV-1 Protease Assay The assay was performed as described by Ma *et al.*[7] with slight modification. One microliter of a dimethyl sulfoxide solution of test compound was mixed with 10.5 μl of the substrate solution (His-Lys-Ala-Arg-Val-Leu-(*p*-NO_2)-Phe-Glu-Ala-Nle-Ser-NH_2, 0.1 mg/ml in HIV-1 protease assay buffer, Bachem AG, Switzerland) and then 0.5 μl of the recombinant protease solution (0.3 mg/ml, Bachem AG) was added to the mixture. After incubation at 37 °C for 15 min, the reaction was stopped by the addition of 1.2 μl of 10% trifluoroacetic acid (TFA) and diluted with 20 μl of water. The hydrolysate and the remaining substrate were quantitatively analyzed by HPLC under the following conditions: column, Inertsil ODS-3 (4.6×150 mm, GL Sciences Inc., Japan); elution, a linear gradient of CH_3CN (15→40%) in 0.1% TFA; injection volume, 20 μl; flow rate, 1.0 ml/min; and detection, 280 nm. The hydrolysate and substrate were eluted at 8.61 and 10.84 min, respectively. The inhibitory activity of the compound in the HIV-1 protease reaction was calculated as follows: % inhibition= $100\times(A_{control}-A_{sample})/(A_{control})$, where A is a relative peak area of the hydrolysate. Acetyl pepstatin (Bachem AG) was used as a positive control in this assay.

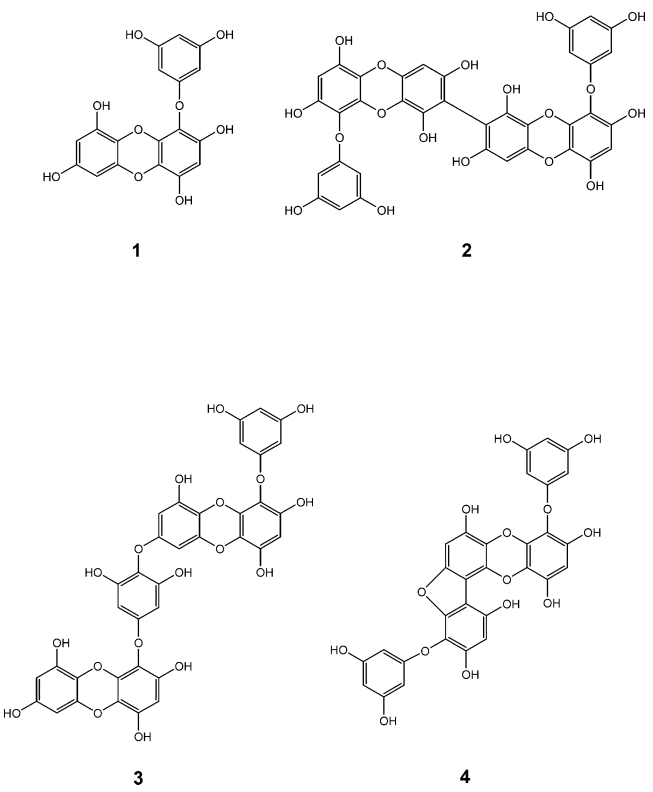

Fig. 1. Structures of Compounds **1**—**4** from *E. cava*

Table 1. Inhibitory Effects of Compounds **1**—**4** on HIV-1 Reverse Transcriptase (RT) and Protease[a]

Compound	IC_{50} (μM)[b]	
	RT	Protease
Eckol (**1**)	>100	>100
8,8′-Bieckol (**2**)	0.51±0.14	81.5±9.6
8,4‴-Dieckol (**3**)	5.31±2.04	36.9±5.4
Phlorofucofuroeckol A (**4**)	>100	>100
Nevirapine	0.28±0.02	NT[c]
Acetyl pepstatin	NT[c]	0.34±0.11

a) Data are expressed as mean±S.E. of three independent experiments. *b*) Inhibitor concentration required to reduce by 50% the HIV-1 RT and protease activity, respectively. *c*) Not tested.

RESULTS

Isolation of Phlorotannins from *E. cava* In our screening system, the EtOAc fraction of *E. cava* was found to inhibit the activity of HIV-1 RT (≥80% inhibition at the concentration of 100 μg/ml). Four phloroglucinol derivatives, eckol (**1**), 8,8′-bieckol (**2**), 8,4‴-dieckol (**3**), and phlorofucofuroeckol A (**4**) were isolated from this fraction using a bioassay-directed fractionation and isolation technique (Fig. 1). The structures of these compounds were determined by comparison of UV, MS, and ^{1}H- and ^{13}C-NMR data with reported values.[8—11]

Inhibitory Effects on HIV-1 RT and Protease Inhibitory activities of these compounds against HIV-1 RT and protease are shown in Table 1. 8,8′-Bieckol (**2**) and 8,4‴-dieckol (**3**) strongly inhibited HIV-1 RT activity, whereas eckol (**1**) and phlorofucofuroeckol A (**4**) did not exhibit inhibitory activity against this enzyme. Compounds **2** and **3** showed moderate inhibitory activity against HIV-1 protease.

Kinetic Study of HIV-1 RT Inhibition by 8,8′-Bieckol (2) The inhibitory mechanism against HIV-1 RT was evaluated in the most potent compound, 8,8′-bieckol (**2**), using a homopolymeric template/primer under steady-state condition. As shown in Fig. 2, compound **2** inhibited the RDDP activity of HIV-1 RT noncompetitively with respect to the triphosphate substrate, dUTP/dTTP, and uncompetitively with respect to the template/primer, $(rA)_n(dT)_{15}$. The K_i values of this compound were determined to be 0.78±0.20 μM against the dUTP/dTTP substrate based on Michaelis-Menten kinetics and 0.23±0.05 μM against the $(rA)_n(dT)_{15}$ substrate in a Dixon plot.

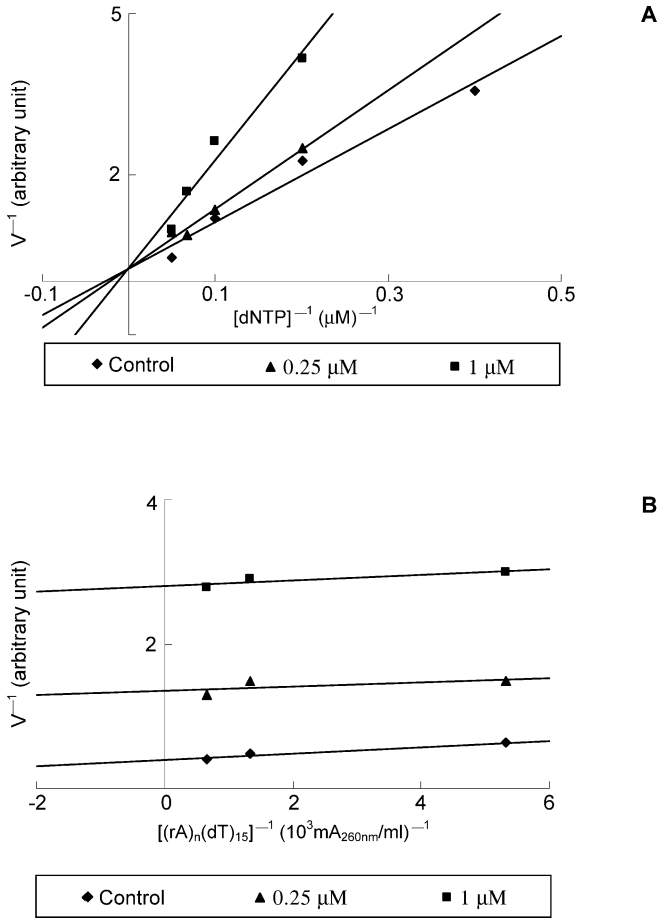

Fig. 2. Kinetic Study of HIV-1 RT Inhibition by 8,8′-Bieckol (**2**)

A, Double reciprocal plot of the inhibition of HIV-1 RT by compound **2** using a saturated ribosomal RNA template with oligo DNA-primer [a homogenous template/primer hybrid, $(rA)_n(dT)_{15}$] in the presence of various dUTP/dTTP concentrations. B, Double reciprocal plot of the inhibition of HIV-1 RT by compound **2** using various concentrations of the template/primer in the presence of saturated dUTP/dTTP concentrations. Each RT activity was measured in triplicate and standard deviations were below 10.4% in all cases. Similar results were observed in two other experiments.

DISCUSSION

E. cava is a brown alga (Laminariaceae) that is abundant in the subtidal regions of Jeju Island, Korea. Eisenine, biotin, and laminine are known components of this species[12] and eckol (**1**), 8,8′-bieckol (**2**), and phlorofucofuroeckol A (**4**) have been isolated from *Ecklonia kurome* OKAMURA as antiplasmin inhibitors.[8—10] Recently, it has been reported that phlorotannins from *Ecklonia* species are radical scavengers.[13,14] In this report, four phlorotannin compounds (**1**—**4**) containing the dibenzo[1,4]dioxin element as the core structure were isolated from *E. cava* and their inhibitory activities against HIV-1 RT and protease were evaluated.

8,8′-Bieckol (**2**) and 8,4‴-dieckol (**3**) showed an inhibitory effect on HIV-1 RT and protease. The inhibitory activity against HIV-1 RT of compound **2** with a biaryl linkage (IC_{50}, 0.51 μM) was 10-fold greater than that of compound **3** with a diphenyl ether linkage (IC_{50}, 5.3 μM), although these two compounds are dimers of eckol (**1**). This difference in the inhibition potential appears to be due to the steric hindrance of the hydroxyl and aryl groups near the biaryl linkage of compound **2**. Although compound **2** showed an inhibitory effect on both HIV-1 RT and protease, 8,8′-bieckol (**2**) selectively inhibited RT over protease. Moreover, the inhibitory effect of compound **2** (IC_{50}, 0.51 μM) was comparable to that of a NNRTI, nevirapine (IC_{50}, 0.28 μM). To our knowledge, it has not been reported that a tannin compound isolated from natural sources has HIV-1 RT inhibitory activity comparable to that of this positive control.[15,16]

Eckol (**1**) and phlorofucofuroeckol A (**4**) did not show anti-HIV-1 RT activity, although they were isolated from the RT inhibitory fractions (≥80% inhibition at the concentration of 100 μg/ml), f. 4 and f. 6, respectively. These results suggest that the inhibitory activities may be due to the potent compounds **2** and **3** present as minor components in these fractions.

Kinetic study of HIV-1 RT with 8,8′-bieckol (**2**) in a non-radioactive HIV-1 RT assay showed that this compound inhibited the RDDP activity of HIV-1 RT noncompetitively with respect to dUTP/dTTP (Fig. 2A). This kinetic pattern of inhibition is consistent with those of other NNRTIs, which inhibited RT activity noncompetitively with respect to dGTP and dTTP in the radioactive HIV-1 RT assay.[17—19] Meanwhile, 8,8′-bieckol (**2**) displayed an uncompetitive type of inhibition with respect to a homopolymeric template/primer, which indicates that this compound binds to HIV-1 RT only after the template/primer initially binds to the enzyme (Fig. 2B). This result agrees with previously reported results of NNRTIs, such as trovirdine, 9-CI-TIBO, and HEPT.[19,20]

Although the antiviral activities of Korean seaweed have been studied previously,[21,22] isolation of the responsible compounds has not been reported. Our findings suggest that 8,8′-bieckol might offer a structural lead in the discovery of new nonnucleoside HIV-1 RT inhibitors.

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