JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

HIV-1 Integrase and Neuraminidase Inhibitors from *Alpinia zerumbet*†

Atul Upadhyay,[§] Jamnian Chompoo,[§] Wataru Kishimoto,[⊥] Tadahirio Makise,[#] and Shinkichi Tawata*[,⊥]

[§]Department of Biochemistry and Applied Bioscience, The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan

[#]Makise Clinic, 750 Minamiuehara, Nakagusuku, Okinawa 901-2424, Japan

[⊥]Faculty of Agriculture, University of the Ryukyus, 1 Senbaru, Nishihara-cho, Okinawa 903-0213, Japan

ABSTRACT: AIDS and influenza are viral pandemics and remain one of the leading causes of human deaths worldwide. The increasing resistance of these diseases to synthetic drugs demands the search for novel compounds from plant-based sources. In this regard, the leaves and rhizomes of *Alpinia zerumbet*, a traditionally important economic plant in Okinawa, were investigated for activity against HIV-1 integrase (IN) and neuraminidase (NA). The aqueous extracts of leaves and rhizomes had IN inhibitory activity with IC_{50} values of 30 and 188 μg/mL, whereas against NA they showed 50% inhibition at concentrations of 43 and 57 μg/mL, respectively. 5,6-Dehydrokawain (DK), dihydro-5,6-dehydrokawain (DDK), and 8(17),12-labdadiene-15,16-dial (labdadiene) were isolated from the rhizomes and were tested for enzyme inhibitions. DK and DDK strongly inhibited IN with IC_{50} of 4.4 and 3.6 μg/mL, respectively. Against NA, DK, DDK, and labdadiene exhibited mixed type of inhibition with respective IC_{50} values of 25.5, 24.6, and 36.6 μM and K_i values ranging from 0.3 to 2.8 μM. It was found that DDK is a slow and time-dependent reversible inhibitor of NA, probably with a methoxy group as its functionally active site. These results suggest that alpinia could be used as a source of bioactive compounds against IN and NA and that DK and DDK may have possibilities in the design of drugs against these viral diseases.

KEYWORDS: *Alpinia zerumbet*, 5,6-dehydrokawain, dihydro-5,6-dehydrokawain, 8(17),12-labdadiene-15,16-dial, anti-HIV-1 integrase, antineuraminidase

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) represents one of the most important modern epidemics, with over 40 million people infected worldwide. The replication of HIV requires three enzymes: protease, integrase (IN), and reverses transcriptase. Insertion of a viral genome inside the infected cell is mediated through the encoded enzyme IN, which has been a rational target for treating HIV inhibition.[1] Synthetic IN inhibitors such as raltegravir (also known as isentress or MK-0518) are being used in reducing HIV load.[2] On the other hand, the impingement of influenza on human health is undeniably escalating, its impact being more serious during the winter season.[3] Human cases of avian influenza and, more recently, the outbreak of the aggressive porcine A/H1N1 strain in 2009 have heightened awareness of the threat of pandemic. The disease is associated with a RNA virus that contains hemagglutinin and neuraminidase (NA) as surface antigens. NA is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles.[4] Specifically, NA cleaves the α-ketosidic bond that links a terminal neuraminic acid residue to the adjacent oligosaccharide moiety. NA is therefore essential for the movement of the virus to and from sites of infection in the respiratory tract.[5,6] With the ever-present threat of a pandemic derived from the HIV and influenza viruses and the emergence of strains resistant to synthetic drugs, the importance of the search for novel compounds from plant-based source intensifies.

Alpinia [*Alpinia zerumbet* (family Zingiberaceae)] is a perennial ginger growing widely in the subtropics and tropics. It is used in folk medicine for its anti-inflammatory, bacteriostatic, and fungistatic properties.[7] The essential oil extracted from its leaves possessed both relaxant and antispasmodic actions in rat ileum.[8] Early reports have shown the α-pyrones, dihydro-5,6-dehydrokawain (DDK), and 5,6-dehydrokawain (DK), are major compounds in alpinia leaves, and they have shown plant growth inhibition against lettuce seeds,[9] insecticidal activity against *Coptotermes formosanus*, and antifungal activity against *Pythium* sp. and *Corticium rolfsii*.[10] The aqueous extract of its leaves has demonstrated hypotensive activity,[11] mainly due to flavonoids and kava pyrones.[12] Furthermore, DK and DDK are reported to inhibit the aggregation of ATP release from rabbit platelets.[13] DK and DDK are described to have antiulcerogenic and antithrombotic activities.[12] The inhibitory properties of DK on human platelet aggregation, anti-inflammatory, and cancer chemopreventive therapeutic properties are reported.[14] Labdadiene was traditionally used as a medicine against inflammatory diseases.[15] The cardiovascular effects induced by labdadiene were evaluated in male Wistar rats.[16] Moreover, labdadiene has also been reported to inhibit lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation.[17] Our laboratory has

† Part of the Symposium on Pesticide Toxicology in Honor of Professor John Casida.

Special Issue: Casida Symposium

Received: May 11, 2010
Accepted: December 27, 2010
Published: February 09, 2011

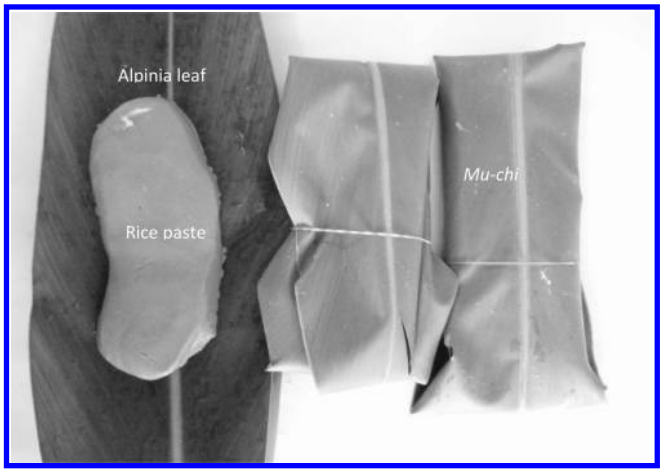

Figure 1. Mu-chi, a traditional food of Okinawa. A rice ball or paste is wrapped in alpinia leaves and served after steaming.

reported DDK and phenolic compounds and their antioxidant activities in the leaves and rhizomes of the plant.[18]

In Okinawa, alpinia leaves have been used to prepare a traditional food, *mu-chi* (see Figure 1), and it is common folklore that it prevents the common cold.[19] In this study, we primarily focused on investigating the IN and NA inhibitory activities of aqueous extracts of alpinia leaves and rhizomes and three compounds isolated from rhizomes. To the best of our knowledge this is the first report on IN and NA inhibitory activities of alpinia and the associated secondary metabolites. Furthermore, in this study, we also exhibited the probable mechanism of NA inhibition by DDK.

MATERIALS AND METHODS

Preparation of Plant Extracts and Isolation of Compounds. The leaves and rhizomes of alpinia were collected from the University of the Ryukyus campus, Okinawa. Twenty grams of fresh leaves or rhizomes was separately boiled in 1 L of water for 30 min, and the cooled extract was filtered and dried under vacuum at 40 °C. All of the residues were weighed and dissolved in 50% DMSO to make a stock solution of 1 mg/mL before the IN and NA inhibition of crude extracts was tested. To isolate the active compounds, we used the rhizomes of alpinia. Our laboratory has been working with two pyrone compounds[10,18−20] (see Figure 2), and thus we chose first to investigate the anti-IN and -NA properties of these compounds. For the isolation of these compounds, 2 kg of fresh alpinia rhizomes was boiled in 10 L of water for 20 min. The cooled extract was filtered and reduced to 1 L under vacuum at 40 °C. Further extraction with hexane (500 mL × 3) was done, and the hexane fraction was evaporated to complete dryness under vacuum. The dried extract was boiled in water and filtered hot. The residue obtained was purified by preparative HPLC to obtain DK using a TSK gel ODS-100Z column (Tosoh Corp., Japan) (15 × 0.46 cm i.d.; 5 μm particle size) monitored continuously at 280 nm. The mobile phase consisted of 0.1% acetic acid and MeOH, which was increased from 50 to 100% in 20 min at a flow rate of 0.8 mL/min. For DDK, the filtrate was crystallized at 4 °C, which was further purified using preparative HPLC as above. Compounds were identified using NMR and GC-MS. The amounts of the purified compounds were weighed and expressed as milligrams per 100 g of fresh rhizomes. DDK and DK were determined to be 24.1 and 18.8 mg/100 g fresh rhizomes. The ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were

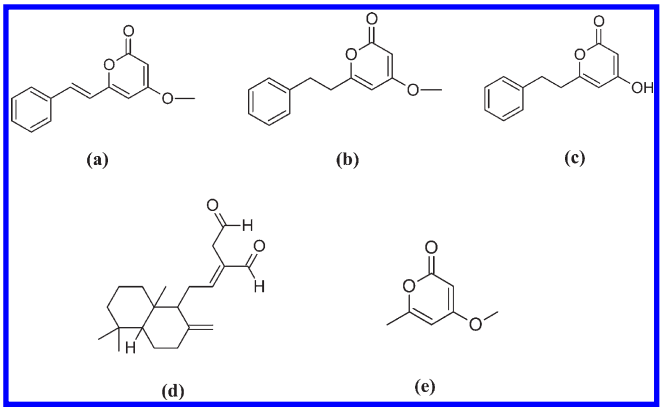

Figure 2. Chemical structures of (a) DK, (b) DDK, (c) DDK-OH, (d) labdadiene, and (e) 4MHP.

recorded on a JEOL JNM-ECA600 (JEOL, Japan) in $CDCl_3$. Chemical shifts are expressed in parts per million (δ) relative to TMS. 2D NMR experiments (H, C-COSY, HMQC, HMBC) were obtained using standard pulse sequences. DDK: electron ionization mass spectrometry (EIMS), m/z 230 $[M]^+$ (30), 202 (8), 125 (30), 111 (28), 91 (100), 69 (12); ^{1}H ($CDCl_3$), δ 2.73−2.76 (m, 2H, CH_2), 2.96−2.97 (m, 2H, CH_2), 3.77 (s, 3H, CH_3), 5.42 (s, 1H, *CH*), 5.72 (s, 1H, *CH*), 7.18−7.29 (m, 5H, aromatic); ^{13}C NMR ($CDCl_3$), δ 32.80 (CH_2), 35.42 (CH_2), 55.80 (OCH_3), 87.68 (*CH*), 100.25 (*CH*), 126.41, 128.57, 128.57, 128.27, 128.27, 139.82 (aromatic), 164.32 (*CH*), 164.93 (*CH*), 170.0 (*C*).[21] DK was characterized by the following data: EIMS, m/z 228 $[M]^+$ (60), 200 (20), 157 (35), 129 (20), 69 (20), 44 (35), 40 (100); ^{1}H ($CDCl_3$), δ 3.79 (s, 3H, CH_3), 5.51 (d, 1H, *CH*), 5.97 (s, 1H, *CH*), 6.81 (d, 1H, *CH*), 7.31 (d, 1H, *CH*), 7.32 (m, 5H, aromatic); ^{13}C NMR ($CDCl_3$), δ 163.28 (*C*), 88.65 (*CH*), 171.21 (*C*), 100.95 (*CH*), 160.3 (*C*), 118.53 (*CH*), 135.36 (*C*), 131.88, 128.27, 129.75, 129.27, 127.98 (aromatic), 56.24 (OCH_3).[22] Labdadiene was isolated by another group in our laboratory from the rhizomes of alpinia (unpublished results). DDK-OH was synthesized by stirring DDK in concentrated HCl for 18 h followed by overnight crystallization at 8 °C.[10]

HIV-1 Integrase Assays. IN inhibition was determined using a multiplate integration assay.[23] Fifty microliters of biotinylated-LTR donor DNA was added to a streptavidin-coated 96-well microtiter plate and incubated for 60 min at room temperature. After discarding the solution, IN buffer (12 μL), target DNA (5 pmol), sterilized water (32 μL), inhibitors (6 μL), and IN (180 ng) were added to wells and incubated at 37 °C for 80 min. After the completion of reaction, unbound DNA was removed by washing with PBS containing 0.5% Tween 20 and PBS alone. The relative activity of bound DNA was determined using an alkaline phosphatase labeled anti-digoxigenin antibody (50 mU) after incubation at 37 °C for 1 h. The resulting yellow color was measured using a microplate reader at 405 nm. A control contained enzyme without sample, whereas the blank did not contain enzyme.

The percent inhibition against IN was calculated as

$$\% \text{ inhibition} = [OD_{control} - OD_{sample}]/OD_{control} \times 100$$

where OD = absorbance detected from each well.

NA (*Clostridium perfringens*) Inhibition Assay. The enzyme assay was performed as reported with slight modifications.[24] Briefly, 4-methylumbellifery-l-α-D-*N*-acetylneuramic acid sodium salt hydrate (Sigma, M8639), 0.1 mM in 50 mM sodium acetate buffer (pH 5.0), was used as substrate. NA (Sigma, N2876), 0.1 U/mL in acetate buffer, was used as enzyme source. All of the inhibitors were dissolved in MeOH and diluted to the appropriate concentration in acetate buffer. Fifty microliters of enzyme was added to 20 μL of inhibitor mixed with 80 μL of

Table 1. IN and NA Inhibitory Activities of Different Extracts and Isolated Compounds[a]

	IN inhibition	NA inhibition		
test sample	IC_{50}	IC_{50}	type of inhibition	K_i
leaf	30 ± 1 μg/mL	43 ± 1 μg/mL	nt	nt
rhizomes	188 ± 2 μg/mL	57 ± 1 μg/mL	nt	nt
DK	4.4 ± 0.5 μg/mL a	25.5 ± 0.7 μM a	mixed	0.3 μM
DDK	3.6 ± 0.9 μg/mL a	24.6 ± 0.4 μM a	mixed	2.8 μM
labdadiene	nt	36.6 ± 1 μM b	mixed	0.6 μM
DDK-OH	nt	525.6 ± 11.3 μM	nt	nt
4MHP	nt	822.9 ± 7.2 μM	nt	nt
suramin	2.3 ± 0.7 μg/mL a	nt	nt	nt
quercetin	nt	34.7 ± 0.9 μM b	nt	nt

[a] Different letters in the same column indicate the existence of significant difference (Tukey test). nt, not tested.

acetate buffer in a microplate. Reaction was started by adding 50 μL of substrate, and fluorescence was measured using a GENIOS fluorescence meter, Wako, Japan. The excitation wavelength was set at 360 nm, and the emission wavelength was set at 450 nm. For kinetic studies, we used a time-driven protocol with initial velocity recorded over a range of substrate concentrations for different inhibitor concentrations (0, 10, 15, and 20 μM). Dixon plots were obtained by plotting the slopes of the obtained line (K_m/V_m) against substrate concentrations. For time-dependent studies, we obtained progress curves for 600 s at several preincubation times using 25 μM DDK, and the slopes of lines obtained were plotted against preincubation time. To obtain the effect of enzyme concentrations, we used different enzyme concentrations over a range of inhibitor concentrations. All of the data were analyzed using Microsoft Excel Office, 2007.

The inhibition was calculated using

$$\% \text{ inhibition} = [1-(S - S_0)/(C - C_0)] \times 100$$

where S and C represent relative fluorescence units (RFU) for sample and control after reaction time and S_0 and C_0 are RFU at zero time.

Statistical Treatment. All of the experiments were conducted in triplicates and repeated twice. The data represent the mean ± the standard deviation (SD) of six results. The IC_{50} value was determined graphically as the concentration of each sample required to give 50% inhibition activity. For kinetic studies of NA inhibition, all calculations were performed in Excel, Microsoft Office 2007. For significance analysis, the data were analyzed by one-way ANOVA and the means were separated using Tukey's HSD range test at $p = 0.01$. All statistical analyses were performed using SPSS version 16.0 for Windows Vista.

RESULTS

IN Inhibition Assays. The IN inhibitory activities of different extracts and the isolated compounds are shown in Table 1. It was found that the aqueous extract of the leaves of alpinia had stronger activity than the rhizomes. When inhibition by the isolated compounds was carried out, IC_{50} values of DK and DDK against IN were 4.4 ± 0.5 and 3.6 ± 0.9 μg/mL, respectively. Suramin had an IC_{50} of 2.3 ± 0.7 μg/mL under the stated conditions.

NA Inhibition Activities. The water extracts of alpinia leaves and rhizomes showed considerable NA activity, with IC_{50} values of 42.7 ± 1.2 and 57.1 ± 1.1 μg/mL, respectively. In the case of NA inhibition, we also used labdadiene isolated from the rhizomes of alpinia. The IC_{50} values of the extract and the isolated compounds are shown in Table 1. When the inhibition by isolated compounds was checked, DDK (IC_{50} = 24.6 ± 0.4 μM) and DK (IC_{50} = 25.5 ± 0.7) both had more potent activity than the positive control quercetin[25] with an IC_{50} of 34.7 ± 0.9 μM under these conditions (see Table 1). Labdadiene (IC_{50} = 36.6 ± 1.0) had activity similar to that of quercetin. The kinetic studies of individual compounds showed mixed type of inhibition (see Figure 3) with estimated K_i values ranging from 0.2 to 2.8 μM.

DISCUSSION

The present study examines the inhibitory properties of aqueous extract of leaves and rhizomes of alpinia against IN and NA. DK, DDK, and labdadiene were isolated from the rhizomes. To determine the inhibitory activity against IN, we carried out strand transfer inhibition assay using a microtiter plate method. Strand transfer is the second step of the integration reaction, which corresponds to the ligation of the viral 3′-OH cDNA ends to the 5′-DNA phosphate of an acceptor DNA.[1] In our result we found the leaf aqueous extract had higher activity than the rhizome extract. This was also true with NA inhibition (Table 1). The leaves and rhizomes of alpinia have been a source of a wide variety of bioactive constituents. The content of phenolics and flavonoids in the leaves and rhizomes of alpinia have been extensively investigated.[9−12,19] Our previous work reported the presence of phenolic compounds in the leaves and rhizomes of alpinia.[18] These groups of compounds have always been a promising class of molecules against a variety of diseases. Therefore, we have supposed that IN or NA inhibitory activities of alpinia leaves and rhizomes may be attributed to the phenolic compounds. Besides, several compounds present in other species of alpinia have been reported to have IN or NA inhibitory properties.[26−28] Furthermore, isolation and identification of specific compounds active against IN and/or NA from alpinia are being undertaken in our laboratory. Hence, once it was confirmed that the aqueous extract of the leaves and rhizomes of alpinia had considerable activity, we explored the secondary metabolites present in the plant.

Two compounds, DK and DDK, were tested against IN activity. Both of these compounds had activities similar to that of suramin ($p = 0.01$), which is widely used as a positive control against IN inhibition assays in vitro.[23] Our results indicated that both DK and DDK could be used as possible candidates for IN inhibition (Table 1). Furthermore, the simple structures and low molecular weights of DK and DDK would certainly add to their merits over complex synthetic inhibitors. Besides, we had previously shown that DK could be metabolized to hispidin in vitro using CYP2C9.[29] Because hispidin has been shown to have IN inhibitory activity,[10] DK seems to act as an anti-IN in two forms, first as DK itself and second after it has been catalyzed to hispidin by CYP2C9. The mechanism of IN inhibition by DK and DDK is still unclear, whereas the activity of labdadiene against IN is yet to be determined.

The IC_{50} values of the investigated compounds against NA indicate that DK and DDK have significantly better inhibition properties, whereas labdadiene had similar activity against NA when compared to quercetin (Table 1). Our results therefore

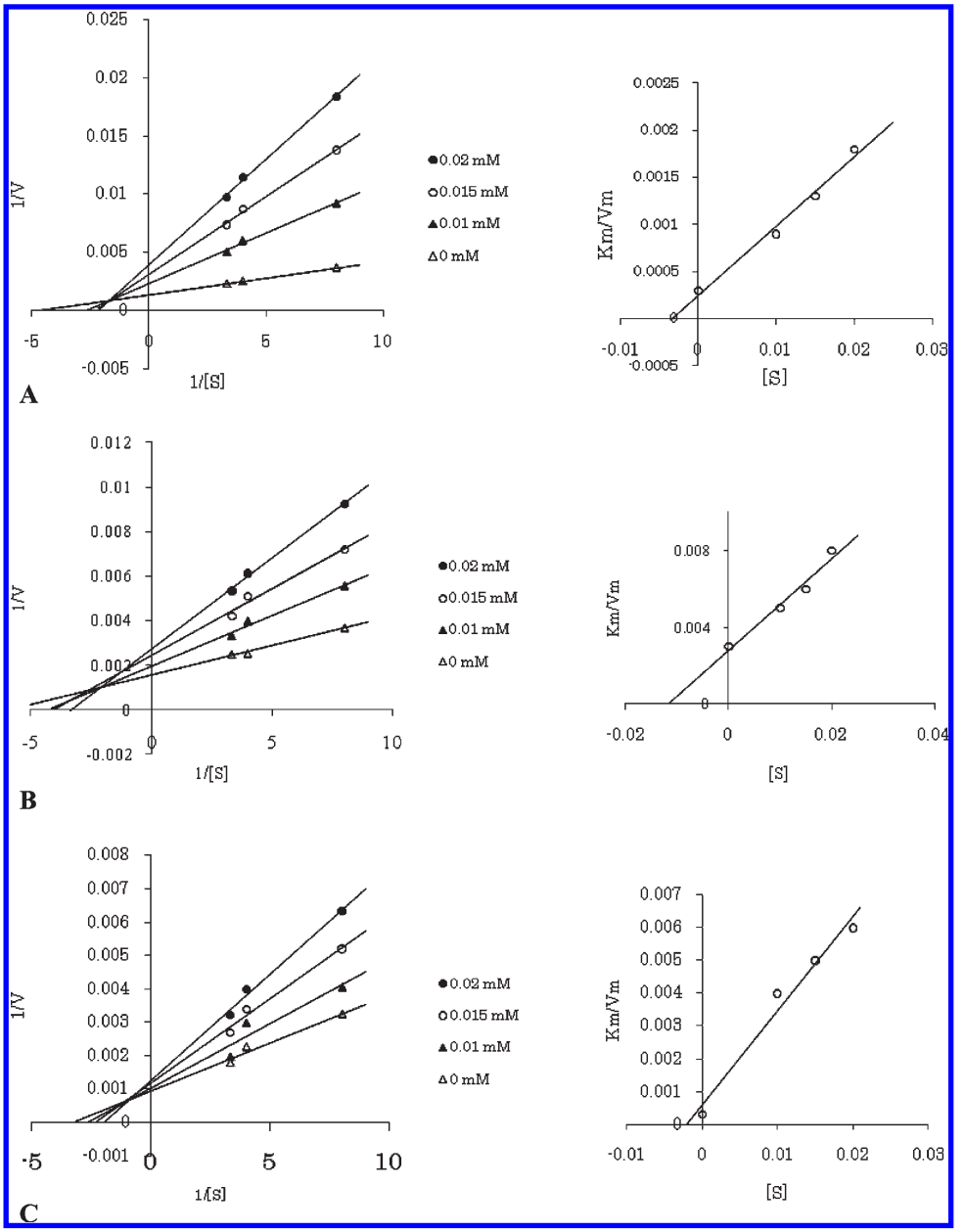

Figure 3. Effect of DK (A), DDK (B), and labdadiene (C) on neuraminidase inhibition. (Left) Lineweaver−Burk plot in the presence of compounds at concentrations of 0, 10, 15, and 20 μM. Neuraminidase inhibition was assayed as described in the text. (Right) Secondary plot of Lineweaver−Burk plot. The slopes were plotted against respective compound concentrations. The intercept on the *x*-axis gives an estimate of K_i.

suggest that DK, DDK, and labdadiene could be used effectively against NA.

The role of pyrone in inhibiting IN and NA is reported. Four naphtha-γ-pyrones belonging to the chaetochromin and ustilaginoidin family were identified as IN inhibitors.[30] On the other hand, all compounds containing pyrone rings do not show inhibitory properties. A structure−activity relationship study of 32 different flavones with γ-pyrone showed that the activity depends on the charge of the γ-oxygen atom of the pyrone ring.[31] However, in our study we used DK and DDK, which are α-pyrone compounds. Tipranavir, an α-pyrone compounds, has been shown to have potent HIV protease inhibition[32] and is a drug approved by the U.S. FDA. Although yet to be confirmed, this information might suggest that the IN inhibition of DK and DDK may be attributed to the presence of the α-pyrone group.

Furthermore, in the case of NA inhibition by α-pyrone, Grienke et al. have reported that of four diarylheptanoids isolated from *A. katsumadai*, the most potent NA inhibitory compound had an α-pyrone moiety along with an additional phenyl group.[27] However, the authors have not discussed whether the inhibition was due to the pyrone moiety or some other functional groups. To investigate the active functional group of DDK, we examined NA inhibition by the α-pyrone compound 4-methoxy-6-methyl-2*H*-pyran-2-one (4MHP), a moiety present in DDK. Interestingly, the activity of 4MHP against NA was very low, with an IC_{50} = 822.9 ± 7.2 μM. This made us think that, for DDK, the pyrone group may not have an effective role in inhibiting NA. Our next step was to determine whether the ethylbenzene group or the methoxy group of DDK is active against NA. For this, we synthesized a compound, DDK-OH (see Figure 2), and exam-

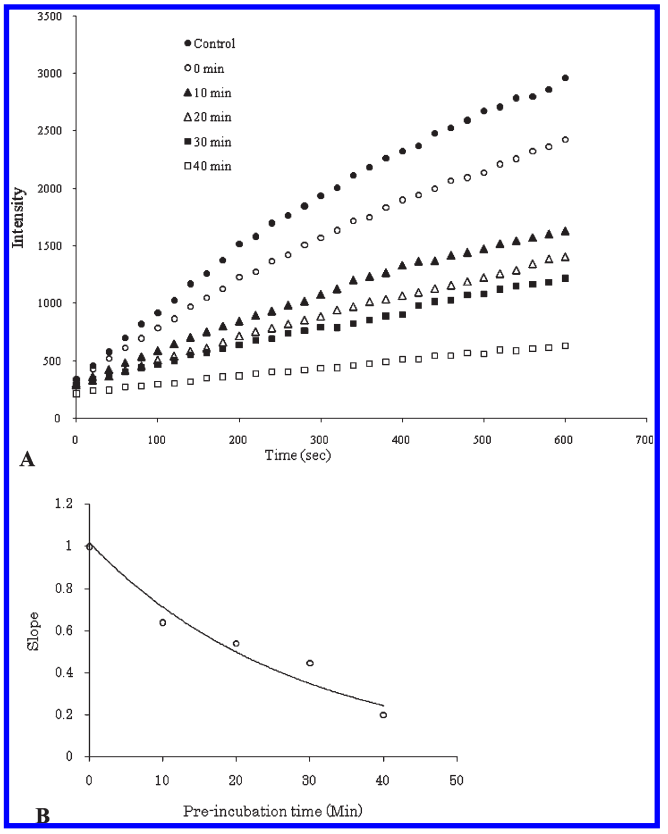

Figure 4. Effect of preincubation time on hydrolysis of substrate by NA: (A) time-dependent inhibition of NA in the presence of 25 μM DDK; (B) decrease in slopes of the lines of panel A as a function of time.

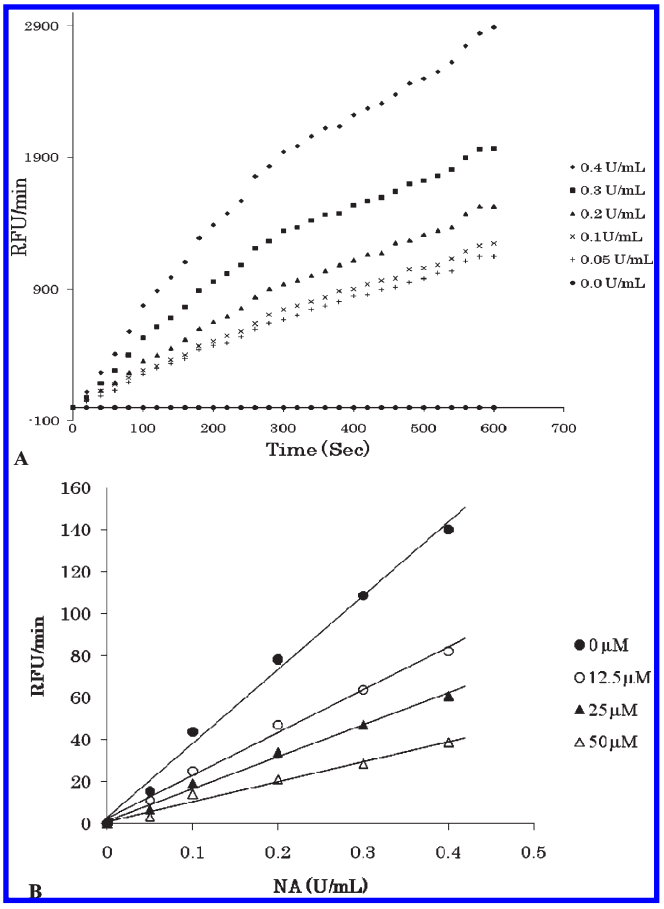

Figure 5. Effect of enzyme concentration on NA inhibition: (A) typical plot of residual activity of NA at various concentrations (0−0.4 U/mL) in the presence of DDK at 25 μM; (B) hydrolytic activity of NA as a function of enzyme concentration at different concentrations of DDK.

ined its activity against NA. The IC_{50} of DDK-OH (525.6 ± 11.3 μM) was better than that of 4MHP but was >100-fold weaker than DDK. These two results suggest that the active functional group in DDK is probably the methoxy group present in the C-5. Hence, it is likely that for DDK α-pyrone has very less functionality in inhibiting NA.

The plausible explanations of inhibition of NA by labdadiene are still under investigation. However, it is noteworthy that this compound contains double aldehyde groups in C-15 and 16 and that the commercial influenza drug Tamiflu also has an aldehyde group. Researchers have tried to develop active compounds against NA by adding various aldehyde groups to the carboxylate and acetomido moiety of Tamiflu to increase the inhibitory properties.[33]

The kinetics studies of individual compounds showed mixed type of inhibition (see Figure 3). These types of inhibition are quite common with natural compounds. The predominant inhibition mode shown by naturally occurring NA inhibitors is noncompetitive. However, the compounds isolated from alpinia exhibited a mixed type of inhibition, thereby indicating a different class of compounds identified against NA. The estimated K_i values for DK, DDK, and labdadiene are 0.2, 2.8, and 0.6, respectively (Table 1). The low K_i of DK along with its low IC_{50} value certainly makes it a potent compound against NA.

We further investigated the inhibitory mechanism of DDK at its IC_{50} concentration. We explored the effect of preincubation time on the inhibition of the hydrolysis of neuramic acid. Because the decrease in residual activity was observed with increasing preincubation time, DDK emerged as a slow-binding inhibitor at low concentrations (see Figure 4A). Furthermore, increasing preincubation time of DDK also led to a decrease in the slope, thereby indicating reduction in both initial and steady state velocity (see Figure 4B). This result along with low K_i indicates that DDK is a slow binder to NA. DDK therefore is more like the drug Tamiflu, which is also a slow and time-dependent inhibtior of NA.[34] Moreover, when the effect of enzyme concentration on the inhibition was probed, it was found that the residual activity of NA increased with the enzyme concentration at fixed substrate amount (see Figure 5A). Plots of residual enzyme activity versus enzyme concentration at different concentrations of DDK gave a family of straight lines passing through the origin, indicating that DDK is a reversible inhibitor (see Figure 5B). Hence, we may say that in our study DDK acted as a time-dependent, slow, reversible inhibitor of NA.

To sum up, we found that the leaves and rhizomes of alpinia had IN and NA inhibitory activities. We showed that compounds isolated from alpinia had significant properties against these enzymes and discussed the mechanism of inhibition of NA by DDK. Our results indicate that DK and DDK could be used as sources of IN and NA inhibitors; however, further research is necessary to use them as lead candidates in drug design.

AUTHOR INFORMATION

Corresponding Author

*E-mail: b986097@agr.u-ryukyu.ac.jp.

■ ACKNOWLEDGMENT

We express sincere thanks to Professor John E. Casida at the University of California, Berkeley, for his useful comments and suggestions.

■ REFERENCES

(1) Pommier, Y.; Jhonson, A. A.; Marchand, C. Integrase inhibitors to treat HIV/AIDS. *Nat. Rev. Drug Discovery* **2005**, *2*, 236–247.

(2) Hanley, W. D.; Wenning, L. A.; Moreau, A.; Kost, J. T.; Mangin, E.; Shamp, T.; Stone, J. A.; Gottsdiener, K. M. Effect of tipranavir-ritonavir on pharmacokinetics of raltegravir. *Antimicrob. Agents Chemother.* **2009**, *53*, 2752–2755.

(3) Moscona, A. Neuraminidase inhibitors for influenza. *N. Engl. J. Med.* **2005**, *353*, 1363–1373.

(4) Chandrasekaran, A.; Srinivasan, A.; Raman, R.; Viswanathan, K.; Raguram, S.; Tumpey, T. M.; Sasisekharan, V.; Sasisekharan, R. Glycan topology determines human adaptation of avian influenza H5N1 virus hemagglutinin. *Nat. Biotechnol.* **2008**, *26*, 107–113.

(5) De Clercq, E. Antiviral agents active against influenza A viruses. *Nat. Rev. Drug Discovery* **2006**, *5*, 1015–1025.

(6) Von Itzstein, M. The war against influenza: discovery and development of sialidase inhibitors. *Nat. Rev. Drug Discovery* **2007**, *6*, 967–974.

(7) Zoghbi, M. G. B.; Andrade, E. H. A.; Maia, J. G. S. Volatile constituents from leaves and flowers of *Alpinia speciosa* K. Schum. and *A. purpurata* (Viell.) Schum. *Flavour Fragrance J.* **1999**, *14*, 411–414.

(8) Bezerra, M. A. C.; Leal-Cardoso, J. H.; Coelho-de-Souza, A. N.; Criddle, D. N.; Fonteles, M. C. Myorelaxant and antispasmodic effects of the essential oil of *Alpinia speciosa* on rat ileum. *Phytother. Res.* **2000**, *14*, 549–551.

(9) Fujita, T.; Nishimura, H.; Kaburagi, K.; Mizutani, J. Plant growth inhibiting α-pyrones from *Alpinia speciosa*. *Phytochemistry* **1994**, *36*, 23–27.

(10) Tawata, S.; Taira, S.; Kobamoto, N.; Ishihara, M.; Toyama, S. Syntheses and biological activities of dihydro-5,6-dehydrokawain derivatives. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1643–1645.

(11) Laranja, S. M.; Bergamaschi, C. M.; Schor, N. Evaluation of acute administration of natural products with potential diuretic effects, in humans. *Mem. Inst. Oswaldo Cruz* **1991**, *86*, 237–240.

(12) Mpalantinos, M. A.; de Moura, R. S.; Parente, J. P.; Kuster, R. M. Biologically active flavonoids and kava pyrones from the aqueous extract of *Alpinia zerumbet*. *Phytother. Res.* **1998**, *12*, 442–444.

(13) Teng, C. M.; Hsu, S. Y.; Lin, C. H.; Yu, S. M.; Wang, K. J.; Lin, M. H.; Chen, C. F. Antiplatelet action of dehydrokawain derivatives isolated from *Alpinia speciosa* rhizome. *Chin. J. Physiol.* **1990**, *33*, 41–48.

(14) Jantan, I.; Raweh, S. M.; Sirat, H. M.; Jamil, S.; Yasin, Y. H. M.; Jalil, J.; Jamal, J. A. Inhibitory effect of compounds from Zingiberaceae species on human platelet aggregation. *Phytomedicine* **2008**, *15*, 306–309.

(15) Kunnumakkara, A. B.; Ichikawa, H.; Anand, P.; Mohankumar, C. J.; Hema, P. S.; Nai, M. S.; Aggarwal, B. B. Coronarin D, a labdane diterpene, inhibits both constitutive and inducible nuclear factor-κB pathway activation, leading to potentiation of apoptosis, inhibition of invasion, and suppression of osteoclastogenesis. *Mol. Cancer Ther.* **2008**, *7*, 3306–3317.

(16) Oliveira, A. P.; Furtado, F. F.; Silva, M. S.; Tavares, J. F.; Mafra, R. A.; Araujo, D. A. M.; Cruz, J. S.; Medeiros, I. A. Calcium channel blockade as a target for the cardiovascular effects induced by 8(17),12*E*,14-labdatrien-18-oic acid (labdane-302). *Vasc. Pharmacol.* **2006**, *44*, 338–344.

(17) Liu, Y; Nair, M. G. Labdane diterpenes in *Curcuma mangga* rhizomes inhibit lipid peroxidation, cycloxygenase enzymes and human tumor cell proliferation. *Food Chem.* **2011**, *124*, 527–532.

(18) Elzaawely, A. A.; Xuan, T. D.; Tawata, S. Essential oils, kava pyrones and phenolic compounds from leaves and rhizomes of *Alpinia zerumbet* (Pers.) B.L. Burtt. & R.M. Sm. and their antioxidant activity. *Food Chem.* **2007**, *103*, 486–494.

(19) Tawata, S.; Fukuta, M.; Xuan, T. D.; Deba, F. Total utilization of tropical plants *Leucaena leucocephala* and *Alpinia zerumbet*. *J. Pestic. Sci.* **2008**, *33*, 40–43.

(20) Elzaawely, A. A.; Xuan, T. D.; Tawata, S. Changes in essential oil, kava pyrones and total phenolics of *Alpinia zerumbet* (Pers.) B.L. Burtt. & R.M. Sm. leaves exposed to copper sulphate. *Environ. Exp. Bot.* **2007**, *59*, 347–353.

(21) Takashi, F.; Nishimura, H.; Kaburagi, K.; Mizutani, J. Plant growth inhibiting a-pyrones from *Alpinia speciosa*. *Phytochemistry* **1994**, *36*, 23–27.

(22) Dharmaratne, H. R. W.; Nanayakkara, N. P. D.; Khan, I. A. Kavalcatones from *Piper methysticum*, and their ^{13}C NMR spectroscopic analyses. *Phytochemsitry* **2002**, *59*, 429–433.

(23) Tewtrakul, S.; Itharat, A.; Rattanasuwan, P. Anti-HIV-1 protease- and HIV-1 integrase activities of Thai medicinal plants known as Hua-Khao-Yen. *J. Ethnopharmacol.* **2006**, *105*, 312–315.

(24) Jeong, H. J.; Ryu, Y. B.; Park, S.; Kim, J. H.; Kwon, H.; Kim, J. H.; Park, K. H.; Rho, M.; Lee, W. S. Neuraminidase inhibitory activities of flavonols isolated from *Rhodiola rosea* roots and their in vitro anti-influenzal viral activities. *Bioorg. Med. Chem.* **2009**, *17*, 6816–6823.

(25) Nguyen, P. H.; Nguyen, T. N. A.; Kang, K. W.; Ndineth, D. T.; Mbafor, J. T.; Kim, Y. R.; Oh, W. K. Prenylated pterocarpans as bacterial neuraminidase inhibitors. *Bioorg. Med. Chem.* **2010**, *18*, 3335–3344.

(26) Ye, Y.; Li, B. 1′*S*-1′-Acetoxychavicol acetate isolated from *Alpinia galanga* inhibits human immunodeficiency virus type 1 replication by blocking Rev transport. *J. Gen. Virol.* **2006**, *87*, 2047–2053.

(27) Grienke, U.; Schmidtke, M.; Kirchmair, J.; Pfarr, K.; Wutzler, P.; Durrwald, R.; Wolber, G.; Liedl, R.; Stuppner, H.; Rollinger, J. M. Antiviral potential and molecular insight into neuraminidase inhibiting diarylheptanoids from *Alpinia katsumadai*. *J. Med. Chem.* **2010**, *53*, 778–786.

(28) Sawamura, R.; Sun, Y.; Yasukawa, K.; Shimizu, T.; Watanabe, W.; Kurokawa, M. Antiviral activities of diarylheptanoids against influenza virus in vitro. *J. Nat. Med.* **2010**, *64*, 117–120.

(29) Upadhyay, A.; Uezato, Y.; Tawata, S.; Ohkawa, H. CYP2C9 catalyzed bioconversion of secondary metabolites of three Okinawan plants. In *Proceedings of 16th International Conference on Cytochrome P450*; Shoun, H., Ohkawa, H., Eds.; Nago: Okinawa, Japan, 2009; pp 31−34.

(30) Singh, S. B.; Zink, D. L.; Bills, G. F.; Teran, A.; Silverman, K. C.; Lingham, R. B.; Felock, P.; Hazuda, D. J. Four novel bis-(naphtha-γ-pyrones) isolated from *Fusarium* species as inhibitors of HIV-1 integrase. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 713–717.

(31) Lameira, J.; Medeiros, I. G.; Reis, M.; Santos, A. S.; Alves, C. N. Structure−activity relationship study of flavones compounds with anti-HIV-1 integrase activity: a density functional theory study. *Bioorg. Med. Chem.* **2006**, *14*, 7105–7112.

(32) Turner, S. R.; Strohbach, J. W.; Tommasi, R. A.; Arsitoff, P. A.; Johnson, P. D.; Skulnick, H. I.; Dolak, L. A.; Seest, E. P.; Tomich, P. K.; Bohanon, M. J.; Horng, M. M.; Lynn, J. C.; Chong, K. T.; Hinshaw, R. R.; Watenpauhg, K. D.; Janakiraman, M. N.; Thaisrivongs, S. Tipranavir (PNU-140690): a potent, orally bioavailable nonpeptidic HIV protease inhibitor of the 5,6-dihydro-4-hydroxy-2-pyrone sulfonamide class. *J. Med. Chem.* **1998**, *41*, 3467–3476.

(33) Hochgurtel, M.; Kroth, H.; Piecha, D.; Hofmann, M. W.; Nicolau, C.; Krause, S.; Schaaf, O.; Sonnenmoser, G.; Eliseev, A. Target-induced formation of neuraminidase inhibitors from in vitro virtual combinatorial libraries. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 3382–3387.

(34) Kati, W. M.; Saldivar, A. S.; Fariborz, M.; Sham, H. L.; Laver, W. G.; Kohlbrenner, W. E. GS4071 is a slow-binding inhibitor of influenza neuraminidase from both A and B strains. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 408–413.