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Flupyranochromene, a novel inhibitor of influenza virus cap-dependent endonuclease, from *Penicillium* sp. f28743

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Abstract

Influenza virus RNA polymerase has cap-dependent endonuclease activity that produces capped RNA fragments for priming viral mRNA synthesis. This enzymatic activity is essential for viral propagation, but it is not present in any host cellular enzyme, making it an attractive target for the development of anti-influenza drugs. Here, we isolated a novel inhibitor of cap-dependent endonuclease, named flupyranochromene, from the fermentation broth of the fungus *Penicillium* sp. f28743. Structural analysis revealed that this compound bears a putative pharmacophore that chelates divalent metal ion(s) present in the endonuclease active site in the PA subunit of the polymerase. Consistently, in vitro endonuclease assays showed that flupyranochromene exerts its inhibitory effects by blocking endonucleolytic cleavage by the PA subunit of viral RNA polymerase complex.

Introduction

Influenza viruses are the causative agents of seasonal epidemics of acute respiratory disease in humans, and are responsible for substantial morbidity and mortality in susceptible patients [1]. Occasionally, antigenically novel virus strains emerge from different animal hosts, such as avian and swine hosts, through adaptation to humans, this poses a risk of a pandemic with the potential to cause widespread illness and death [2]. Although current standard antiviral drugs, including neuraminidase inhibitors such as oseltamivir and zanamivir, are extremely useful as treatments to reduce the duration of illness and alleviate clinical symptoms, the emergence of drug-resistant strains has been reported,

raising significant concerns [3–5]. Thus, there remains an unmet need for the development of novel anti-influenza drugs, preferably directed against other viral targets.

Influenza virus contains a segmented, single-stranded RNA genome with negative polarity. Each segment of the viral genome forms the viral ribonucleoprotein (vRNP), which is composed of multiple copies of a nucleoprotein and a viral RNA-dependent RNA polymerase. The viral polymerase consists of a heterotrimeric complex of PA, PB1, and PB2 subunits, which are highly conserved among different strains, and it is responsible for the replication and transcription of the viral genome in the nucleus of infected cells. During transcription, the viral polymerase uses its cap-dependent endonuclease activity to produce capped RNA fragments for priming viral mRNA synthesis via the following process [6–8]. The polymerase binds to the 5'-cap of host pre-mRNAs, through recognition by the PB2 subunit [9], and then the endonuclease in the PA subunit cleaves 10–13 nucleotides downstream from the cap [10, 11]. The resultant capped RNA fragment, containing a 3'-hydroxyl group, serves as a primer to initiate viral mRNA synthesis by the PB1 subunit. This unique endonuclease activity is essential for viral propagation, and is not found in host cell enzymes, making it a promising target for novel anti-influenza drugs [12–14]. In fact, a series of 2,4-dioxo-4-phenylbutanoic acids with PA endonuclease inhibitory activity have been shown to be effective against

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influenza virus in cell culture assays and in an in vivo mouse challenge model [15–17]. In addition, VX-787, a PB2 cap-binding inhibitor, is currently undergoing clinical trials [18, 19]. S-03318 (also known as baloxavir), a PA endonuclease inhibitor, has recently been approved in Japan for the treatment of influenza infections [20]. These reports strongly support the clinical efficacy of inhibiting cap-dependent endonuclease against flu.

Natural compounds possess structural diversity and a variety of biological activities, and have led to the development of approximately one-third of all marketed drugs [21]. Despite a decline in natural products research at many pharmaceutical companies, the prominent role of natural resources in the discovery of new lead compounds is still unparalleled because of the vast untouched diversity of biological activities and chemical structures in nature. Various cap-dependent endonuclease inhibitors have been reported; however, only a few are derived from natural resources (flutimide [22] and marchantins [23]). In this study, we screened a microbial fermentation broth from the fungus, *Penicillium* sp. f28743 using an in vitro enzyme assay, and isolated a novel cap-dependent endonuclease inhibitor, which we named flupyranochromene (Fig. 1a). We report the isolation, structural determination, and biological activities of flupyranochromene and two alcohol-derivative forms.

Results and discussion

Screening for inhibitors of influenza virus cap-dependent endonuclease

Previous studies have successfully identified several inhibitors of influenza virus cap-dependent endonuclease by screening for the inhibitory potency of compounds against mRNA synthesis primed with capped alfalfa mosaic virus RNA [15, 16]. Hence, in this study, we initially performed an in vitro transcriptase assay with detergent-disrupted virions and cap1-RNA ($m^7GpppGm$ -RNA, 39 nt) in a 96-well format to allow rapid screening of microbial metabolites. The screening hits were then subjected to a counter-screen with T7 RNA polymerase, a bacteriophage DNA-dependent RNA polymerase, to eliminate hits with low specificity. Finally, the inhibitory effects of selected hits on the cap-dependent endonucleolytic process were verified using purified vRNP and ^{32}P -radiolabeled cap1-RNA. In the course of our screening, we found that the fermentation broth of fungal strain f28743 inhibited cap-dependent endonuclease activity. Its inhibitory activity toward the viral polymerase was approximately tenfold greater than its activity toward T7 RNA polymerase (data not shown). This finding prompted us to isolate and characterize the active compound from the broth for further experiments.

Taxonomy of the producing strain

A scanning electron micrograph of strain f28743 is shown in Fig. 1b. The ITS-5.8 S rRNA [24] and 28 S rRNA-D1/D2 [25] sequences of the strain were identical to that of *Penicillium janthinellum* VI2R3M (100%) and *Penicillium limosum* HK1–23 (99.6%), respectively. Therefore, this strain was tentatively designated as *Penicillium* sp. f28743.

Detection of flupyranochromene and isolation of butyl flupyranochromene

In preliminary LC/MS assays, the active compound was unstable in an alcohol solution and an alcohol derivative was more stable. For this reason, we performed the isolation and structural determination of flupyranochromene using an alcohol derivative.

Culture supernatant containing flupyranochromene was loaded onto a HP-20 column and eluted with 50% aq. CH_3CN . The eluted fraction containing flupyranochromene was extracted with an equal amount of *n*-BuOH under acidic conditions. The BuOH solution was washed with distilled water and concentrated under reduced pressure to yield a dark-yellowish brown oil. By monitoring with LC/MS, we confirmed that most of the flupyranochromene was converted into butyl flupyranochromene. The crude butyl flupyranochromene was purified by low-pressure reverse-phase C18 chromatography to yield 26.7 mg of pure butyl flupyranochromene (pale-yellow crystal; mp, 175–176 °C [dec]).

Butyl flupyranochromene (10 mg) was converted into flupyranochromene by acid hydrolysis with CH_3CN in 0.1 M HCl, and was monitored by HPLC. The acid hydrolysate reaction mixture was concentrated to yield 8.5 mg of dull-yellow solid. The concentrate was purified by reverse-phase C18 HPLC to yield 2.0 mg of flupyranochromene as a pale-yellow amorphous solid.

Structure determination of butyl flupyranochromene

The molecular formulae of butyl flupyranochromene and flupyranochromene were determined as $C_{18}H_{18}O_8$ and $C_{14}H_{10}O_8$, respectively, based on high-resolution electrospray ionization MS (HRESI-MS), which yielded a cationized molecule ($M+Na$)⁺ at m/z 385.0924 (calculated as 385.0894 for $C_{18}H_{18}O_8 Na^+$) and at m/z 329.0248 (calculated as 329.0268 for $C_{14}H_{10}O_8 Na^+$), respectively.

The 1H and ^{13}C -NMR spectral data for butyl flupyranochromene are shown in Supplementary Figure S1. The DEPT and HMQC spectra revealed two methyl-, three methylene-, three methine-, eight fully substituted sp^2 -carbons, and two carbonyl-carbon(s) (Supplementary Figure S1 and S3). The connectivities in the 1H - 1H COSY only revealed the

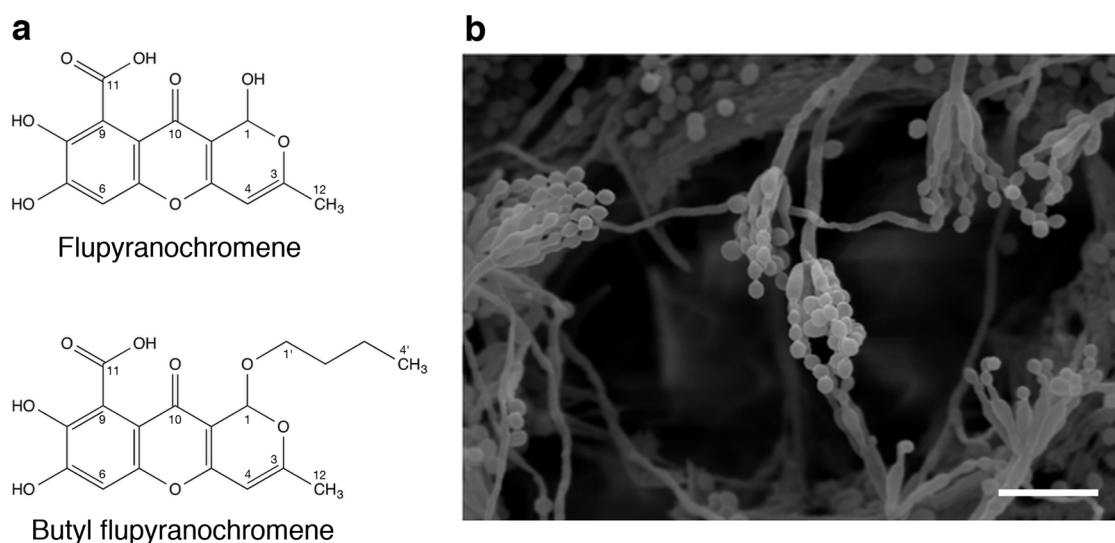


Fig. 1 **a** Structures of flupyranochromene and butyl flupyranochromene. **b** Scanning electron micrograph of *Penicillium* sp. f28743. The fungus was grown on Miura's medium at 25 °C for 12 days. Bar, 10 μm

presence of butyl group from the hydroxy methylene of H-1' (δ_{H} 3.64 and 3.79) to methyl group of H-4' (δ_{H} 0.85) (Supplementary Figure S2). The long-range correlations in the HMBC (Supplementary Figure S4) observed from the methine of H-1 (δ_{H} 6.25) to butyl group of C-1' (δ_{C} 67.4), the carbonyl of C-10 (δ_{C} 171.6), the sp^2 carbons of C-3 (δ_{C} 163.0), C-4a (δ_{C} 158.1), C-10a (δ_{C} 103.4), from the methyl of H-12 (δ_{H} 2.08) to the sp^2 carbons of C-3 and C-4 (δ_{C} 94.1), from the methine of H-4 (δ_{H} 5.88) to C-3, C-4a, C-10a, and the methyl of C-12 (δ_{C} 20.2), established the right side of the structure as shown in Fig. 1a. Unfortunately, the structure of flupyranochromene could not be determined by the HMBC spectrum, because the methine proton of H-6 (δ_{H} 6.87), which exists only one in the left part, was not correlated to two of the remaining six carbons (δ_{C} 119.1 and 168.0). The structure was established unequivocally by single-crystal X-ray analysis. The ORTEP drawing of butyl flupyranochromene is shown in Fig. 2.

Butyl flupyranochromene was easily converted into flupyranochromene under the acidic conditions described above. The derived flupyranochromene was in complete agreement with the active compound in preliminary tests by LC/MS analysis. The NMR spectra of flupyranochromene are shown in Supplementary Figures S5–7. The proposed gross structure of flupyranochromene is shown in Fig. 1a.

Inhibitory effects of flupyranochromene on influenza cap-dependent endonuclease activity

To evaluate the inhibitory potencies of flupyranochromene and the two alcohol derivatives toward cap-dependent endonuclease, we performed an *in vitro* cap-dependent endonuclease assay using vRNP and ^{32}P -labeled cap1-RNA

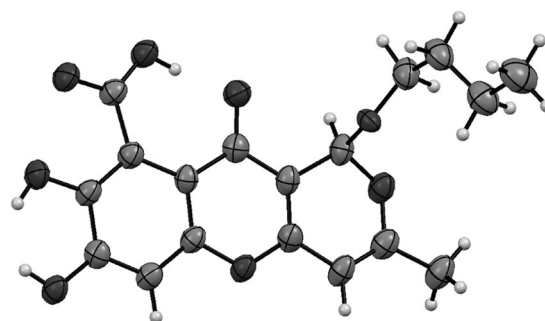


Fig. 2 ORTEP drawing of butyl flupyranochromene

(with ^{32}P in the cap). As shown in Fig. 3 and Table 1, flupyranochromene inhibited the cleavage of capped RNA in a dose-dependent manner, with an IC_{50} value of 0.8 μM . In addition, butyl flupyranochromene and SB238569, a methylated form of flupyranochromene that was previously reported as a metallo- β -lactamase inhibitor [26], showed an obvious inhibitory effect on the endonuclease, with IC_{50} values of 7.0 and 6.6 μM , respectively (Table 1, Supplementary Figure S8). Although there was an approximately ninefold difference in the IC_{50} values for flupyranochromene and the alcohol derivatives, their inhibitory potencies were comparable with those of two previously reported inhibitors, L-735,882 [15] and flutimide [22] (IC_{50} : 1.1 and 6.8 μM , respectively). We also examined the inhibitory potencies of these compounds toward T7 RNA polymerase to estimate their specificities. Flupyranochromene and the butylated and methylated forms exhibited IC_{50} values of 5.0, 12.3, and 101.9 μM , yielding selectivity indexes of 6.6, 1.8, and 15.3, respectively (Table 1, Supplementary Figure S9). These results indicate

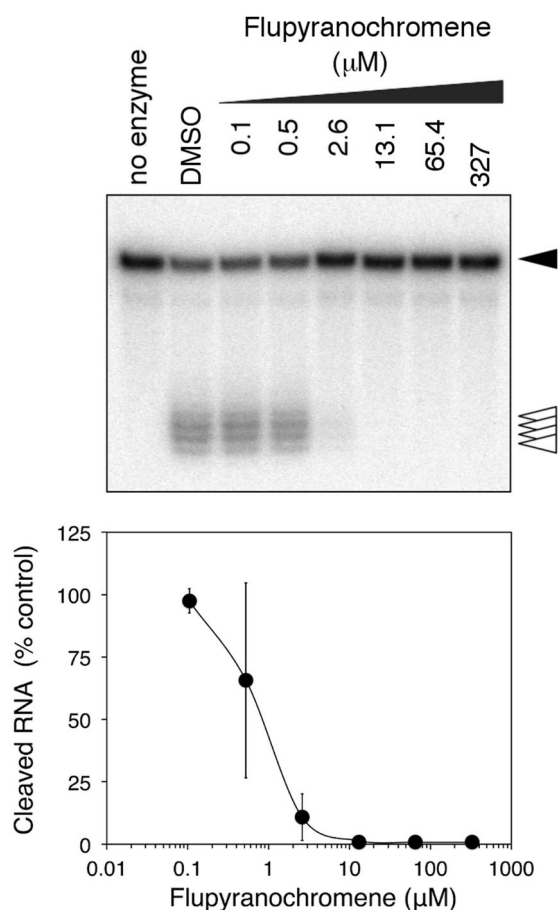


Fig. 3 Effects of flupyranochromene on the cap-dependent endonuclease activity of influenza virus RNP. Purified vRNP was pre-incubated with flupyranochromene, and then the RNA cleavage reaction was started by adding ^{32}P -radiolabeled cap1-RNA. After incubation, the cleaved RNAs were separated by urea-PAGE (upper panel) and quantified by phosphoimaging (lower panel). The added RNA substrates and cleaved products are indicated by closed and opened arrowheads, respectively. The relative amounts of cleaved RNA are shown as the percentage of those in the untreated control (DMSO). The results are shown as the means \pm standard deviations of three independent experiments

Table 1 Inhibitory activities of flupyranochromene and related compounds toward cap-dependent endonuclease of vRNP, T7 RNA polymerase, and PA endonuclease

Compound	IC ₅₀ (μM)			SI ^a
	vRNP	T7RP	PA endonuclease	
Flupyranochromene	0.8	5.0	2.0	6.6
Butyl flupyranochromene	7.0	12.3	1.4	1.8
SB238569 (methylated form)	6.6	101.9	5.0	15.3
DPBA	ND ^b	ND	0.6	ND

^aSI, selectivity index as determined by the ratio of IC₅₀ between vRNP and T7PR

^bND, not determined

that flupyranochromene is a novel inhibitor of influenza virus cap-dependent endonuclease, with moderate but significant selectivity, and suggest that the alkyl group size on the hydroxyl group at position 1 might affect its inhibitory activity.

The previously reported inhibitors described above have a common pharmacophoric motif that chelates the divalent metal ion(s) present in the endonuclease active site, i.e., the butanoic acid chain in L-735,882 and *N*-hydroxyimide in flutimide [12]. Similarly, flupyranochromene was found to bear a functionally equivalent chemical group, i.e., a dihydroxybenzoic acid moiety (Fig. 1a). Based on this finding, we hypothesized that flupyranochromene exerts its inhibitory action by interacting with the active site in a similar manner to the metal-chelating inhibitors, and we tested this hypothesis in vitro using a protein containing an endonuclease domain at the N-terminal end of the PA subunit, termed PA-Nter [10, 11]. As shown in Fig. 4, treatment with flupyranochromene inhibited cleavage of the RNA substrate by PA-Nter in a dose-dependent manner. In addition, we observed that the two alcohol derivatives also inhibited RNA cleavage (Supplementary Figure S10). The respective IC₅₀ values of flupyranochromene and the butylated and methylated forms were 2.0, 1.4, and 5.0 μM, respectively (Table 1). These data were thought to be reliable because the IC₅₀ value we obtained for DPBA, a known inhibitor of the viral endonuclease, was similar to that reported by others (Table 1) [27, 28]. The results indicate that these compounds inhibit PA endonuclease activity, with potencies comparable to those for cap-dependent endonuclease by vRNP. Therefore, we conclude that flupyranochromene exerts inhibitory activity toward cap-dependent endonuclease by blocking endonucleolytic RNA cleavage by the PA subunit.

Conclusions

We identified a novel inhibitor of influenza virus cap-dependent endonuclease activity, flupyranochromene, from the fermentation broth of the fungus *Penicillium* sp. f28743. Flupyranochromene was classified as a pyrano [4,3-*b*]chromene-containing antibiotic. This antibiotic group includes SB238569 [26], fulvic acid [29, 30], and citromycin [31], which are microbial metabolites with various biological activities, such as metallo-β-lactamase inhibitory activity, antibacterial and antifungal activities, and anti-mycobacterial activity, respectively. To our knowledge, this is the first report showing that an antibiotic in this group has inhibitory activity toward cap-dependent endonuclease. Flupyranochromene showed inhibitory potencies comparable to those of two well-known inhibitors, the 2, 4-diketobutanoic acid compound L-735,882 and

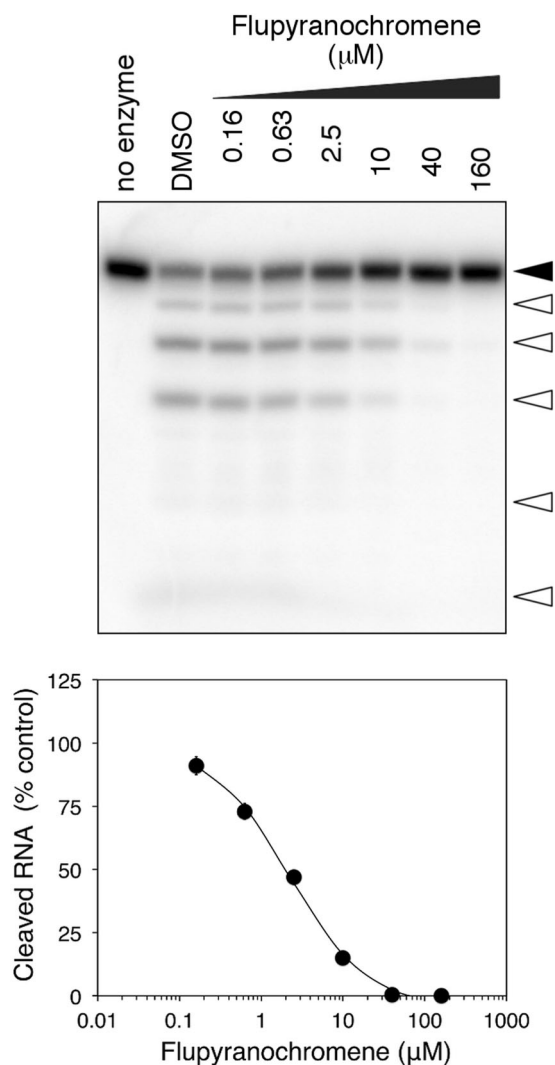


Fig. 4 Effects of flupyranochromene on PA endonuclease activity. PA-Nter, the endonuclease domain of the PA subunit, was preincubated with flupyranochromene, and then the RNA cleavage reaction was started by adding ^{32}P -triphosphate-ended RNA. After incubation, the cleaved RNAs were separated by urea-PAGE (upper panel) and quantified by phosphoimaging (lower panel). The RNA substrates and cleaved products are indicated by closed and opened arrowheads, respectively. The relative amounts of cleaved RNA are shown as the percentages of those in the untreated control (DMSO). The results are shown as the means \pm standard deviations of three independent experiments

flutimide [15, 22], and structural analysis revealed that it has a putative pharmacophore that might chelate the divalent metal ion(s) present in the endonuclease active site of the polymerase [12]. Consistently, flupyranochromene, as well as two alcohol derivatives, inhibited RNA cleavage by the endonuclease domain in the PA subunit, with efficacies comparable to those toward cap-dependent endonuclease by vRNP, indicating that inhibition of cap-dependent endonuclease is mediated by interaction with this domain. Unfortunately, we could not observe a significant reduction in virus replication by these compounds at non-cytotoxic

concentrations in a plaque assay using influenza virus strain A/WSN/33 and MDCK cells (Supplementary Figure S11). Nevertheless, the high potency of flupyranochromene for cap-dependent endonuclease in vitro not only makes it a promising lead compound for anti-influenza drug design in medicinal chemistry but also reinforces that microbial metabolites are a useful screening resource for a class of inhibitors targeting this enzymatic activity.

Materials and methods

Materials

Influenza virus strain A/PR/8/34 (H1N1) was kindly provided by Professor Kyosuke Nagata (Department of Infection Biology, Graduate School of Comprehensive Human Sciences, University of Tsukuba). vRNP was prepared from purified A/PR/8/34 virus on glycerol gradients as previously described [32]. 2,4-dioxo-4-phenylbutanoic acid (DPBA) was purchased from Namiki Shoji Co., Ltd. (Tokyo, Japan).

General experimental procedures

UV spectra were measured using a Hitachi U-2800 spectrometer (Hitachi High-Technologies, Tokyo, Japan). Infrared (IR) spectra were recorded on a FT/IR-4100 Fourier-transform infrared spectrometer (JASCO Corporation, Tokyo, Japan). ^1H - and ^{13}C -NMR spectra were measured using a JNM-ECA600 spectrometer (JEOL, Tokyo, Japan) or a Bruker AVANCE III 500 or a Bruker AVANCE III 600 spectrometer (Bruker Bio-Spin, Billerica, MA, USA). Chemical shifts are adjusted with solvent signal. High-resolution ESI-MS spectra were recorded on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Taxonomic studies of strain f28743

Flupyranochromene-producing strain f28743 was isolated from a moss sample collected in Itoh city, Shizuoka Prefecture, Japan and was maintained on potato-dextrose agar (PDA). The morphological properties of the strain were observed by scanning electron microscopy (Hitachi SU1510; Hitachi High-Technologies) after incubation on Miura's medium at 25 °C for 12 days. The rDNA internal transcribed spacer (rDNA ITS) regions, including the 5.8S rDNA and 28S rDNA of the strain, were amplified by PCR from genomic DNA prepared using a Genomic DNA Extraction Kit Mini (RBC Bioscience Co., New Taipei, Taiwan). The sequence data were deposited in GenBank under accession numbers LC128711 (ITS-5.8 S rRNA) and LC128712 (28S rRNA-D1/D2).

Analysis of flupyranochromene and butyl flupyranochromene

The fermentation broth was separated from the mycelium by centrifugation. After discarding the mycelium, the supernatant was loaded onto a Sep-Pak plus C18 column (Waters Co., Milford, MA, USA), which was prewashed with 3 ml of CH₃CN and equilibrated with distilled water before sample loading to condition the C18 for binding. After 10 ml of supernatant was applied, the Sep-Pak column was washed with 3 ml of distilled water and then eluted with the 50% CH₃CN in water. The eluent was fractionated on a Capcell Pak UG column (2.1 × 150 mm; Shiseido Co., Ltd., Tokyo, Japan) by HPLC under the following conditions: mobile phase A, 0.1% formic acid in CH₃CN; mobile phase B, 0.1% formic acid in water; flow rate, 0.4 ml min⁻¹; column temperature, 40 °C; detection, at 368 nm (UV). The gradient system was a linear gradient from 5% A (at 0–2 min) to 100% A (at 17–20 min). Flupyranochromene eluted at 9.49 min, SB238569 at 11.12 min, and butyl flupyranochromene at 13.84 min (Supplementary Figure S12).

Fermentation and isolation of flupyranochromene

Strain f28743 was inoculated into seed medium consisting of 2% potato starch (Yoshida Pharmaceutical Co., Tokyo, Japan), 1% glucose, 2% soy bean meal (Showa Sangyo Co., Tokyo, Japan), 0.1% KH₂PO₄, and 0.05% MgSO₄·7H₂O and cultured at 25 °C on a rotary shaker at 220 rpm. After 3 days of incubation, 1 ml of the seed culture was inoculated into production medium consisting of 3% glucose, 3% lactose monohydrate, 1% malt extract (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA), 1% corn gluten meal (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 1% yeast extract (Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan), 0.5% NaCl, 0.05% KH₂PO₄, and 0.3% CaCO₃ (pH 6.4). The fermentation was carried out for 4 days under the conditions described above for the seed culture.

The fermentation broth (2 l) was separated into the mycelial cake and supernatant by centrifugation. The supernatant was adjusted to pH 3 with 1 M HCl and loaded onto an HP-20 column (40 × 110 mm; Mitsubishi Chemical Co., Tokyo, Japan). The active compound was eluted with 50% aq. CH₃CN (420 ml) after washing with distilled water (420 ml). The 50% aq. CH₃CN fraction containing the active compound was collected, and CH₃CN was removed under reduced pressure. The concentrated solution was adjusted to pH 3 with 1 M HCl and extracted with an equal volume of BuOH (50 ml). The BuOH solution was washed with distilled water and concentrated under reduced pressure to yield a dark-yellowish brown oil (1.31 g). The BuOH extract was chromatographed on a low-pressure ODS reverse-phase column (ODS-7515–12A, 20 × 200 mm; Senshu Scientific

Co., Ltd., Tokyo, Japan) under the following stepwise conditions: 0.01% trifluoroacetic acid in 20% aq. CH₃CN (500 ml), 0.01% trifluoroacetic acid in 50% aq. CH₃CN (500 ml), and 0.01% trifluoroacetic acid in 100% aq. CH₃CN (500 ml). The target compound was eluted with 0.01% trifluoroacetic acid in 100% aq. CH₃CN and concentrated in vacuo to yield 26.7 mg of pure butyl flupyranochromene as a pale-yellow crystal. The UV spectral data for butyl flupyranochromene were UV λ_{max} (ε) 216 (14,400), 231 (13,400), 290 (5100), 330 (8200), and 368 (11,000) in 0.005 M HCl–CH₃CN. The IR spectral data were ν_{max} (KBr) cm⁻¹ 3500–3000, 2958, 2934, 2873, 1715, 1637, 1596, 1558, 1490, 1462, 1390, 1285, 1085, and 968. The ¹H-NMR data were (600 MHz, DMSO-*d*₆) δ 0.85 (3H, t, *J* = 7.2 Hz), 1.28 (2H, m), 1.48 (2H, m), 2.08 (3H, s), 3.64 (1H, m), 3.79 (1H, m), 5.88 (1H, s), 6.25 (1H, s), and 6.87 (1H, br s). The ¹³C-NMR data were (150 MHz, DMSO-*d*₆) δ 13.6, 18.6, 20.2, 31.2, 67.4, 94.1, 96.0, 102.7, 103.4, 112.8, 119.1, 141.9, 149.5, 152.0, 158.1, 163.0, 168.0, and 171.6.

The butyl flupyranochromene was recrystallized from BuOH (10.5 mg). Conversion of butyl flupyranochromene (10 mg) into flupyranochromene was carried out by acidic hydrolysis with 0.1 M HCl in CH₃CN (10 ml) at room temperature for 3 h, and was monitored by HPLC. The reaction mixture containing the acid hydrolysate was concentrated to yield 8.5 mg of dull-yellow solid. The concentrate was subjected to HPLC on a Capcell Pak C18 column (10 × 250 mm; Shiseido Co., Ltd.) under the following conditions: mobile phase, 20% aq. CH₃CN; flow rate, 3.0 ml min⁻¹; column temperature, 25 °C; detection, at 368 nm (UV). Flupyranochromene was eluted at 31–37 min, and the eluent was collected and concentrated in vacuo to obtain 2.0 mg of flupyranochromene as a yellow amorphous solid. UV λ_{max} (ε) 216 (23,000), 228 (21,000), 290 (7600), 331 (12,300), and 370 (17,200) in 0.005 M HCl–CH₃CN. The IR spectral data were ν_{max} (KBr) cm⁻¹ 3500–2300, 1716, 1640, 1596, 1561, 1490, 1465, 1279, 1222, 1184, 1018, and 954. The ¹H-NMR data were (500 MHz, DMSO-*d*₆) δ 2.05 (3H, br s), 6.40 (1H, brd, *J* = 4.0 Hz), 5.81 (1H, br d, *J* = 1.0 Hz), 6.89 (1H, s), and 7.48 (1H, br d, *J* = 4.0 Hz). The ¹³C-NMR data were (125 MHz, DMSO-*d*₆) δ 20.6, 90.2, 93.3, 102.8, 104.6, 113.0, 118.8, 142.2, 149.5, 151.8, 157.9, 163.2, 168.3, and 171.9.

X-ray structure analysis

Single-crystal X-ray data for butyl flupyranochromene were collected on a Rigaku VariMax with a RAPID imaging plate area detector by graphite-monochromated Cu-Kα radiation (Rigaku Co., Tokyo, Japan) at 93 K. The structure was analyzed by direct methods and refined using full-matrix least-squares in SHELXL (G.M. Sheldrick, Version 2014/7). All non-hydrogen atoms were refined anisotropically. All

hydrogen atoms were placed in standard calculated positions and were refined isotropically. The final cycle of full-matrix least-squares refinement on F was based on 3853 observed reflections and 326 variable parameters, and converged with unweighted agreement factors of $R1 = 0.0690$ and $wR2 = 0.2175$. The X-ray crystallographic data for butyl flupyranochromene were deposited in the Cambridge Crystallographic Data Centre under deposition number CCDC 1826727.

Preparation of SB238569

Conversion of flupyranochromene (3 mg) into SB238569 was carried out by an addition reaction between flupyranochromene and MeOH in 0.1 M HCl (3 ml) at room temperature for 3 h. The reaction mixture was concentrated to yield 7 mg of a dull-yellow solid. Then, the concentrate was separated by HPLC on a Capcell Pak UG column (4.6 × 150 mm; Shiseido Co., Ltd.) under the following conditions: mobile phase A, 0.1% formic acid in CH₃CN; mobile phase B, 0.1% formic acid in water; flow rate, 2 ml min⁻¹; column temperature, 40 °C; detection, at 368 nm (UV). The gradient system is a linear gradient from 5% A (at 0–2 min) to 100% A (at 17–20 min). SB238569 was eluted at 11–12 min, and the eluent was collected and concentrated in vacuo to yield 2.1 mg of SB238569 as a pale-yellow amorphous solid. The ¹H-NMR data were (600 MHz, DMSO-*d*₆) δ 2.10 (3H, s), 3.43 (3H, s), 5.88 (1H, br d, $J = 1.0$ Hz), 6.18 (1H, s), and 6.88 (1H, s). The ¹³C-NMR data were (150 MHz, DMSO-*d*₆) δ 20.0, 54.9, 94.1, 97.0, 102.7, 103.2, 112.8, 119.1, 141.8, 149.5, 151.9, 158.1, 163.1, 167.9, and 171.6

Preparation of RNA substrates

Cap1-RNA, a substrate for the in vitro transcriptase and cap-dependent endonuclease assays described below, was prepared by capping and methylation of a triphosphate-ended RNA (39 nucleotides [nt]: 5'-GAAAAAAAAAAAAAAAAAAAAAAAAAAAGCGGCC-3'), which was synthesized via in vitro transcription from the appropriate linearized plasmid. Briefly, the oligonucleotide 5'-GGTACCTAATACGACTCACTATAGAAAAAAAAAAAAAAAAAAAAAAAAAAAGCGGCCGCAAGCTT-3' (the underlined sequence corresponds to the T7 promoter) was annealed to the complementary oligonucleotide 5'-AAGCTTGCGGCCGCTTTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTATAGTGAGTCGTATTAGGTACC-3', digested with *Kpn*I and *Hind*III, and then ligated into pUC19 digested with the same enzymes. After in vitro transcription of the *Not*I-digested plasmid with T7 RNA polymerase (TAKARA, Shiga, Japan), the resultant transcript was isolated by electrophoresis on a

polyacrylamide gel containing 8 M urea (urea-PAGE). Addition of the Cap1 structure (m⁷GpppGm) to the transcript was performed using the mScript mRNA Production System (CellScript Inc., Madison, WI, USA), and the capped RNA was purified by phenol–chloroform extraction and ethanol precipitation. The substrate for the cap-dependent endonuclease assay was generated as a ³²P-radiolabeled cap1 (m⁷G[³²P]pppGm)-RNA by addition of [α-³²P]GTP to the capping reaction buffer.

Triphosphate-ended RNA (26 nt: 5'-GAAUACU-CAAGCUAUGCAUCGCGGCC-3'), the substrate for the PA endonuclease assay containing efficient cleavage sites [33], was prepared as described above, except that the plasmid template was constructed using the oligonucleotide 5'-GGTACCTAATACGACTCACTATAGAACTCAAGCTT-3' (the underlined sequence corresponds to the T7 promoter) and the complementary oligonucleotide 5'-AAGCTTGCGGCCGCGCATGATAGCTTGTAGTATTCTATAGTGAGTCGTATTAGGTACC-3'. The RNA was radiolabeled at the 5'-terminus with [γ-³²P]GTP by in vitro transcription.

In vitro transcriptase assay

An in vitro transcriptase assay with detergent-disrupted virions was performed in a 96-well microplate for the initial screening of microbial metabolites. Aliquots (22 μl) of a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 100 mM ammonium acetate, 5 mM MgCl₂, 2.5 mM DTT, 0.1% Nonidet P-40, 1 U of RNase inhibitor (TAKARA), and 0.25 μg of purified influenza strain A/PR/8/34 were dispensed into each well, and then 0.5 μl of each metabolite was added to the mixture. After preincubation at 30 °C for 10 min, the reaction was started by adding 2.5 μl of the substrate mixture containing 10 fmol of cap1-RNA; 100 μM ATP, UTP, and CTP; 3 μM GTP; and 0.05 μCi of [α-³²P]GTP. After incubation at 30 °C for 2 h, 10 μg of glycogen and 5% trichloroacetic acid (TCA) were added, and the mixtures were incubated at 4 °C for 30 min. The precipitate was collected on a glass fiber filter (MultiScreen_{HTS}-FB; Millipore, Burlington, MA, USA) and washed with 5% TCA. After the filters were dried, the radioactivity of the product was analyzed by phosphoimaging using a Typhoon Variable Model Imager (GE Healthcare, Piscataway, NJ, USA).

In vitro cap-dependent endonuclease assay

An in vitro cap-dependent endonuclease assay was performed, as described by Wakai et al. [34] with a minor modification. A reaction mixture containing 50 mM Tris-HCl (pH 7.9), 100 mM ammonium acetate, 5 mM MgCl₂, 2.5 mM DTT, 0.1% Nonidet P-40, 4 U of RNase inhibitor, and 12.5 μg of purified vRNPs in a final volume of 25 μl

was preincubated with the compounds, and then the reaction was started by adding 10 fmol of ^{32}P -radiolabeled cap1-RNA. After incubation at 30 °C for 30 min, the RNAs were extracted and separated by 20% urea-PAGE. The band intensity was quantitated by phosphoimaging, and the 50% inhibitory concentration (IC_{50}) was calculated by four-parameter logistic curve fitting.

T7 RNA polymerase assay

The T7 RNA polymerase reaction was performed as described by the manufacturer (TAKARA) using 150 ng of BamHI-digested pTNTTM (Promega, Madison, WI, USA); 200 μM ATP, UTP, and CTP; 50 μM GTP; and 0.01 μCi of [α - ^{32}P]GTP in a final reaction volume of 25 μl . After incubation with the compounds at 37 °C for 1 h, the RNAs were extracted, separated by 10% urea-PAGE, and quantitated as described above.

PA endonuclease assay

The recombinant PA endonuclease domain (PA-Nter; residues 1–220) was prepared as an N-terminal hexahistidine (His)-tagged protein [9, 23]. The cDNA encoding PA-Nter was amplified from vRNP prepared from A/PR/8/34 virus by RT-PCR using the following primers: 5'-CATATGGAAGATTTTGTGCGACAA-3' and 5'-GGATCCCTACGGAGACTTTGGTCGGCAAG-3'. The fragment was cloned into pCold II (TAKARA) between the *NdeI* and *BamHI* sites. The resultant construct was introduced into *Escherichia coli* strain Rosetta-gami B (Novagen), and the recombinant protein was expressed by culturing overnight at 16 °C with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside. After cell lysis by sonication, the solubilized protein was purified by Ni-NTA agarose chromatography (Qiagen, Hilden, Germany).

The PA endonuclease assay was performed as described by Dias et al. [7] with a minor modification. A reaction mixture containing 50 mM Tris-HCl (pH 7.9), 100 mM ammonium acetate, 1 mM MnCl_2 , 2.5 mM DTT, 0.1% Nonidet P-40, and 1 μM of PA-Nter in a final volume of 25 μl was preincubated with the compound, and then the reaction was started by adding 7.5 pmol of ^{32}P -radiolabeled triphosphate-ended RNA. After incubation at 30 °C for 30 min, the RNAs were extracted, separated by 20% urea-PAGE, and quantitated as described above. DPBA was included as a positive control.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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