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Halogenated analogs of 1′-acetoxychavicol acetate, Rev-export inhibitor from *Alpinia galanga*, designed from mechanism of action

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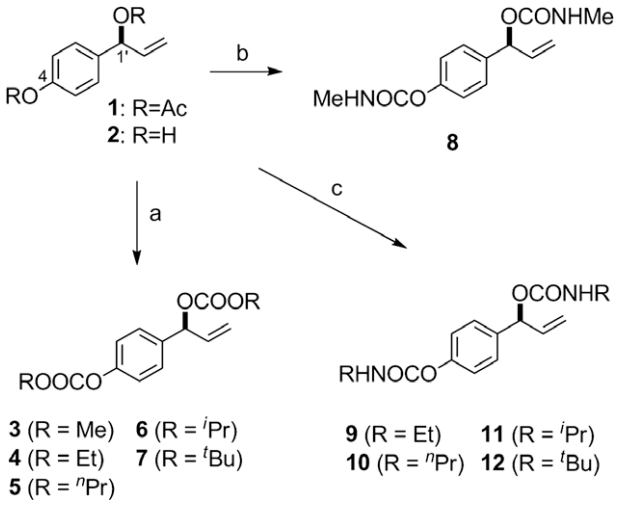
ABSTRACT

In the course of search for the robust analogs of 1′-acetoxychavicol acetate (ACA, **1**), the Rev-export inhibitor from the medicinal plant *Alpinia galanga*, we clarified formation of the quinone methide intermediate **ii** to be essential for exerting the inhibitory activity of **1**. Based on this mechanism of action, the rational design from the MO calculation of the conclusive activation energy to **ii** resulted in the four halogenated analogs with more potent activity than ACA (**1**). In particular, the difluoroanalog **20d** exhibited approximately four-fold potent activity as compared with **1**.

Rev is required for expression of the majority of HIV-1 proteins necessary for formation of infectious virus particles by promoting nuclear-cytoplasm export of virus mRNA.[1] Since the export is critical for viral replication, inhibition for this step has been recognized as an attractive strategy for therapeutic intervention for acquired immunodeficiency syndrome (AIDS).[2] Previously, we found out 1′-acetoxychavicol acetate (ACA, **1**) as an inhibitor for nuclear export of Rev from the medicinal plant *Alpinia galanga*. In addition, the structural requirement for the biological activity of **1** was revealed to be the configuration at C-1′, the 1-acetoxy-2-ene moiety, and the two acetyl functions linked to the hydroxyl groups.[3]

Indeed, ACA (**1**) exhibited potent activity in vitro, while in vivo efficacy of **1** will be expected to be significantly reduced because of facile deacetylation leading to the inactive diol by esterase in serum. On the other hand, we presented carbonates and carbamates as not only bioisosteric but also robust functions of esters; stability in serum increased in order of the ester, the carbonate, and the carbamate.[4,5] Hence, we synthesized several carbonates and carbamates from the optical active diol (**2**)[3] and evaluated them for Rev-export inhibitory activity by using the fission yeast,[6] which express the model fusion protein consisting of glutathione *S*-transferase (GST), SV40 T antigen nuclear localization signal (NLS), green fluorescent protein (GFP), and Rev nuclear export signal (NES) (Scheme 1).[7] Among the synthesized analogs, the methyl, ethyl,



Scheme 1. Synthesis of carbonates and carbamates of **1**. Reagents and conditions: (a) MeOCOCl, pyridine, rt, 88%; EtOCOCl, pyridine, rt, 71%; *n*PrOCOCl, pyridine, rt, 68%; *i*PrOCOCl, pyridine, rt, 87%; $(t\text{BuO})_2\text{CO}$, DMAP, pyridine, rt, 64%; (b) AcOH, DPPA, Et_3N, rt to 55 °C, then CuCl, rt, 88%; (c) EtNCO, CuCl, DMF, rt, 67%; *n*PrNCO, CuCl, DMF, rt, 76%; *i*PrNCO, CuCl, DMF, rt, 58%; *t*BuNCO, CuCl, DMF, rt, 70%.

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Table 1
Inhibitory activity of carbonates and carbamates of **1** for nuclear export of Rev

Compound	MIC (μM)
1	4.3
3	25
4	25
5	10
6	>50
7	>50
8	>50
9	>50
10	>50
11	>50
12	>50

and *n*-propyl carbonates (**3–5**) exhibited moderate inhibitory potency, while the activities of the other analogs were fairly reduced as compared with that of ACA (**1**) as shown in Table 1.

This unexpected outcome in the biological activity directed us to examine stability of the carbonates and carbamates (**3–12**) in fetal bovine serum.[8] In the case of the analogs of **1**, stability of all the carbamates were enhanced as expected, while all carbonates except for the bulky *t*-butyl congener (**7**) were facilely subjected to enzymatic hydrolysis to afford the diol in the serum. Curiously, the three carbonates (**3–5**) showed moderate activity in spite of nearly complete conversion to the inactive diol (Fig. 1).

Recently, the first low-molecular Rev-export inhibitor, leptomycin B (LMB), was clarified to be bound to Cys-529 of CRM1, the receptor of NES, in the yeast to inhibit nuclear export of Rev.[9] In this mechanism of action, the thiol function of cysteine was shown to be linked to the α,β-unsaturated lactone moiety in LMB by a covalent bond. In this context, we synthesized the biotinylated LMB probe[10] capturing CRM1 by biotin-avidin affinity technique and elucidated ACA (**1**) to inhibit Rev-export through the same fashion as LMB.[3] Furthermore, treatment of **1** with *N*-acetyl-L-cysteine methyl ester was found to provide the two adducts (**13** and **14**) readily.

To consider the disappointing biological activity of the carbamates (**8–12**), the most potent carbonate **5** and inactive carbamate **10** were also treated with *N*-acetyl-L-cysteine methyl ester. In regard to **5**, the same adducts were similarly afforded, while the carbamate **10** robuster in the serum than **1** suffered no modification upon this treatment. In addition to this chemical outcome, taking removal of the 4-*O*-acetyl group as well as equal formation of the diastereomers of **14** from treatment of **1** and **5** with *N*-acetyl-L-cysteine methyl ester into account, the two adducts would be provided through sequential formation of the monocarbonate **i** and quinone methide intermediate **ii** as depicted Scheme 2. Exertion of biological activity of ACA (**1**) and its analogs was, therefore, presumed to be significantly concerned with formation of these intermediates.

To confirm this hypothesis, some 4-*O*-acetyl analogs (**16–19**) were synthesized from the optical active acetate **15**[3] and evaluated for Rev-export inhibitory activity. Consequently, the analogs except for **19** bearing the isopropoxycarbonyl group inhibited Rev-export moderately (Fig. 2). Next, we planned to design the analogs of **1** by ingenious use of the above hypothesis. In general, the quinone methide **ii** was assumed to be formed through hydrolysis of the functional groups at C-4 (STEP 1) followed by elimination of the substituents at C-1′ (STEP 2) by way of the 4-hydroxy-intermediates **i**. Thus, we intended to design more potent analogs than **1** by utilizing the activation energy for STEP 1 and 2. As a result of calculating the activation energy for both steps with respect to ACA (**1**) and the 4-*O*-acetyl analogs (**16–19**), the hydrolysis step must be the rate-determining step to the quinone methide **ii**.[11] In addition, the activation energy for STEP 1 (E1) was found to show favorable correlation with Rev-export inhibitory activity as shown in Figure 2.

This finding led us to introduce electron-withdrawing halogen substituents at the ortho position of C-4 bearing the hydrolyzed functions to explore more potent analogs of **1**. Table 2 summarizes the activation energy (E1 and E2) of some ortho-halogenated analogs (**20a**–**20d**). As expected, introduction of the halogen atoms at the meta position of ACA (**1**) reduces E1 and noticeable decrease in E1 was observed for the 2,3-difluoroanalog (**20d**) in particular. Hence, we embarked on the synthesis of the four analogs and evaluation for their Rev-export inhibitory potency.

The synthesis of **20a**–**20d**[12] was executed as depicted in Scheme 3. Successive protection of the hydroxyl group in 3-fluoro-4-hydroxybenzaldehyde (**23a**) as *t*-butyldimethylsilyl (TBS) ether and introduction of the vinyl group by vinylmagnesium bromide afforded allyl alcohol **24a**. Dess–Martin periodinane oxidation of the alcohol provided ketone **25a** in 59% yield for three steps. Enantioselective reduction of **25a** with the complex of (*R*)-2-methyl-CBS-oxazaborolidine and $BH_3{\cdot}SMe_2$[13] afforded (*S*)-secondary alcohol **26a** in 72% yield with 90% ee. Removal of the TBS group by

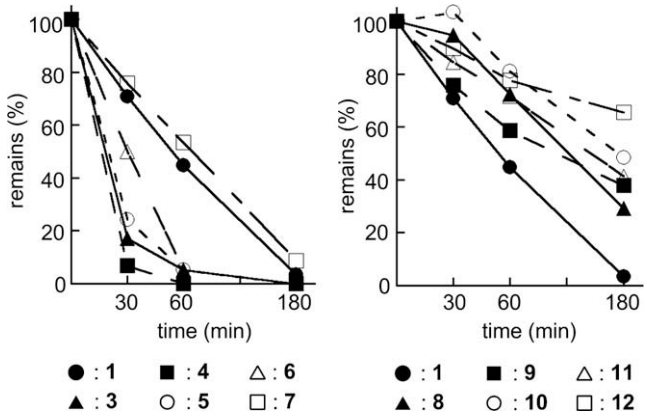
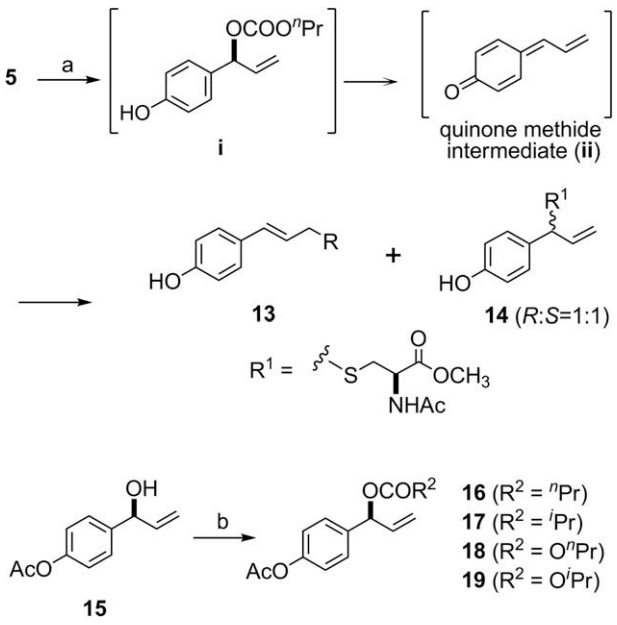
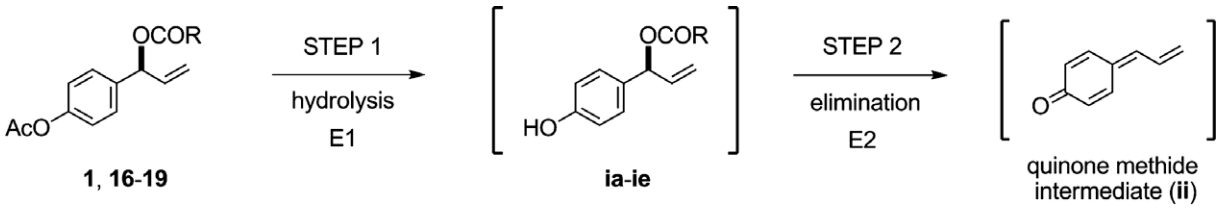


Figure 1. Stability of carbonates and carbamates of **1** in serum.



Scheme 2. Reagents and conditions: (a) *N*-acetyl-L-cysteine methyl ester, 1,4-dioxane, Tris buffer (pH 7.5), rt, 47% for **13**, 43% for **14**; (b) *n*PrCOCl, pyridine, rt, 74%; *i*PrCOCl, pyridine, rt, 73%; *n*PrOCOCl, pyridine, rt, 73%; *i*PrOCOCl, pyridine, rt, 72%.



compound	R	activation energy (kcal/mol) E1	E2	inhibitory activity for nuclear export of Rev (MIC)
1	Me	48.4	21.2	4.3 μM
16	^{n}Pr	49.1	21.8	19 μM
17	^{i}Pr	49.3	22.3	19 μM
18	O^{n}Pr	49.6	14.6	26 μM
19	O^{i}Pr	49.8	10.7	> 50 μM

Figure 2. Relationship between activation energy and Rev-export inhibitory activity in 4-*O*-acetyl analogs of **1**.

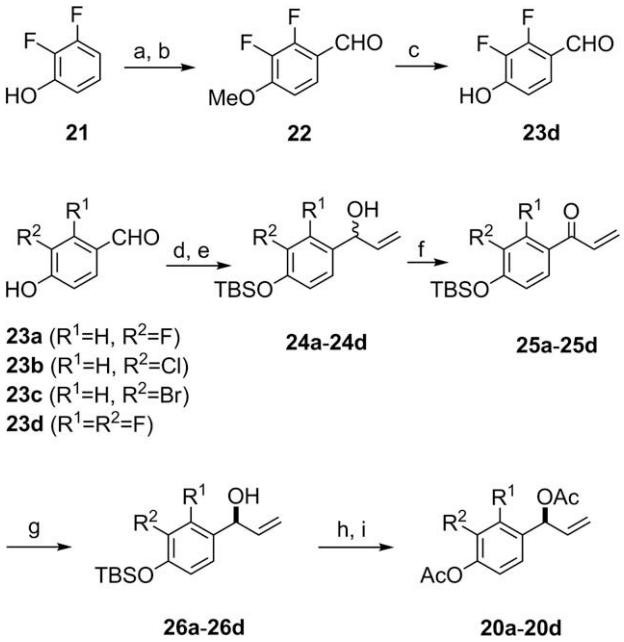
n-Bu_4NF followed by acetylation of the resulting diol with Ac_2O in pyridine furnished 1′-acetoxy-3-fluorochavicol acetate (**20a**) quantitatively from **26a**. The chloro and bromo analogs (**20b** and **20c**) were synthesized from the corresponding aldehydes (**23b** and **23c**) in the same manner as **20a**. The synthesis of the 2,3-difluoroanalog **20d** was similarly carried out after preparation for the starting aldehyde **23d** described as follows. Methylation of 2,3-difluorophenol (**21**) by MeI and K_2CO_3 followed by introduction of the formyl residue, which was achieved by treatment with dichloromethyl methyl ether and $TiCl_4$,[14] provided the hydroxyl-protected aldehyde **22**. Removal of the methyl group in **22** by treatment with BBr_3 afforded 2,3-difluoro-4-hydroxybenzaldehyde (**23a**).

The synthesized analogs were assessed for Rev-export inhibitory activity by using the fission yeast expressing the fusion protein of GST-NLS-GFP-RevNES. All the halogenated analogs more potently inhibited Rev-export than ACA (**1**). Among them, the difluoroanalog **20d** exhibited the most potent inhibitory activity. Moreover, we evaluated inhibitory effect for nuclear export of nearly genuine HIV-Rev in HeLa cells, transfected with HA-tagged Rev plasmids,[15] by indirect fluorescent antibody technique with regard to **1** and the four halogenated analogs.[3,16] In this bioassay, all the analogs (**20a**–**20d**) also showed more potent inhibitory activity than ACA (**1**) as listed in Table 3. Notably, the difluoroanalog **20d** with the lowest E1 most potently inhibited Rev-export in both assays and exhibited approximately eight-fold potent activity in the genuine system in comparison with **1**.

In the course of search for the robust analogs of ACA (**1**), we clarified formation of the quinone methide intermediate **ii** to be crucial for exerting Rev-export inhibitory activity of **1**. Based on this mechanism of action, we disclosed the four halogenated analogs **20a**–**20d** as the more potent active inhibitors than **1** by the rational design from the activation energy in the rate-determining step to **ii** by MO calculation. So far, several biological activities of **1**, in addition to inhibition for nuclear export of Rev, have been found and loss of the two acetyl functions has almost brought about significant reduction in the activities.[17–19] These findings

Table 2
Calculated activation energy of halogenated analogs of **1**

	Activation energy (kcal/mol)	
	E1	E2
ACA (**1**)	48.4	21.2
3-FluoroACA (**20a**)	43.6	23.4
3-ChloroACA (**20b**)	45.1	23.6
3-BromoACA (**20c**)	44.9	24.1
2,3-DifluoroACA (**20d**)	39.2	26.4



Scheme 3. Synthesis of halogenated analogs of **1**. Reagents and conditions: (a) MeI, K_2CO_3, DMSO, rt, quant.; (b) $TiCl_4$, $CHCl_2OMe$, CH_2Cl_2, rt, 75%; (c) BBr_3, CH_2Cl_2, rt, 52%; (d) TBSCl, imidazole, CH_2Cl_2, rt; (e) vinylmagnesium bromide, THF, rt, 71% for **24a**, 79% for **24b**, 77% for **24c**, 63% for **24d**, two steps; (f) Dess–Martin periodinane, CH_2Cl_2, rt, 83% for **25a**, quant. for **25b**, 88% for **25c**, 74% for **25d**; (g) (*R*)-2-methyl-CBS-oxazaborolidine, $BH_3{\cdot}SMe_2$, THF, −40 °C, 72% (90% ee) for **26a**, 69% (96% ee) for **26b**, 78% (99% ee) for **26c**, 33% (60% ee) for **26d**; (h) *n*Bu_4NF, THF, rt; (i) Ac_2O, pyridine, rt, quant. for **20a**, 91% for **20b**, 93% for **20c**, 88% for **20d**, two steps.

Table 3
Inhibitory activity of halogenated analogs of **1** for nuclear export of Rev

Compound	Model fusion protein in fission yeast (MIC, μM)	HA-Rev in HeLa cell (IC_{50}, μM)
ACA (**1**)	4.3	0.98
3-FluoroACA (**20a**)	1.7	0.48
3-ChloroACA (**20b**)	2.2	0.62
3-BromoACA (**20c**)	1.7	0.49
2,3-DifluoroACA (**20d**)	1.4	0.13

would indicate that the activities share the mechanism of action involving formation of the quinone methide intermediate **ii**. Thus, the logical design by the activation energy E1 would open up a new avenue to develop new agents using ACA (**1**) as a scaffold.

Acknowledgments

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7. After picking of an aliquot of colony of *S. pombe* on the agar, the yeasts were transferred and cultured in the thiamine-free MM-medium to induce the fusion protein for 24 h at 37 °C. Then the cells were seeded in 96-well microplates along with the test samples in the medium containing 1% DMSO and incubated at 37 °C for further 3 h. Distribution of the GST-NLS-GFP-RevNES-fused protein was monitored with a fluorescence microscope to determine MIC values.
8. Each sample (20 μL of 0.2 mM EtOH solution) was treated with RPMI1640 medium containing 10% fetal bovine serum (2 mL) and the whole was incubated at 37 °C for 0, 30, 60, 180 min, respectively. After extraction of the treated mixture with EtOAc (2 mL), each extract was concentrated under reduced pressure. The residue was dissolved with 100 μL of CH_3CN, then an aliquot (20 μL) of the solution was analyzed by reversed-phase HPLC [column: Mightysil RP-18 (5 μm) 4.6 × 250 mm, mobile phase: CH_3CN–H_2O (1:9→2:8, liner gradient, 5 min; 2:8, 5 min; 2:8→8:2, liner gradient, 20 min), flow rate = 1.0 mL/min, detection: UV 220 nm] to determine the remaining amount of the tested sample at the indicated times in triplicate. In this experiment, fetal bovine serum was diluted with RPMI1640 medium to modulate enzymatic activity of esterase.
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11. The activation energy for the E1 and E2 steps was calculated by MO calculation using SPARTAN '02 (Wavefunction) in the following protocol. At first, the chemical structures of ACA (**1**), the analogs, and H_2O were fully optimized by the semiempirical PM3 method. The oxygen atom of H_2O was vertically located to the carbonyl carbon linked to the oxygen at C-4 at a distance of 1.95 Å, then the potential energy of the stationary state was calculated. According to the reaction mechanism of hydrolysis of the acetyl group,[20] the primary structure of the transition state was respectively generated. Each transition state was further optimized by the semiempirical PM3 method to calculate the potential energy. The activation energy (E1 and E2) was determined by difference of the potential energy between the stationary state and transition state.
12. *Compound* **20a**: colorless oil, $[\alpha]_D^{24}$ −34.2 (*c* 1.0, EtOH), IR ν_{max} (KBr) cm^{-1}: 1765, 1740, 1608, ^1H NMR (300 MHz, $CDCl_3$) δ: 7.17 (1H, brd, *J* = 10.9 Hz, 2-H), 7.13 (1H, br d, *J* = 8.5 Hz, 6-H), 6.98 (1H, dd, *J* = 8.5, 6.1 Hz, 5-H), 6.23 (1H, d, *J* = 6.1 Hz, 1′-H), 5.95 (1H, ddd, *J* = 17.0, 10.4, 6.1 Hz, 2′-H), 5.32 (1H, d, *J* = 17.0 Hz, 3′-Ha), 5.28 (1H, d, *J* = 10.4 Hz, 3′-Hb), 2.33 (3H, s, 4-OAc), 2.12 (3H, s, 1′-OAc), FAB-MS (*m*/*z*): 253 $[M+H]^+$, HR FAB-MS (*m*/*z*): calcd for $C_{13}H_{13}FO_4$+H; 253.0876, found 253.0873.
Compound **20b**: colorless oil, $[\alpha]_D^{24}$ −40.8 (*c* 1.0, EtOH), IR ν_{max} (KBr) cm^{-1}: 1763, 1740, 1605, ^1H NMR (300 MHz, $CDCl_3$) δ: 7.44 (1H, d, *J* = 2.0 Hz, 2-H), 7.27 (1H, d, *J* = 8.3, 2.0 Hz, 6-H), 7.12 (1H, d, *J* = 8.3 Hz, 5-H), 6.22 (1H, br d, *J* = 5.9 Hz, 1′-H), 5.95 (1H, ddd, *J* = 17.1, 10.4, 5.9 Hz, 2′-H), 5.33 (1H, ddd, *J* = 17.1, 1.3, 1.1 Hz, 3′-Ha), 5.28 (1H, ddd, *J* = 10.4, 1.3, 1.1 Hz, 3′-Hb), 2.36 (3H, s, 4-OAc), 2.13 (3H, s, 1′-OAc), FAB-MS (*m*/*z*): 269 $[M+H]^+$, HR FAB-MS (*m*/*z*): calcd for $C_{13}H_{13}{}^{35}ClO_4$+H; 269.0581, found 269.0586, calcd for $C_{13}H_{13}{}^{37}ClO_4$+H; 271.0552, found 271.0554.
Compound **20c**: colorless oil, $[\alpha]_D^{24}$ −39.9 (*c* 1.0, EtOH), IR ν_{max} (KBr) cm^{-1}: 1766, 1738, 1610, ^1H NMR (300 MHz, $CDCl_3$) δ: 7.60 (1H, d, *J* = 2.0 Hz, 2-H), 7.31 (1H, dd, *J* = 8.3, 2.0 Hz, 6-H), 7.12 (1H, d, *J* = 8.3 Hz, 5-H), 6.22 (1H, d, *J* = 6.0 Hz, 1′-H), 5.95 (1H, ddd, *J* = 16.9, 10.2, 6.0 Hz, 2′-H), 5.33 (1H, d, *J* = 16.9 Hz, 3′-Ha), 5.28 (1H, d, *J* = 10.2 Hz, 3′-Hb), 2.36 (3H, s, 4-OAc), 2.13 (3H, s, 1′-OAc), FAB-MS (*m*/*z*): 313 $[M+H]^+$, HR FAB-MS (*m*/*z*): calcd for $C_{13}H_{13}{}^{79}BrO_4$+H; 313.0075, found 313.0071, calcd for $C_{13}H_{13}{}^{81}BrO_4$+H; 315.0056, found 315.0053.
Compound **20d**: colorless oil, $[\alpha]_D^{24}$ −21.6 (*c* 1.0, EtOH), IR ν_{max} (KBr) cm^{-1}: 1763, 1736, 1610, ^1H NMR (300 MHz, $CDCl_3$) δ: 7.13 (1H, ddd, *J* = 8.9, 6.9, 2.3 Hz, 5-H), 6.92 (1H, ddd, *J* = 8.9, 6.6, 2.1 Hz, 6-H), 6.48 (1H, br d, *J* = 6.0 Hz, 1′-H), 5.99 (1H, ddd, *J* = 17.2, 10.4, 6.0 Hz, 2′-H), 5.32 (1H, dd, *J* = 17.2, 1.1 Hz, 3′-Ha), 5.29 (1H, ddd, *J* = 10.4, 1.1, 1.1 Hz, 3′-Hb), 2.35 (3H, s, 4-OAc), 2.12 (3H, s, 1′-OAc), FAB-MS (*m*/*z*): 271 $[M+H]^+$, HR FAB-MS (*m*/*z*): calcd for $C_{13}H_{12}F_2O_4$+H; 271.0782, found 271.0785.
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16. HeLa cells (1.0×10^5 cells) were maintained on coverslips in 24-well microplate with 1 mL of Dulbecco's MEM medium supplemented with 10% FBS at 37 °C in 5% CO_2 for 24 h. Transfection of pCG-HA-Rev (plasmid encoding HA-tagged Rev protein) and pCRRE/ΔRev (plasmid encoding Gag protein) plasmids into HeLa cells were performed using PolyFect transfection reagent kit (QIAGEN) for 16 h according to the manufacturer's instructions. After the cells were washed, each solution of tested sample at an appropriate concentration in the medium containing 1% DMSO was inoculated and the whole was incubated at 37 °C for further 12 h. Cells were rinsed with cold D-PBS (−) twice and fixed with 4% formaldehyde/D-PBS (−) for 20 min. Then the cells were defatted with MeOH under shaking for 10 min and washed with cold D-PBS (−) thrice. After treatment with 10% FBS in Dulbecco's MEM medium for 30 min, the samples were incubated with anti-HA antibody (Roche) for 45 min followed by incubation with FITC-labeled anti-mouse IgG antibody (Vector) for 45 min. Localization of the HA-tagged Rev protein in the cells was examined with a fluorescence microscope, then image analysis was conducted by Scion image software (Scion) to determine Rev-export inhibitory activity. In the depicted pictures, several cells free from transfection displayed disperse weak fluorescence due to nonspecific binding of the antibodies.
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