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New Rev-export inhibitor from *Alpinia galanga* and structure–activity relationship

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ABSTRACT

Bioassay-guided separation by use of the fission yeast expressing NES of Rev, an HIV-1 viral regulatory protein, disclosed 1′-acetoxychavicol acetate (ACA, **1**) as a new inhibitor for nuclear export of Rev from the roots of *Alpinia galanga*. Both analysis for mechanism of action with biotinylated probe (**2**) and several synthesized analogs established crucial portions in **1** for Rev-export inhibitory activity.

The acquired immunodeficiency syndrome (AIDS) is a life-threatening disease caused by HIV-1[1] and the HIV pandemic remains one of the most serious threats to worldwide public health.[2] The HIV-Rev protein is an essential factor for viral replication and promotes nuclear-cytoplasm export of mRNA responsible for the production of viral structural proteins.[3] This export was shown to be mediated by the receptor protein, chromosomal region maintenance 1 (CRM1), through the direct binding to the nuclear export signal (NES) of Rev.[4] Since export of Rev is critical for viral replication, inhibition of this switch is an attractive strategy for therapeutic intervention.[5] Thus, we have been engaged in exploration for Rev-export inhibitors from medicinal plants. Previously, we revealed valtrate from Valerianae Radix as an inhibitor with anti-HIV activity.[6] Further exploration disclosed 1′-acetoxychavicol acetate (ACA, **1**)[7] to be another inhibitor for nuclear export of Rev from *Alpinia galanga*. Herein, we describe not only biological potency of **1** but also elucidation of pharmacophore and structure–activity relationship in **1** by use of a biotinylated probe (**2**) derived from a known inhibitor, leptomycin B (LMB)[4] and several synthesized analogs.

In search for Rev-export inhibitors, fission yeast *Schizosaccharomyces pombe*,[4] which express a model fusion protein consisting of glutathione *S*-transferase (GST), SV40 T antigen nuclear localization signal (NLS), green fluorescent protein (GFP), and Rev-NES, was utilized.[8] Among about 300 medicinal plants tested, the MeOH extract from *A. galanga* showed high inhibitory potency for nuclear export of Rev. Bioassay-guided separation of the extract led to the isolation of ACA (**1**) as a principle responsible for the biological activity with MIC of 4.7 μM. Recently, LMB was established 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 (**2**)[6] capturing CRM1 by biotin-avidin affinity technique and clarified valtrate to inhibit Rev-export through the same fashion as LMB. With respect to ACA (**1**), comparative analysis for mechanism of action between **1** and LMB was conducted in the same manner.[10] As shown in Figure 1, pre-treatment of HeLa cells with **1** at a concentration of 5 μM apparently reduced intensity of the band of CRM1 on SDS–PAGE, furthermore the band completely disappeared in the presence of not less than 10 μM of **1** in analogy with pre-treatment of LMB. Thus, ACA (**1**) was clarified to inhibit nuclear transport of Rev by direct binding of Cys-529 in CRM1 through the same mechanism of action as LMB. Next, the reactants of **1** and *N*-acetyl-cysteine methyl ester were examined to reveal the pharmacophore in **1** to bind to Cys-529 in CRM1. Treatment of **1** with *N*-acetyl-cysteine methyl ester in Tris–HCl buffer (pH 7.5) provided two sulfides **3** and **4** (a mixture of diastereomers in a ratio of 1:1) in 49% and 42% yield as depicted in Scheme 1. Consequently, the pharmacophore in **1** was unambiguously revealed to be 1′-acetoxyl-2′-ene moiety.

In order to elucidate participation of absolute configuration on C-1′ and the two acetyl functions into the biological activity, structure–activity relationship of ACA (**1**) was analyzed by synthesized analogs. In the first instance, enantioselective synthesis of **1** was executed as depicted in Scheme 2. Protection of a hydroxyl group

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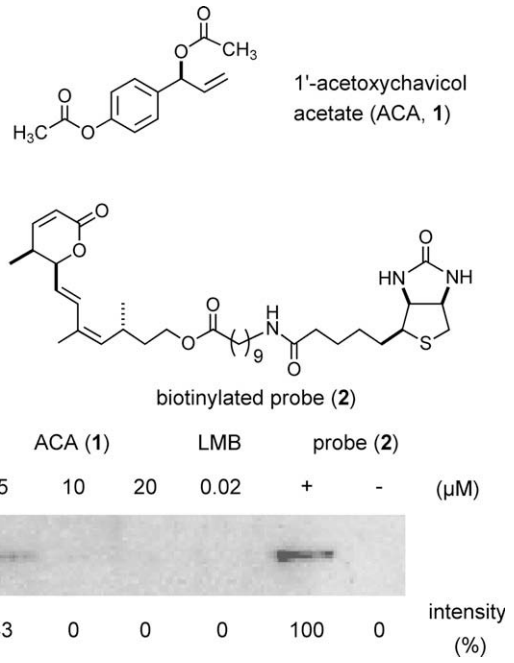
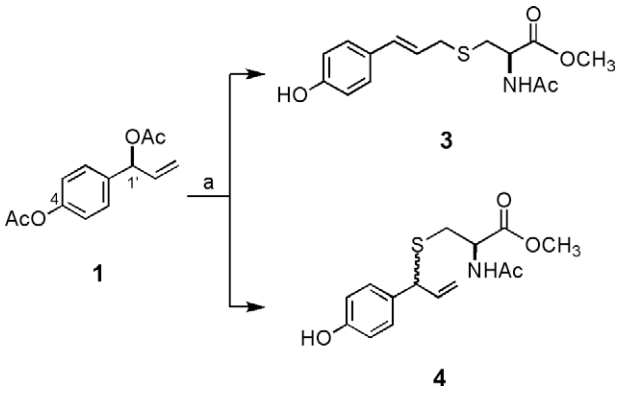


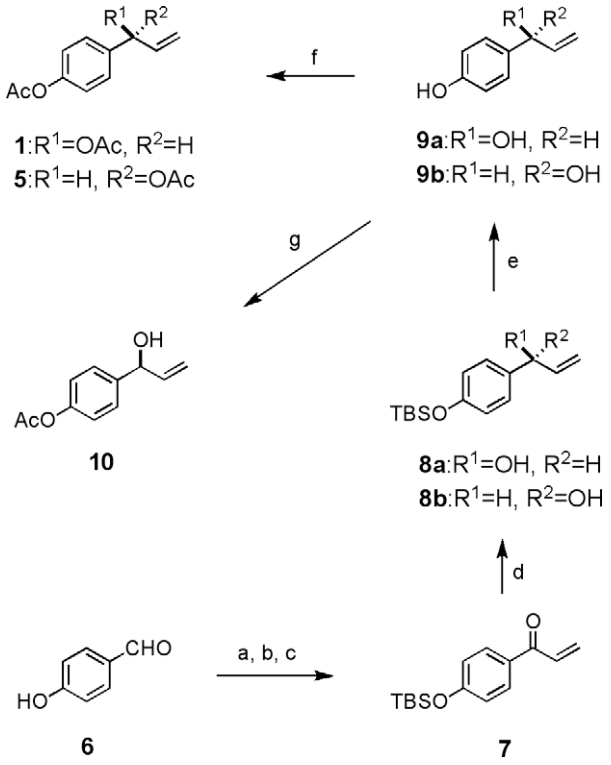
Figure 1. Comparative analysis for mechanism of action between ACA and LMB.



Scheme 1. Reagents and conditions: (a) *N*-acetyl-L-cysteine methyl ester, 1,4-dioxane, 0.2 M Tris–HCl buffer (pH 7.5), 49% for **3**, 42% for **4** (1:1 diastereomeric mixture).

in 4-hydroxybenzaldehyde (**6**) as a *t*-butyldimethylsilyl (TBS) ether followed by Grignard reaction with vinylmagnesium bromide to give a secondary alcohol. Dess–Martin periodinane oxidation of the alcohol provided a ketone **7** in 92% yield for three steps. Enantioselective reduction of **7** with borane dimethylsulfide complex in the presence of (*R*)-oxazaborolidine[11] afforded a secondary alcohol **8a** with desired *S*-configuration in 84% yield with 94% ee. Removal of the TBS group with *n*-Bu_4NF and successive acetylation of a resulting diol **9a** with Ac_2O in pyridine furnished (*S*)-1′-acetoxychavicol acetate (**1**) in 99% yield from **8a**. Synthesized ACA (**1**) was identified with the natural product isolated from *A. galanga* in comparison with both physicochemical and biological properties. On the other hand, treatment of **9a** with Ac_2O in pyridine at 0 °C gave 1′-*O*-deacetyl analog **10** quantitatively. Application of (*S*)-oxazaborolidine to asymmetric reduction of **7** gave a 1′-*R* alcohol **8b** in 81% yield with 94% ee. The alcohol **8b** was converted to (*R*)-1′-acetoxychavicol acetate (**5**) in 90% yield in the same manner.

Of the three analogs (**5**, **9a**, **10**),[12] 1′-*R* congener **5** only showed 10-fold less potent activity than ACA (**1**), while the remaining two analogs displayed little inhibition for Rev-export even at a concentration of 50 μM. Based on these biological outcomes, both 1′-*S* configuration and the two acetyl functions were clarified to be concerned with inhibitory potency for nuclear export of Rev together with 1′-acetoxyl-2′-ene moiety.



Scheme 2. Reagents and conditions: (a) *tert*-butyldimethylsilyl chloride, imidazole, CH_2Cl_2; (b) vinylmagnesium bromide, THF; (c) Dess–Martin periodinane, CH_2Cl_2, 92%; (d) (*R*)-oxazaborolidine, $BH_3{\cdot}SMe_2$, THF, −40 °C, 84% (94% ee) for **8a**; (e) *n*-Bu_4NF, THF, 99%; (f) Ac_2O, pyridine, quant.; (g) Ac_2O, pyridine, 0 °C, quant.

Finally, we evaluated inhibitory effect of ACA (**1**) for nuclear export of genuine HIV-Rev in HeLa cells transfected with HA (haemagglutinin)-tagged Rev plasmids[13] by indirect fluorescent antibody technique.[14] As depicted in Figure 2, Rev protein are distributed in both cytoplasm and nuclei in the absence of ACA (**1**), while treatment with 5 μM of **1** brings about localization of Rev in only nuclei. This result suggests that ACA (**1**) securely inhibits export of the genuine Rev protein from nucleus to cytoplasm.

In conclusion, we have disclosed the new Rev-export inhibitor from the nucleus to cytoplasm, 1′-acetoxychavicol acetate (ACA, **1**) from *A. galanga* according to bioassay-guided separation by use of fission yeast expressing the fusion proteins of GST-NLS-GFP-RevNES. Comparative analysis for mechanism of action using the biotinylated LMB probe **2** revealed **1** to inhibit nuclear export of Rev by linking to Cys-529 in CRM1, the receptor of NES in Rev.

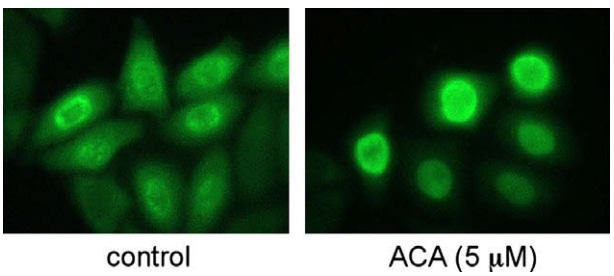


Figure 2. Inhibition for nuclear export of HA-tagged-Rev by ACA.

Taking the reactants of **1** with *N*-acetyl-cysteine methyl ester and synthesis of several analogs into consideration, 1′-*S* configuration and the two acetyl functions as well as 1′-acetoxyl-2′-ene moiety proved crucial for Rev-export inhibitory activity of **1**. In addition, ACA (**1**) was shown to inhibit export of the genuine Rev protein in HeLa cells expressing HA-tagged Rev by indirect fluorescent antibody technique. Exploration for synthetic leads possessing more potent activity than **1** is in progress in our laboratory.

Acknowledgments

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8. After picking of an aliquot of colony of *S. pombe* on the agar, the yeasts were transferred and cultured in thiamine-free MM-medium with inducing the fusion protein for 24 h at 37 °C. Then the cells were seeded in 96-well plates along with test samples in the medium containing 1% DMSO and incubated at 37 °C for further 3 h. The distribution of the GST-NLS-GFP-RevNES-fused protein was monitored by fluorescence microscope to determine MIC values.
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10. In culture dishes (3 cm i.d.), HeLa cells (6.0×10^5 cells) were cultured in 3 mL of Dulbecco's MEM medium containing with 10% fetal bovine serum at 37 °C in 5% CO_2 for 24 h. After the whole was washed, the cells were treated with 3 μM concentration of biotinylated LMB probe **2** in 1 mL of the medium containing 1% DMSO for 3 h. For competitive experiments, ACA (**1**) and LMB were injected 1 h prior to addition of **2**, respectively. The cells were harvested, then 0.2 mL of TBS lysis buffer (pH 7.5, 20 mM Tris–HCl, 0.1% NonidetP40, 0.15 mM NaCl, 2 M 2-mercaptoethanol, 1% protease inhibitor cocktail-DMSO) was added and the mixture was sonicated for 10 min at 0 °C. After centrifugation at 15000 rpm for 30 min, the supernatant was treated with 50 μL of 50% (v/v) beads immobilized with streptavidin in TBS lysis buffer under rotation at 4 °C overnight. The beads were rinsed thrice by the lysis buffer, then the bound proteins were eluted by SDS–PAGE sample buffer (50 μL) under boiling at 95 °C for 5 min. Each eluate was separated by 5–20% SDS–PAGE, then the proteins were transferred to PVDF membrane and the blot was blocked with 5% milk in TBS-T at 4 °C overnight. The membrane was incubated with primary antibody to CRM1 (Santa Cruz Biotech) at room temperature for 1 h. The bound antibodies were detected by treatment with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at room temperature for 1 h, then the blots were visualized using enhanced chemiluminescence.
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12. Compound **5**: colorless oil. $[\alpha]_D^{24}$ +49.5 (*c* 1.0, EtOH). IR (KBr): 3019, 1765, 1741, 1606 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.37 (2H, d, *J* = 8.5 Hz, 2-H), 7.07 (2H, d, *J* = 8.5 Hz, 3-H), 6.26 (1H, d, *J* = 6.1 Hz, 1′-H), 5.98 (1H, ddd, *J* = 6.1, 11.0, 17.1 Hz, 2′-H), 5.30 (1H, dd, *J* = 1.2, 17.1 Hz, 3′-Ha), 5.25 (1H, dd, *J* = 1.2, 11.0 Hz, 3′-Hb), 2.30 (3H, s, 4-OAc), 2.11 (3H, s, 1′-OAc). FAB-MS *m/z*: 235 [M+H$^+$]. FAB-HRMS *m/z*: Calcd for $C_{13}H_{15}O_4$: 235.0970, Found: 235.0966.
Compound **9a**: colorless oil. $[\alpha]_D^{24}$ −33.5 (*c* 1.0, EtOH). IR (KBr): 3350, 3025, 1608 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.24 (2H, d, *J* = 8.3 Hz, 2-H), 6.80 (2H, d, *J* = 8.3 Hz, 3-H), 6.04 (1H, ddd, *J* = 16.5, 10.5, 6.0 Hz, 2′-H), 5.33 (1H, d, *J* = 16.5 Hz, 3′-Ha), 5.17 (1H, d, *J* = 10.5 Hz, 3′-Hb), 5.16 (1H, d, *J* = 6.0 Hz, 1′-H). FAB-MS *m/z*: 151 [M+H]$^+$. FAB-HRMS *m/z*: Calcd for $C_9H_{11}O_2$: 151.0759, Found: 151.0762.
Compound **10**: colorless oil. $[\alpha]_D^{24}$ −36.3 (*c* 1.0, EtOH). IR (KBr): 3265, 3022, 1767, 1606 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.38 (2H, d, *J* = 8.5 Hz, 2-H), 7.07 (2H, d, *J* = 8.5 Hz, 3-H), 6.03 (1H, ddd, *J* = 16.8, 10.0, 5.6 Hz, 2′-H), 5.35 (1H, d, *J* = 16.8 Hz, 3′-Ha), 5.25 (1H, d, *J* = 10.0 Hz, 3′-Hb), 5.21 (1H, d, *J* = 5.6 Hz, 1′-H), 2.30 (3H, s, OAc). FAB-MS *m/z*: 193 [M+H]$^+$. FAB-HRMS *m/z*: Calcd for $C_{11}H_{13}O_3$: 193.0864, Found: 193.0866.
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14. 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 under 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.