



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.

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The acquired immunodeficiency syndrome (AIDS) is a life-threatening disease caused by HIV-1¹ 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 *Valeriana 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 μ M. 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 μ M apparently reduced intensity of the band of CRM1 on SDS-PAGE, furthermore the band completely disappeared in the presence of not less than 10 μ M 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'-acetoxy-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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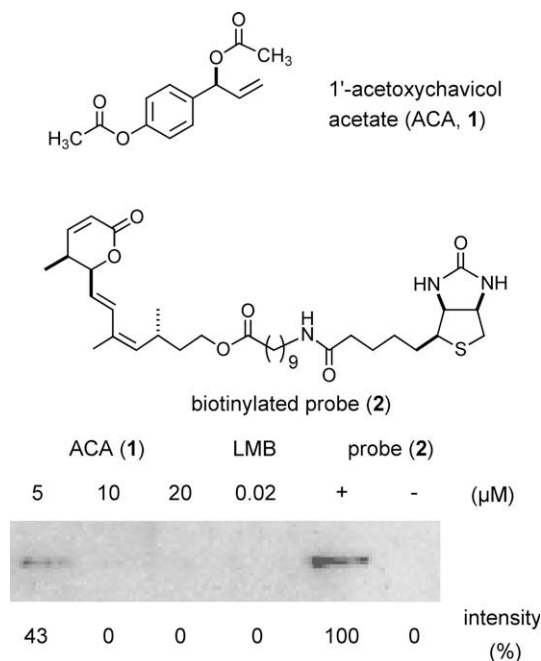
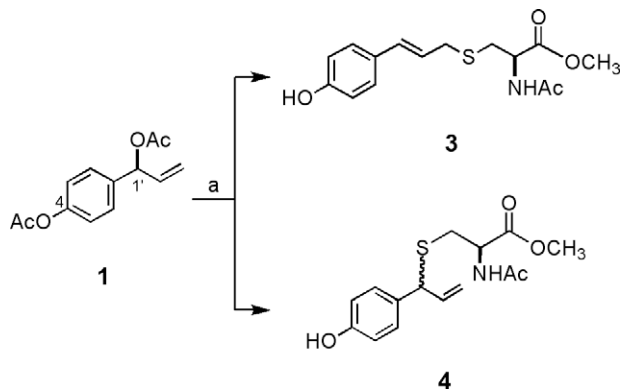


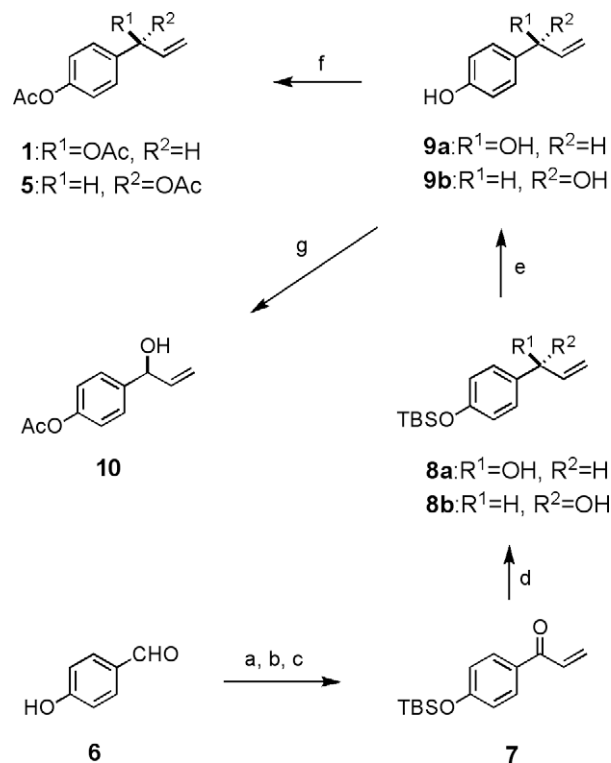
Figure 1. Comparative analysis for mechanism of action between ACA and LMB.



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*)-oxazaborolidine¹¹ afforded a secondary alcohol **8a** with desired *S*-configuration in 84% yield with 94% ee. Removal of the TBS group with *n*-Bu₄NF and successive acetylation of a resulting diol **9a** with Ac₂O 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 Ac₂O 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 concen-



Scheme 2. Reagents and conditions: (a) *tert*-butyldimethylsilyl chloride, imidazole, CH₂Cl₂; (b) vinylmagnesium bromide, THF; (c) Dess–Martin periodinane, CH₂Cl₂, 92%; (d) (*R*)-oxazaborolidine, BH₃·SMe₂, THF, −40 °C, 84% (94% ee) for **8a**; (e) *n*-Bu₄NF, THF, 99%; (f) Ac₂O, pyridine, quant.; (g) Ac₂O, pyridine, 0 °C, quant.

tration 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'-acetoxy-2'-ene moiety.

Finally, we evaluated inhibitory effect of ACA (**1**) for nuclear export of genuine HIV-Rev in HeLa cells transfected with HA (haemagglutinin)-tagged Rev plasmids¹³ 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.

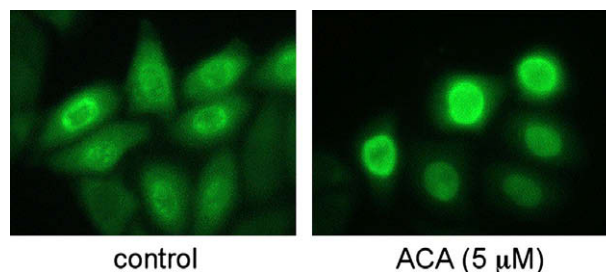


Figure 2. Inhibition for nuclear export of HA-tagged-Rev by ACA.

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.

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- 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, CDCl₃) 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 C₁₃H₁₅O₄: 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, CDCl₃) 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 C₉H₁₁O₂: 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, CDCl₃) 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 C₁₁H₁₃O₃: 193.0864, Found: 193.0866.
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