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Unprecedented NES non-antagonistic inhibitor for nuclear export of Rev from *Sida cordifolia*

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ABSTRACT

Bioassay-guided separation from the MeOH extract of the South American medicinal plant *Sida cordifolia* resulted in isolation of (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**) as an unprecedented NES non-antagonistic inhibitor for nuclear export of Rev. This mechanism of action was established by competitive experiment by the biotinylated probe derived from leptomycin B, the known NES antagonistic inhibitor. Additionally, structure–activity relationship analysis by use of the synthesized analogs clarified cooperation of several functionalities in the Rev-export inhibitory activity of **1**.

The acquired immunodeficiency syndrome (AIDS) is a life-threatening disease caused by HIV-1 virus.[1] In recently, the HIV pandemic has remained one of the most serious threat to worldwide public health.[2] Replication of HIV-1 entails an ordered pattern of the viral gene expression dependent on the viral regulatory protein, Rev. Namely, the Rev protein facilitated nuclear-cytoplasm export of mRNA responsible for production of the viral proteins and is regarded as an essential factor for viral replication.[3] Recently, transport of Rev was shown to be mediated by the receptor protein, chromosomal region maintenance 1 (CRM1), through tight interaction to a specific leucine-rich nuclear export signal (NES) of Rev.[4] Since export of Rev was crucial for viral proliferation, an inhibition for this function of Rev 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 and 1′-acetoxychavicol acetate as active principles with anti-HIV activity. Moreover, they were shown to inhibit export of Rev due to NES antagonistic mode as well as the known inhibitor leptomycin B,[6] the metabolite of actinomyces.[7,8] On the other hand, no NES non-antagonistic Rev-export inhibitors have been found out in spite of promising potentiality for anti-HIV leads with novel mechanism of actions. This circumstance prompted us to explore unprecedented NES non-antagonistic inhibitors for nuclear export of Rev from medicinal plants. Here, we deal with not only biological behavior of (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**), the first NES non-antagonistic inhibitor for Rev-export, isolated from the South American medicinal plant *Sida cordifolia* but also structural requirement for biological potency of **1** by structure–activity relationship analysis among the synthesized analogs.

In the course of search for Rev-export inhibitors, we utilized fission yeast[4] expressing the fusion protein, which consists of glutathione *S*-transferase (GST), SV40 T-antigen nuclear localization signal (NLS), green fluorescent protein (GFP), and Rev-NES.[7] The fusion protein possesses fairly different sequence of amino acids and conformation except for the region around Rev-NES. Accordingly, the bioassay using this fission yeast is intensively anticipated to bring about the inhibitors with NES antagonistic mode. Based on this consideration, we designed to enroll another bioassay, in which distribution of the whole Rev protein tagged with HA in HeLa cells was monitored by indirect fluorescent antibody technique,[8,9] to search for NES non-antagonistic inhibitors for nuclear export of Rev. In brief, the desired NES non-antagonistic inhibitor interrupted only nuclear export of HA-Rev in HeLa cells.

As a result of screening about 400 extracts from medicinal plants by combination of the two bioassays, the MeOH extract of the South American medicinal plant *S. cordifolia* (powder of the

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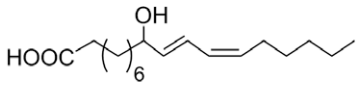


(10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**)

Figure 1. NES non-antagonistic inhibitor for nuclear export of Rev from *S. cordifolia*.

whole plants) was found out as a promising candidate. After this extract was successively partitioned between EtOAc and H_2O, *n*-BuOH and H_2O, the resulting EtOAc extract was found to exhibit the most potent activity among the three extracts. Subsequently, the EtOAc extract was subjected to successive separation by SiO_2 column, ODS column chromatography, and reversed phase HPLC under the guidance of bioassay to give the active principle exhibiting 54.7% of inhibition for nuclear export of Rev at the concentration of 10 μg/mL. On the basis of comparison of spectroscopic data such as ^1H, ^{13}C NMR, and IR with those reported[10] as well as the molecular formula of $C_{18}H_{40}O_3$ determined by HR FAB-MS, this active principle was identified to be (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**) (Fig. 1). To examine optical purity of **1**, the optical rotation of the corresponding methyl ester **1a** was compared with that reported after conversion by trimethylsilyldiazomethane treatment in MeOH. However, optical rotation of **1a** is +1.5° far from the reported datum[11] of *S*-**1a**, +8.3° in measurement under the same condition (*c* 0.3, $CHCl_3$). In addition, HPLC analysis by chiral column presented **1** to be a mixture of *R*- and *S*-isomers in a ratio of 40:60. On the other hand, both optical pure *R*- and *S*-isomers separated by chiral HPLC exhibited as potent activity as the isolated **1**, indicative of no participation of the configuration at C-9 in the biological potency.

Next, we performed comparative analysis of the mechanism of action of (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**) in the competitive experiment using our biotinylated probe **2**[7] derived from leptomycin B, the NES antagonistic inhibitor. As depicted in Figure 2, pre-incubation of **1** prior to addition of **2** resulted in little reduction of intensity of CRM1 captured by the probe **2** even at the concentration of 100 μM. Consequently, **1** was clarified to inhibit nuclear export of Rev with NES non-antagonistic mode, completely distinct from the known NES antagonistic inhibitors, leptomycin B, valtrate, and 1′-acetoxychavicol acetate. Since (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**) was previously reported as an anti-HIV principle,[12] inhibition for nuclear export of Rev with NES non-antagonistic mode was revealed to be one of the mechanism of actions related to anti-HIV activity of **1**.

Subsequently, structure–activity relationship was examined to elucidate crucial partial structures for Rev-export inhibitory activity of **1**. Namely, we designed two analogs **3** and **4** containing the shortened alkyl chains between the hydroxyl and carboxylic groups, the hydroxycarboxylic acid **5** lacking the nona-1,3-diene moiety, the hydroxydiene **6** devoid of the 1-carboxyhexyl portion, and the fully saturated analog **7** along with the commercially available deoxoanalog **8** (Fig. 3).

The synthesis of these analogs was executed as shown in Scheme 1. Condensation of the known aldehydes (**12a** and **b**),[13,14] prepared according to the described procedures, with bis-(tri-*n*-butylstannyl)-ethylene (**13**) in the presence of *n*-BuLi afforded vinylstannyl alcohols (**14a** and **b**). Respective Stille coupling between **14a** or **14b**, and (*Z*)-1-iodo-1-heptene **15**[15] followed by saponification furnished the desired two analogs (**3** and **4**).[16] In the same manner, (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**) was also synthesized from **11** in a racemic form in 29.4% overall yield for four steps. Our synthetic route of **1** was shorter than the previous protocols in spite of similarity in the overall yields.[17–19]

Treatment of aldehyde **12c**[20] with methylmagnesium bromide afforded secondary alcohol **16**, which was subjected to alkaline hydrolysis to provide the analog **5**. On the other hand, Sonogashira coupling between 3-butyn-2-ol (**17**) with iodide **15** and the following Red-Al® reduction gave the analog **6** by way of ene-yne alcohol **18**. Successive treatment of the aldehyde **12c** with ethynylmagnesium chloride followed by introduction of 1-heptenyl function by Sonogashira coupling afforded conjugated ene-yne alcohol **20**. The alcohol was subjected to hydrogenation in the presence of Pd–C under a H_2 atmosphere and subsequent alkaline hydrolysis by aq NaOH to furnish the analog **7**.

Table 1 summarizes inhibitory activity of **1** and the analogs (**3**–**8**) for nuclear export of Rev in HeLa cells. Among the six analogs, the three analogs **4**, **5**, and **8** exhibited about two or threefold less potent activity than **1**, while the other analogs (**3**, **6**, and **7**) showed less than 50% inhibition for Rev-transport even at the concentration of 30 μM. Based on these findings, the carboxyl moiety, the

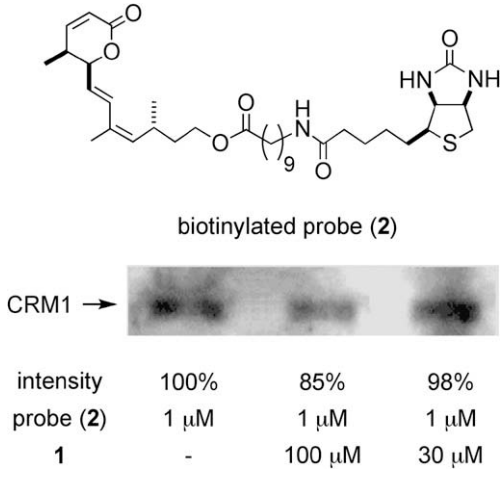


Figure 2. Comparative analysis of mechanism of action between **1** and leptomycin B by use of biotinylated probe.

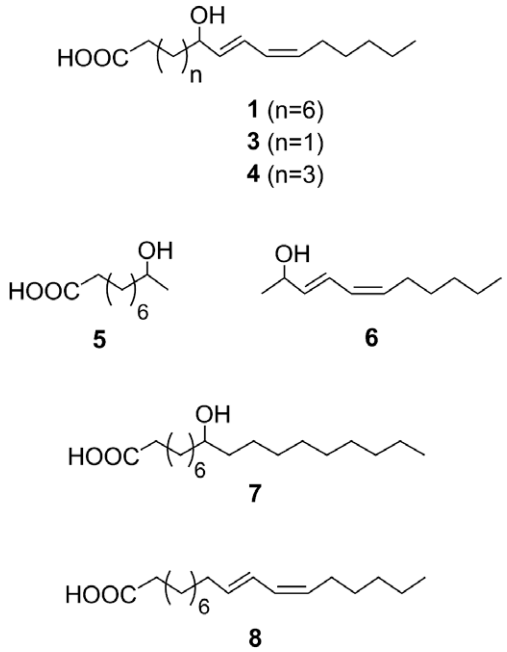
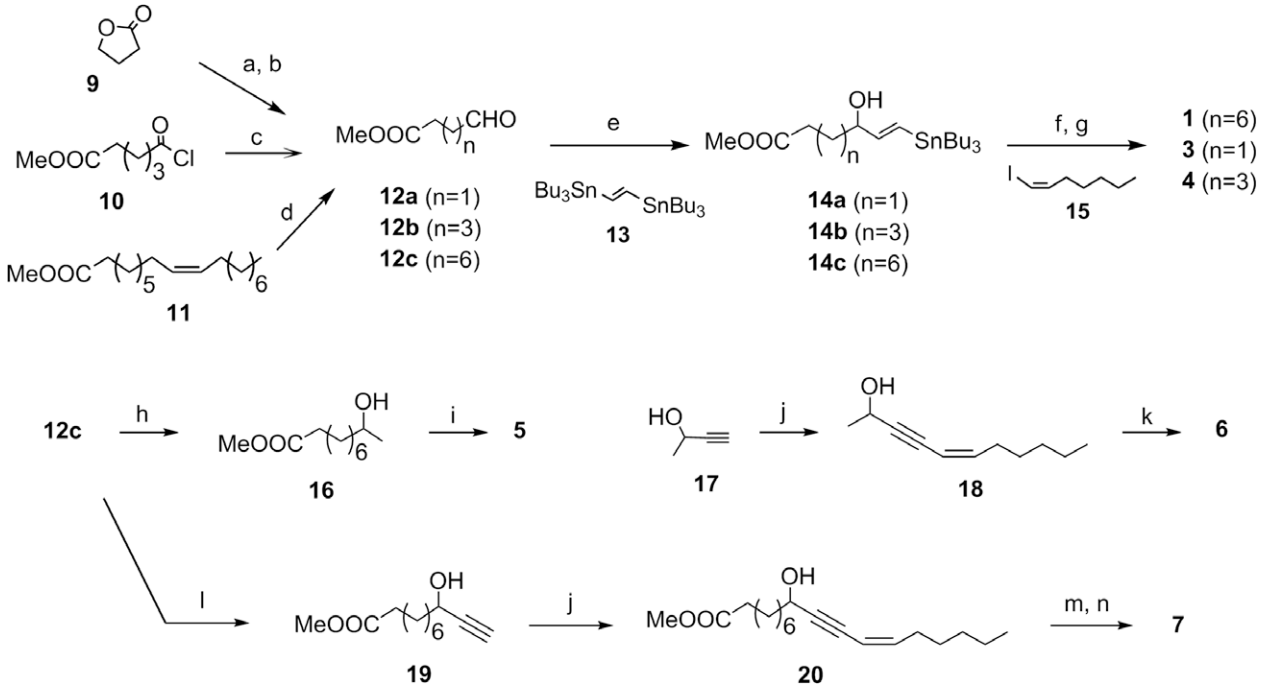


Figure 3. Analogs of **1** for structure–activity relationship analysis.



Scheme 1. Synthesis of analogs of **1**. Reagents and conditions: (a) NaOMe, MeOH; (b) $(COCl)_2$, dimethylsulfoxide, Et_3N, −78 °C, 85% for two steps; (c) H_2, Pd–C, 2,6-lutidine, 73%; (d) O_3, CH_2Cl_2 then Me_2S, 97%; (e) **13**, *n*BuLi, THF, 53% (*n* = 1), 50% (*n* = 3), 55% (*n* = 6); (f) **15**, $PdCl_2(CH_3CN)_2$, DMF, 60% (*n* = 1), 62% (*n* = 3), 67% (*n* = 6); (g) K_2CO_3, aq MeOH, 95% for **3** (*n* = 1), 98% for **4** (*n* = 3), quant. for **1** (*n* = 6); (h) MeMgBr, THF; (i) NaOH, aq MeOH, 70% from **12c**; (j) **15**, $PdCl_2(CH_3CN)_2$, CuI, piperidine, 74% for **18**, 62% for **20**; (k) Red-Al, THF, 67%; (l) ethynylmagnesium chloride, THF, 68%; (m) H_2, Pd–C, MeOH, 78%; and (n) NaOH, aq MeOH, 80%.

Table 1
Inhibitory activity of **1** and analogs for nuclear export of Rev

	IC_{50} (μM)
(10*E*,12*Z*)-9-Hydroxyoctadeca-10,12-dienoic acid (**1**)	7.2
Trideca analog (**3**)	21.4
Pentadeca analog (**4**)	>30
C1–C10 analog (**5**)	20.8
C8–C18 analog (**6**)	>30
Saturated analog (**7**)	>30
Dehydroxyanalog (**8**)	15.0

conjugated diene portion, the distance between these two functions, and the hydroxyl group at C-9 of **1** were presumed to participate in inhibition for nuclear export of Rev, cooperatively.

In conclusion, bioassay-guided separation from the MeOH extract of the South American medicinal plant *S. cordifolia* resulted in isolation of (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid (**1**) as the unprecedented NES non-antagonistic inhibitor for nuclear export of Rev. This mechanism of action was established by the competitive experiment with the biotinylated probe **2** derived from leptomycin B, the known NES antagonistic inhibitor. Additionally, structure–activity relationship analysis by using the synthesized analogs clarified cooperation of the carboxyl moiety, the conjugated diene portion, the distance between these functions, and the 9-hydroxyl group in the Rev-export inhibitory activity of **1**.

Acknowledgments

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16. *Compound* **3**: a white amorphous powder. IR (KBr): 3310, 1758, 1642 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 6.53 (1H, dd, *J* = 15.2, 11.0 Hz, 6-H), 5.97 (1H, dd, *J* = 11.0, 10.5 Hz, 7-H), 5.65 (1H, dd, *J* = 15.2, 7.0 Hz, 5-H), 5.47 (1H, dt, *J* = 10.5, 7.0 Hz, 8-H), 4.25 (1H, m, 4-H), 2.51 (2H, t, *J* = 7.0 Hz, 2-H), 2.16 (2H, m, 9-H), 1.25–1.94 (8H, m, 3-, 10-, 11-, 12-H), 0.89 (3H, t, *J* = 6.9 Hz, 13-H). FAB-MS *m*/*z*: 227 $[M+H]^+$. FAB-HRMS *m*/*z*: Calcd for $C_{13}H_{23}O_3$: 227.1647. Found: 227.1650. *Compound* **4**: a white amorphous powder. IR (KBr): 3280, 1756, 1642 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 6.48 (1H, dd, *J* = 15.5, 11.5 Hz, 8-H), 5.97 (1H, dd, *J* = 11.5, 10.5 Hz, 9-H), 5.65 (1H, dd, *J* = 15.5, 7.5 Hz, 7-H), 5.45 (1H, dt, *J* = 10.5, 7.9 Hz, 10-H), 4.18 (1H, m, 6-H), 2.36 (2H, t, *J* = 7.6 Hz, 2-H), 2.18 (2H, m, 11-H), 1.26–1.71 (12H, m, 3-, 4-, 5-, 12-, 13-, 14-H), 0.89 (3H, t, *J* = 6.7 Hz, 15-H). FAB-MS *m*/*z*: 255 $[M+H]^+$. FAB-HRMS *m*/*z*: Calcd for $C_{10}H_{19}O_3$: 255.1960. Found: 255.1964.
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