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Prenylcoumarin with Rev-export inhibitory activity from Cnidii Monnieris Fructus

Satoru Tamura [a], Toshiaki Fujitani [a], Masafumi Kaneko [b], Nobutoshi Murakami [a,b,*]

[a] Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan
[b] PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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ABSTRACT

By use of the fission yeast expressing the model fusion protein comprised of GST, SV40 T antigen NLS, GFP, and Rev-NES in the bioassay, the prenylcoumarin osthol (**1**) was disclosed as the new Rev-export inhibitor from the MeOH extract of Cnidii Monnieris Fructus. Furthermore, **1** was also found to inhibit export the genuine Rev in HeLa cells by indirect fluorescent antibody technique. By the competitive experiment using the biotinylated probe **3**, osthol (**1**) was revealed to inhibit nuclear export of Rev through a NES non-antagonistic mode. Structure–activity relationship analysis of several analogs of **1** clarified that both prenyl side chain and double bond adjacent to the lactone carbonyl residue play an important role in the Rev-export inhibitory potency of **1**.

The acquired immunodeficiency syndrome (AIDS) is a life-threatening disease caused by HIV-1.[1] Replication of HIV-1 entails an ordered pattern of the viral gene expression, which is dependent on the viral regulatory protein, Rev.[2] Rev acts to increase cytoplasmic accumulation of the viral mRNAs by transport from the nucleus to the cytoplasm with the aid of the cargo protein CRM1.[3,4] Since this process is essential for viral replication, inhibition for export of Rev is an attractive strategy for therapeutic intervention.[5]

In this context, we have been devoted to exploring Rev-export inhibitors from medicinal plants to find out the several active principles.[6–8] In addition, their mechanism of actions were compared with the first low-molecular inhibitor, leptomycin B (LMB),[4] by using the biotinylated probe derived from LMB. Further investigation on search for new Rev-export inhibitors resulted in isolation of the prenylcoumarin, osthol (**1**) from Cnidii Monnieris Fructus. Herein, we describe not only the bioassay-guided isolation, identification, and biological property but also the structural requirement for the Rev-export inhibitory potency with respect to **1**.

To search for the Rev-export inhibitors, the potency was evaluated by using the fission yeast *Schizosaccharomyces pombe*,[4] which express the model fusion protein consisting of glutathione *S*-transferase (GST), SV40 T antigen nuclear localization signal (NLS), green fluorescent protein (GFP), and the nuclear export signal (NES) of Rev. Rev was shown to export from the nucleus to the cytoplasm by recognition of CRM1, the receptor of Rev-NES. In brief, the fusion protein imported by NLS is subjected to interruption of nuclear export to cluster in the nucleus in the presence of inhibitors.

As a result of screening about 400 extracts from medicinal plants by using this bioassay,[9] the MeOH extract of Cnidii Monnieri Fructus (fruits of *Cnidium monnieri* Cusson) was revealed to be 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 and reversed phase HPLC under the guidance of bioassay to give the active principle (**1**, 0.39% from the crude drug) with IC_{50} of 9.8 μM in the bioassay by the fission yeast. Intensive analysis of ^1H and ^{13}C NMR, IR, FAB-MS, and FAB HRMS spectra unambiguously identified the active principle as the prenylcoumarin, osthol (**1**).[10]

Furthermore, we evaluated inhibitory effect of osthol (**1**) for nuclear export of genuine HIV-Rev in HeLa cells, in which the HA-tagged Rev plasmids[11] were transfected, by indirect fluorescent antibody technique.[12] In this precision bioassay, Rev protein is distributed in both cytoplasm and nucleus in the absence of **1**, while treatment with **1** at a concentration of 5 μM brings about localization of Rev in only the nuclei as shown in Figure 1. In addition, osthol (**1**) inhibits nuclear export of Rev in HeLa cells with IC_{50} of 1.6 μM. This result suggests that osthol (**1**) securely inhibits export of genuine Rev protein from the nucleus to the cytoplasm.

* Corresponding author. Present address: Kyoto Pharmaceutical University. Tel.: +81 75 595 4634; fax: +81 75 595 4768.
E-mail address: murakami@poppy.kyoto-phu.ac.jp (N. Murakami).

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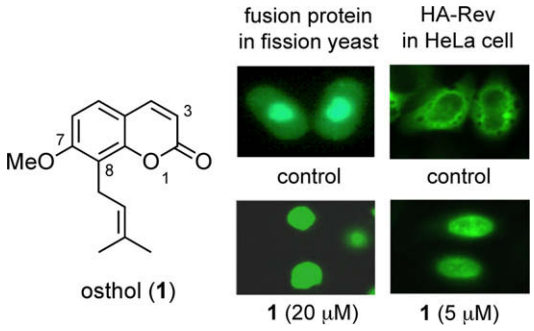


Figure 1. Osthol (**1**), Rev-export inhibitor from Cnidii Monnieris Fructus.

Subsequently, the mechanism of action of osthol (**1**) was compared with that of LMB (**2**), the first low-molecular inhibitor for Rev-export, by use of our synthesized probe **3** designed from **2**. Previously, LMB (**2**) was found to be bound to Cys-529 of CRM1, the receptor of NES, by a covalent bond.[13] Furthermore, the probe **3** was revealed to capture CRM1, whereas the potency almost completely disappeared in the presence of the competitive NES antagonistic inhibitors, valtrate and 1′-acetoxychavicol acetate (ACA) as well as LMB. After prior addition of osthol (**1**) to HeLa cells, the whole was incubated with probe **3** (1.0 μM). Lysate of the harvested cells was treated with streptavidin beads, thereafter the proteins attached to the beads were detected by an immunoblotting technique.[14]

As shown in Figure 2, pre-treatment of osthol (**1**) even at a concentration of 100 μM obviously presents the band due to CRM1 and this behavior is analogous to treatment of only probe **3**. On the other hand, the band of CRM1 completely disappears by pre-treatment of LMB. Taking these experimental outcomes together with the IC_{50} values of **1** (1.6 μM) and **3** (1.2 μM) into account, osthol (**1**) was intensively presumed to inhibit nuclear export of Rev through a NES non-antagonistic mode. Since osthol (**1**) was previously reported as an anti-HIV principle,[15] inhibition for nuclear export of Rev was revealed to be one of the mechanism of actions related to anti-HIV activity of **1**.

Previously, LMB was found to form the stable Michael adduct with *N*-acetyl-L-cysteine methyl ester at the α,β-unsaturated γ-lactone portion.[13] In addition, the NES antagonistic Rev-export inhibitors, valtrate and ACA found by our group, similarly afforded the adducts on treatment with *N*-acetyl-L-cysteine methyl ester.[6,7] On the contrary, osthol (**1**) gave no cysteine adducts under the same treatment in spite of possessing the α,β-unsaturated

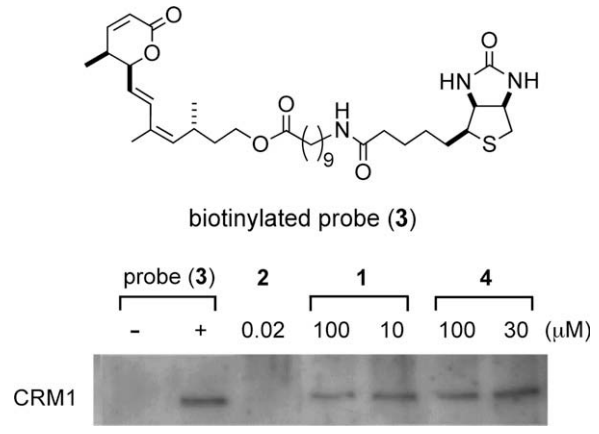


Figure 2. Comparative analysis of mechanism of action of osthol (**1**) and 7-demethoxyanalog **4**.

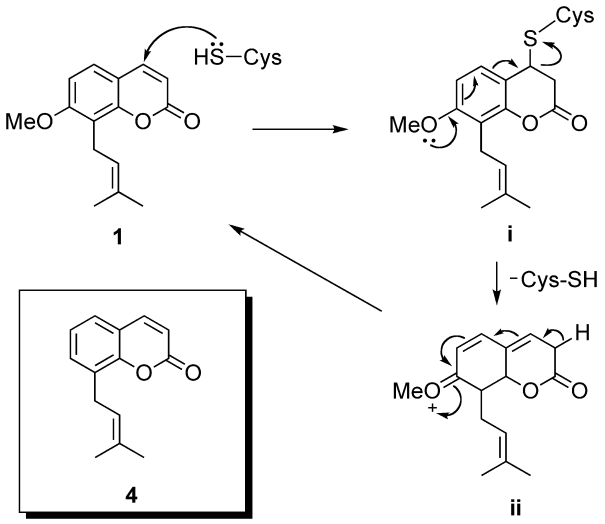
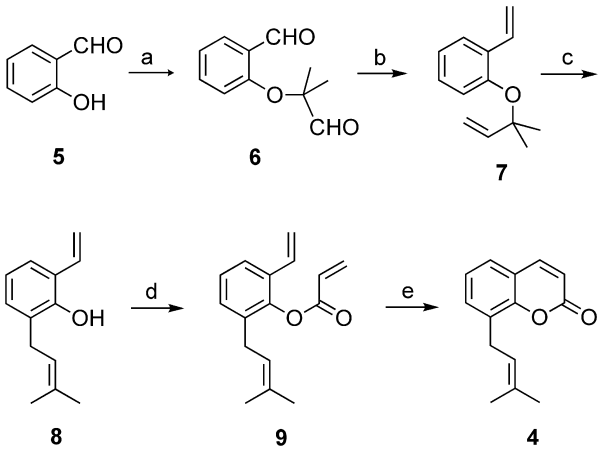


Figure 3. Postulation leading to synthesis of 7-demethoxyanalog **4**.

δ-lactone moiety. In the case of **1**, the plausible cysteine adduct **i** was assumed to revert by way of the cation intermediate **ii** due to the electron-donating methoxyl group at C-7, which would lead to interrupt labeling of CRM1 by the probe **3** in the co-presence of osthol (**1**) (Fig. 3). To examine this possibility, we synthesized the demethoxyanalog **4** and evaluated it for Rev-export inhibitory activity.

The synthesis of the demethoxyanalog **4** was conducted as depicted in Scheme 1. Introduction of the 1-formyl-1-methylethyl group to salicylaldehyde (**5**) was carried out by 2-bromo-2-methylpropanal in the presence of potassium *t*-butoxide and 18-crown-6 to provide dial **6**, which was subjected to Wittig olefination by using methyltriphenylphosphonium bromide (PH_3PCH_3Br) and sodium hexamethyldisilazide (NaHMDS) to give diene **7**. Claisen rearrangement of the diene **7** followed by acylation using acryloyl chloride and triethylamine gave α,β-unsaturated ester **9** via **8**. Ring-closing metathesis of the ester **9** by the second generation of Grubbs' catalyst[16] furnished the desired demethoxyanalog **4** (Scheme 1).[17]

In HeLa cells, the synthesized analog **4** was shown to inhibit export of Rev with IC_{50} of 6.4 μM. This biological outcome indicates that the methoxyl group at C-7 enhances Rev-export inhibitory activity. Despite the moderately potent activity in comparison with



Scheme 1. Synthesis of 7-demethoxyanalog **4**. Reagents and conditions: (a) 2-bromo-2-methylpropanal, *t*-BuOK, 18-crown-6, CH_3CN, rt, 76%; (b) Ph_3PCH_3Br, NaHMDS, THF, rt; (c) 120 °C, DMF; (d) acryloyl chloride, Et_3N, CH_2Cl_2, rt, 50%, three steps; (e) second generation Grubbs' catalyst, CH_2Cl_2, reflux, 65%.

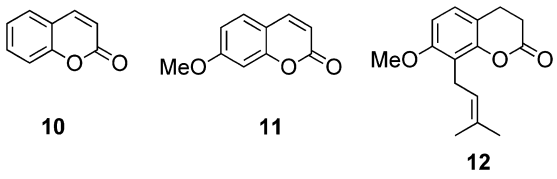


Figure 4. Congeners used for structural requirement of biological potency of **1**.

osthol (**1**), the demethoxyanalog **4** exhibited nearly the same biological behavior as **1** in the competitive experiment with the biotinylated probe **3** (Fig. 2). Thus, osthol (**1**) was clarified to inhibit Rev-export through the NES non-antagonistic mode different from LMB, valtrate, and ACA.

Finally, we analyzed the structural requirement of the biological potency of **1** by use of the two commercially available coumarins (**10** and **11**) and the synthesized analog **12** lacking the double bond conjugated to the lactone carbonyl residue (Fig. 4). The synthesis of 3,4-dihydroanalog is illustrated in Scheme 2. Namely, 2-hydroxy-4-methoxybenzaldehyde (**13**) was subjected to Wittig two-carbon homologation by (carbetoxymethylene)triphenylphosphorane to provide α,β-unsaturated ester **14**. Successive hydrogenation under a H_2 atmosphere and installation of the 1-formyl-1-methylethyl group for the ester **14** afforded aldehyde **15**. Conversion of the formyl function in **15** to the terminal olefin followed by Claisen rearrangement of the resulting allyl ether **16** gave prenylated phenol **17**. Saponification and subsequent lactonization by EDCI·HCl and HOBt furnished the planned 3,4-dihydroanalog **12** (Scheme 2).[18]

Table 1 summarizes inhibitory activity of osthol (**1**) and the four analogs for export of Rev in the bioassay using HeLa cells. As described above, the demethoxyanalog **4** retains the activity in comparison with **1**, whereas the potency of the other analogs are significantly reduced. With regard to the three analogs **10**–**12**, they showed no or little inhibition for Rev-export at a concentration of 25 μM. Judging from these biological scores, both prenyl function and double bond conjugated to the lactone carbonyl residue were revealed to be particularly involved in the Rev-export inhibitory potency of osthol (**1**).

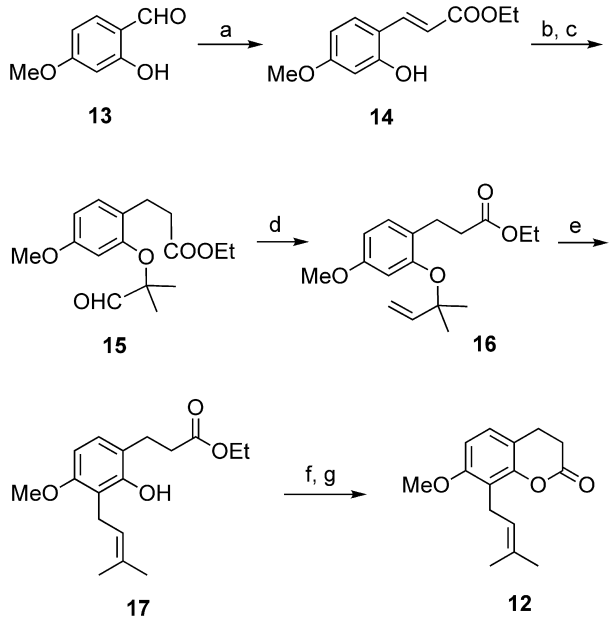
Table 1
Inhibitory activity of osthol (**1**) and its analogs for Rev-export in HeLa cells

Compound	IC_{50} (μM)
Osthol (**1**)	1.6
4	6.4
10	>25*
11	>25*
12	>25*

* The two analogs (**10** and **11**) exhibited no inhibitory activity, while the 3,4-dihydroanalog **12** showed only 2.6% of inhibition for Rev-export at a concentration of 25 μM.

In summary, utilization of the fission yeast, expressing the model fusion protein comprised of GST, SV40 T antigen NLS, GFP, and Rev-NES, in the bioassay disclosed osthol (**1**) as the new Rev-export inhibitor from the MeOH extract of Cnidii Monnieris Fructus. Furthermore, **1** was also found to inhibit export the genuine Rev in HeLa cells by indirect fluorescent antibody technique. By the competitive experiment using the biotinylated probe **3**, osthol (**1**) was revealed to inhibit nuclear export of Rev through the NES non-antagonistic mode. Based on structure–activity relationship analysis of several analogs of **1**, both prenyl side chain and double bond adjacent to the lactone carbonyl residue was shown to be crucial for the biological potency of **1**.

Previously osthol (**1**) was reported to exhibit anti-HIV activity, therefore inhibition for Rev-export was established as one of the mechanism of actions related to anti-HIV activity of **1** from the present study. Up to date, we have presented valtrate, ACA, and (10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid as the Rev-export inhibitors from the medicinal plant resources. The three active principles inhibit Rev-export in the bioassay using HeLa cells with IC_{50} of 2.5, 3.6, and 7.2 μM, respectively. Hence, osthol (**1**) should be noted by not only difference in structural feature but also remarkable Rev-export potency as compared with these precedent inhibitors.



Scheme 2. Synthesis of 3,4-dihydroanalog **12**. Reagents and conditions: (a) $Ph_3P{=}CHCOOEt$, THF, rt, 89%; (b) H_2, Pd–C, MeOH, rt, 95%; (c) 2-bromo-2-methylpropanal, *t*-BuOK, 18-crown-6, CH_3CN, rt, 68%; (d) Ph_3PCH_3Br, NaHMDS, THF, rt; (e) DMF, 120 °C, 41%, two steps; (f) 1 N aq. NaOH, rt; (g) EDCI·HCl, HOBt, CH_2Cl_2, rt, 95%, two steps.

Acknowledgments

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9. 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.

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12. 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.
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14. In culture dishes (3 cm id), HeLa cells (2.0×10^5 cells) were cultured in 1 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 1 μM concentration of biotinylated LMB probe **3** in 1 mL of the medium containing 1% DMSO for 3 h. For competitive experiments, osthol (**1**), 7-demethoxyanalog **4**, and LMB (Cosmo Bio) were injected 1 h prior to addition of **3**, 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 15,000 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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17. Compound **4**: an amorphous white powder, IR ν_{max} (KBr) cm^{-1}: 1734, 1609, ^1H NMR (300 MHz, $CDCl_3$) δ: 7.70 (1H, d, J = 9.5 Hz, 4-H), 7.40, 7.32 (1H both, br d, J = 7.3 Hz, 5- and 7-H), 7.20 (1H, dd, J = 7.3, 7.3 Hz, 6-H), 6.42 (1H, d, J = 9.5 Hz, 3-H), 5.34 (1H, t, J = 7.3 Hz, 2′-H), 3.56 (2H, d, J = 7.3 Hz, 1′-H), 1.76 (6H, s, 4′- and 5′-H), FAB-MS (m/z): 215 $(M+H)^+$, HR FAB-MS (m/z): calcd for $C_{14}H_{14}O_2$+H; 215.1072, found: 215.1098.
18. Compound **12**: an amorphous white powder, IR ν_{max} (KBr) cm^{-1}: 1715, ^1H NMR (300 MHz, $CDCl_3$) δ: 6.96 (1H, d, J = 8.4 Hz, 5-H), 6.61 (1H, d, J = 8.4 Hz, 6-H), 5.18 (1H, br t, J = 7.3 Hz, 2′-H), 3.83 (3H, s, OMe), 3.39 (2H, d, J = 7.3 Hz, 1′-H), 2.92, 2.74 (2H both, m, 3- and 4-H), 1.79, 1.66 (3H both, br s, 4′- and 5′-H), FAB-MS (m/z): 247 $(M+H)^+$, HR FAB-MS (m/z): calcd for $C_{15}H_{18}O_3$+H; 247.1334, found: 247.1332.