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New Rev-Transport Inhibitor with Anti-HIV Activity from Valerianae Radix

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Abstract—Bioassay-guided separation by use of the fission yeast expressing NES of Rev, a HIV-1 viral regulatory protein, resulted in isolation of valtrate (**1**) as a new Rev-transport inhibitor from the nucleus to cytoplasm from Valerianae Radix. Valtrate (**1**) also inhibited the p-24 production of HIV-1 virus without showing any cytotoxicity against the host MT-4 cells.

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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 upon the viral regulatory protein, Rev.² Rev acts to increase cytoplasmic accumulation of the viral mRNAs, which encodes the viral structural proteins, through the transport from the nucleus to cytoplasm.³ As Rev is critical for viral replication, inhibition of the function of Rev is an attractive strategy for therapeutic intervention.⁴ Recently, the transport of Rev 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.⁵ Leptomycin B (**2**) has been shown to inhibit the binding of the NES of Rev (RevNES) to CRM1 and exhibits potent inhibitory effect on the proliferation of HIV-1 virus.^{5,7} Furthermore, the analogous polyketide, callistatin A (**3**), isolated from a marine sponge by our group also inhibited the Rev transport from the nucleus to cytoplasm.⁸ In spite of the potent in vitro biological activity, the significant toxic feature of the two polyketides limited their use as chemotherapeutics. This circumstance urged us to engage in exploring new Rev-transport inhibitors from natural products. Here, we describe the isolation of a new Rev-transport inhibitor, valtrate (**1**), from Valerianae

Radix and a comparative analysis for the mode of action between **1** and callistatin A (**3**) by use of a biotinylated probe **13** (Fig. 1).

In order to search for new Rev-transport inhibitors, we utilized a fission yeast *Schizosaccharomyces pombe*,⁵ which expresses a fusion protein consisting of glutathione *S*-transferase (GST), SV40 T antigen nuclear localization signal (NLS), green fluorescent protein (GFP), and RevNES, in bioassay-directed separation. The practical assay protocol is as follows. After inducing the fusion protein of *S. pombe* in thiamine-free medium for 24 h at 37 °C, 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. Among about 200 kinds of extracts of medicinal plants, the extract of Valerianae Radix (the roots of *Valeriana fauriei* BRIQUET) showed Rev-transport inhibitory activity. Bioassay-guided separation of the extract disclosed valtrate (**1**),⁹ an iridoid ester previously obtained by Thies, as a new Rev-transport inhibitor with moderate lipophilicity.¹⁰ Valtrate (**1**) completely inhibited the transport from the nucleus to cytoplasm of the fused protein of *S. pombe* at the concentration of 3 µg/mL.

Recently, leptomycin B (**2**) was shown to link to the Cys-529 in CRM1 by the α,β -unsaturated lactone moiety through S–C bond formation.¹¹ We clarified that callistatin

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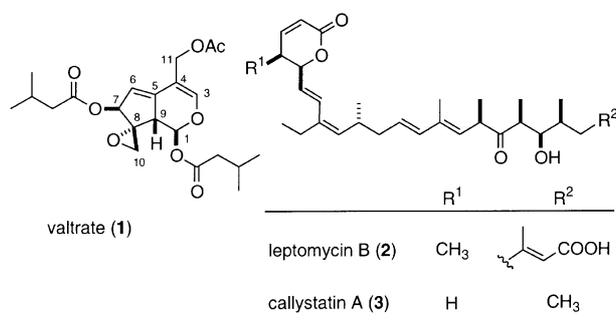
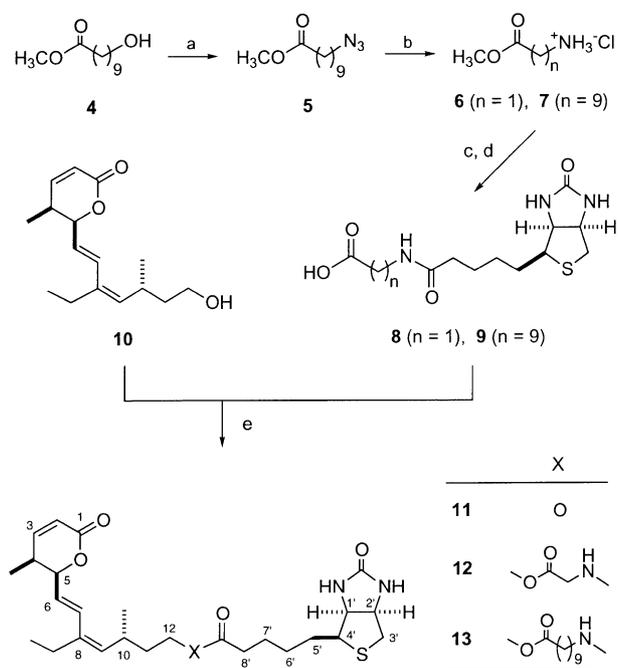


Figure 1. Chemical structures of valtrate (**1**), leptomycin B (**2**), and callystatin A (**3**).

A (3) also binds to CRM1 in the same fashion. Next, we analyzed the mode of action of valtrate (**1**) in comparison with those of **2** and **3**. Prior to the comparative analysis, exploration for a feasible probe was undertaken. During the course of our study on structure activity relationship of **3**, we found a simplified lead compound **10** with moderate Rev-transport inhibitory activity ($\text{MIC} = 3.8 \mu\text{M}$).¹² Consequently, the probes were designed to connect **10** and D-biotin by using amino carboxylic acids as linkers. In the first instance, two biotinylated probe candidates (**12**, **13**) with linker moieties were synthesized as depicted in Scheme 1. One linker is commercially available hydrochloride of glycine methyl ester (**6**), and the other (**7**) was prepared from methyl 1-hydroxydecanoate (**4**) in the following manner. Namely, treatment of **4** with mesylchloride in the presence of Et_3N afforded a mesylate, which was submitted to azidation by NaN_3 and $n\text{Bu}_4\text{NBr}$ to give an azide **5**. Hydration of **5** by use of 10% Pd/C under H_2 atmosphere furnished a hydrochloride salt of methyl 10-aminodecanoate **7** in 56% yield for three steps. Condensation of **6** and D-biotin with EDCI-HCl, HOBT, and Et_3N followed by saponification with 1 N NaOH provided a carboxylic acid **8** quantitatively. The carboxylic acid **8** was coupled with **10** under the same conditions as the condensation of **6** and biotin to furnish a biotinylated probe **12** in 86% yield. In accordance with this protocol, the other probe **13** was synthesized from **7** via **9**. At the same time, readily accessible biotin conjugate **11** was also prepared. Assessment of the biological scores of the three probes revealed that the probe **13** ($\text{MIC} = 1 \mu\text{M}$) completely inhibited Rev-transport with 10-fold more potency than the other two probes (**11**, **12**; $\text{MIC} = 10 \mu\text{M}$). Therefore, the comparative analysis for the mode of action of valtrate (**1**) and callystatin A (**3**) was conducted by using **13**.¹³

First of all, we examined binding affinity of the biotinylated probe **13** against CRM1.¹⁴ SDS-PAGE analysis through streptavidin–biotin affinity disclosed a characteristic protein band around 100 KDa (lane 2), which was assignable to CRM1 (Fig. 2). This protein band was not detected in the absence of **13** (lane 1) or the pre-treatment with callystatin A (**3**) (lane 3). Similarly, the addition of valtrate (**1**) prior to the inoculation of **13** gave rise to the same behavior as that of **3** (lane 4). Valtrate (**1**) is, therefore, presumed to inhibit the Rev-transport from the nucleus to cytoplasm through direct binding to the Cys-529 in CRM1.



Scheme 1. Synthesis of biotinylated probe (**13**) derived from callystatin A (**3**). Reagents and conditions: (a) MsCl , Et_3N , toluene, 0°C then NaN_3 , $n\text{Bu}_4\text{NBr}$, H_2O , 60°C ; (b) H_2 , 10% Pd/C, $\text{MeOH}-\text{CHCl}_3$ (50:1), two steps 56%; (c) D-biotin, EDCI-HCl, HOBT, Et_3N , DMF; (d) 0.5 N aq LiOH, dioxane, two steps quant for **8**, quant for **9**; (e) EDCI-HCl, HOBT, Et_3N , DMF, 83% for **11**, 86% for **12**, 84% for **13**.

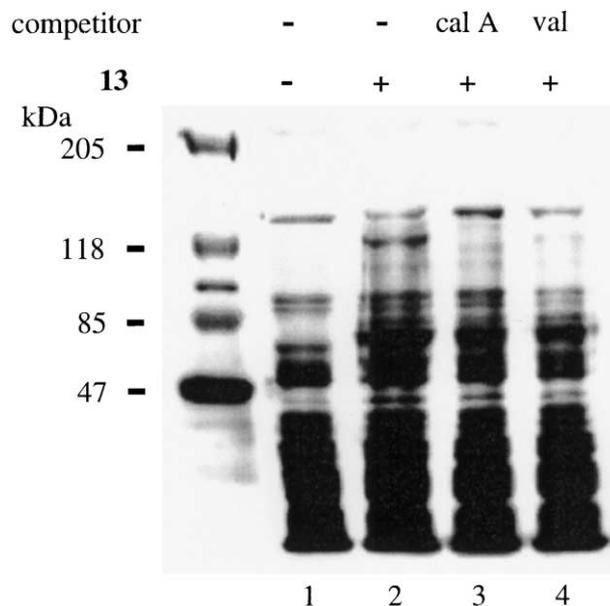


Figure 2. Analysis for the binding of valtrate (**1**) to CRM1 using biotinylated probe (**13**).

In order to confirm this presumption and deduce the reactive site of valtrate (**1**) with the Cys-529 in CRM1, reaction of **1** and *N*-acetyl-cysteine methyl ester (**14**) was examined (Fig. 3). Treatment of **1** with **14** in Tris buffer (pH 7.5) afforded an alcohol **15**.¹⁵ with concomitant epoxy ring cleavage as a major reactant. The ^1H NMR spectrum of **15** exhibited the signals [δ 2.76 (1H, d, $J = 14.0$ Hz), δ 3.36 (1H, d, $J = 14.0$ Hz)] due to the

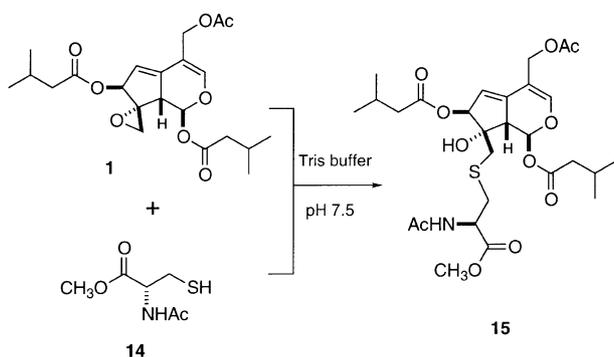


Figure 3.

methylene bearing a sulfur or an oxygen atom instead of the methylene proton signals [δ 2.91 (1H, d, $J=5.0$ Hz), δ 3.03 (1H, d, $J=5.0$ Hz)] of the epoxy portion in **1**. Location of the hydroxyl group of **15** was determined by the isotope effect induced by exchange of an OH group for an OD group in ^{13}C NMR.¹⁶ In comparison of the ^{13}C NMR spectrum of **15** taken in CD_3OH with that in CD_3OD , distinct deuterium shifts were observed with respect to the four carbon signals (C-7, C-8, C-9, and C-10) around C-8. In particular, the chemical shift ascribable to C-8 showed large up-field shift by 0.10 ppm, while the other four carbons were shifted to lower field by 0.02–0.05 ppm. Consequently, the chemical structure of the reactant **15** was unambiguously established as depicted in Figure 3.

Finally, anti-viral activity of valtrate (**1**) was assessed by measurement of HIV-p24 antigen production in the supernatants of the infected MT-4 cell cultures with a commercially available HIV-antigen kit.¹⁷ Valtrate (**1**) showed 44% inhibition on p-24 production at the concentration of 0.5 μM without showing any cytotoxicity against the host MT-4 cells.

In summary, we have elucidated a new Rev-transport inhibitor from the nucleus to cytoplasm with appropriate lipophilicity as medicinal leads, valtrate (**1**), from *Valerianae Radix* according to bioassay-guided separation using fission yeast expressing the fusion proteins of GST-NLS-GFP-RevNES. Furthermore, the biotinylated probe **13** was synthesized in order to compare the modes of action between **1** and **3**. The analysis of the binding protein to **1** using **13** and the reactant **15** of **1** with *N*-acetyl-cystein methyl ester (**14**) demonstrated that both **1** and **3** inhibit Rev-transport in the same fashion. Exploration for synthetic leads having more potent anti-HIV activity than **1** is in progress in our laboratory.

Acknowledgements

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References and Notes

- Popovic, M.; Sarngadharan, M. G.; Read, E.; Gallo, R. C. *Science* **1984**, *224*, 497.
- (a) Zapp, M. L.; Stern, S.; Green, M. R. *Cell* **1993**, *74*, 969. (b) Werstuck, G.; Zapp, M. L.; Green, M. R. *Chem. Biol.* **1996**, *3*, 129.
- Daly, T. J.; Cook, K. S.; Gray, G. S.; Maione, T. E.; Rusche, J. R. *Nature (London)* **1989**, *342*, 816.
- Kjems, J.; Askjaer, P. *Adv. Pharmacol.* **2000**, *48*, 251.
- Kudo, N.; Wolff, B.; Sekimoto, T.; Schreiner, E. P.; Yoneda, Y.; Yanagida, M.; Horinouchi, S.; Yoshida, M. *Exp. Cell Res.* **1998**, *242*, 540.
- Kobayashi, M.; Wang, W.; Tsutsui, Y.; Sugimoto, M.; Murakami, N. *Tetrahedron Lett.* **1998**, *39*, 8291.
- Wolff, B.; Sanglier, J.-J.; Wang, Y. *Chem. Biol.* **1997**, *4*, 139.
- Murakami, N.; Sugimoto, M.; Nakajima, T.; Higuchi, K.; Aoki, S.; Yoshida, M.; Kudo, N.; Kobayashi, M. Abstracts of Papers, 41st Symposium on the Chemistry of Natural Products, Nagoya, Oct., 1999; p 229. *Chem. Abstr.*, 776311.
- (a) Thies, P. W. *Tetrahedron* **1968**, *24*, 313. (b) Konowal, A.; Snatzke, G.; Thies, P. W. *Tetrahedron* **1978**, *34*, 253.
- Leo, A. J. *Chem. Rev.* **1993**, *93*, 1281. ClogP has been taken as a calculated parameter of lipophilicity and utilized as a convenient index to predict in vivo pharmacological potency of medicinal leads. In general, clogPs ranging from -3 to 3 are believed to be favorable for exerting in vivo potency. The two polyketides possessed very large clogPs (**2**: 6.90, **3**: 6.85), while the clogP of **1** is 1.92. These clogPs were calculated using the computer program (version 4.0, Bio Byte Corporation, Claremont, CA 91711, USA).
- Kudo, N.; Matsumori, N.; Taoka, H.; Fujiwara, D.; Schreiner, E. P.; Wolff, B.; Yoshida, M.; Horinouchi, S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9112.
- Murakami, N.; Kawanishi, M.; Sugimoto, M.; Matsui, K.; Aoki, S.; Kobayashi, M. Unpublished results.
- A colorless amorphous solid, $[\alpha]_D^{25} + 38.3^\circ$ (c 0.05, MeOH, 27°C). IR ν_{max} (KBr) cm^{-1} : 1726, 1697, 1680 (sh), 1650, 1575, 1263, 1244. ^1H NMR (500 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD} = 1:1$) δ : 1.02 (3H, d, $J=6.4$ Hz, 10- CH_3), 1.06 (3H, d, $J=6.2$ Hz, 4- CH_3), 1.09 (3H, d, $J=7.2$ Hz, 8- CH_2CH_3), 1.22–1.80 (22H, m, $\text{CH}_2 \times 11$), 2.25 (2H, q, $J=7.2$ Hz, 8- CH_2CH_3), 2.35 (2H, t, $J=7.5$ Hz), 2.37 (2H, t, $J=7.2$ Hz) (CH_2CO_2 , 8'- H_2), 2.64 (1H, m, H-4), 2.74 (1H, dd, $J=12.9, 3.0$ Hz, H-3'), 2.84 (1H, m, H-10), 2.93 (1H, dd, $J=12.9, 5.1$ Hz, H-3''), 3.20 (1H, m, H-4'), 3.39 (2H, CH_2NHCO , overlapped with CH_3OD), 3.95 (1H, dt-like, $J = \text{ca. } 11, 6$ Hz, H-12), 4.10 (1H, dt-like, $J = \text{ca. } 11, 5$ Hz, H-12), 4.33 (1H, ddd, $J=7.8, 5.1, 3.0$ Hz, H-2'), 4.52 (1H, dd, $J=7.8, 4.7$ Hz, H-1'), 5.10 (1H, ddd, $J=6.4, 4.0, 1.1$ Hz, H-5), 5.21 (1H, d, $J=9.4$ Hz, H-9), 5.76 (1H, dd, $J=15.8, 6.4$ Hz, H-6), 6.04 (1H, dd, $J=9.7, 1.1$ Hz, H-2), 6.73 (1H, d, $J=15.8$ Hz, H-7), 7.11 (1H, dd, $J=9.7, 5.6$ Hz, H-3). FAB-MS m/z : 660 ($\text{M}+\text{H}$)⁺. HR FAB-MS m/z : calcd for $\text{C}_{36}\text{H}_{58}\text{N}_3\text{O}_6\text{S}$: 660.4046, found: 660.4050.
- KB 3–1 cells (8.0×10^5 cells) in 10 mL of RPMI medium 1640 containing with 10% fetal bovine serum were cultured in the presence of biotinylated probe **13** at 37°C for 3 h. Valtrate (**1**) and callistatin A (**3**) were injected 1 h prior to addition of **13**, respectively. All compounds were inoculated as 10 μL EtOH solutions and the final concentrations of **1**, **3**, and **13** were 2.0, 5.4, and 10 μM , respectively. After harvesting the cells, 1.6 mL of 0.1% Nonidet P40–Tris buffer saline (TBS, pH 7.4) was added and the mixture was stirred with a vortex mixer for 10 min at 4°C . The whole was centrifuged at 15,000 rpm

for 30 min. This procedure to collect solubilized protein was conducted three times. The combined supernatant was treated with 150 μL of 50% (v/v) immobilized streptavidin (Sigma) in TBS under rotation for 15 h, then the mixture was centrifuged at 10,000 rpm for 5 min. After removing the supernatant and washing the pellets, the bound proteins were eluted from the pellets by SDS-PAGE sample buffer (20 μL) under boiling at 95 $^{\circ}\text{C}$ for 5 min. Each eluate was applied to polyacrylamide gel (Ready Gels J, Bio-Rad), then the gel was stained with Silver Stain II Kit (Wako).

15. Yellow oil, $[\alpha]_{\text{D}}^{25} + 102.3^{\circ}$ (c 0.20, CHCl_3 , 25 $^{\circ}\text{C}$). IR ν_{max} (KBr): 3304, 1740, 1659, 1244. ^1H NMR (500 MHz, CDCl_3) δ : 1.01, 1.00, 0.93, 0.92 (3H each, all d, $J=6.7$ Hz, $\text{CH}(\text{CH}_3)_2 \times 2$), 2.04, 2.01 (3H each, both s, Ac $\times 2$), 2.06 (1H, m, $\text{CH}(\text{CH}_3)_2$), 2.14 (2H, d, $J=6.1$ Hz, COCH_2), 2.15 (1H, m, $\text{CH}(\text{CH}_3)_2$), 2.29 (1H, dd, $J=15.0, 6.7$ Hz, COCH_2), 2.31 (1H, dd, $J=15.0, 8.0$ Hz, COCH_2), 2.76 (1H, d, $J=14.0$ Hz, H-10), 2.82 (1H, dd, $J=14.6, 8.5$ Hz, SCH_2), 2.87 (1H, dd, $J=9.4, 2.4$ Hz, H-9), 2.94 (1H, dd, $J=14.6, 3.3$ Hz, SCH_2), 3.36 (1H, d, $J=14.0$ Hz, H-10), 3.75 (3H, s, OCH_3), 4.66, 4.60 (2H, ABq, $J=12.2$ Hz, H₂-11), 4.88 (1H, ddd, $J=10.4, 8.5, 3.3$ Hz,

CHNHAc), 5.31 (1H, d, $J=3.1$ Hz, H-7), 5.76 (1H, dd, $J=3.1, 2.4$ Hz, H-6), 6.23 (1H, d, $J=10.4$ Hz, NH), 6.25 (1H, d, $J=9.4$ Hz, H-1), 6.63 (1H, s, H-3). ^{13}C NMR (125 MHz, CD_3OD) δ : 174.6, 173.4 (2C), 173.4, 173.1 ($\text{COCH}_2\text{CH}(\text{CH}_3)_2 \times 2$, Ac $\times 2$), 150.1 (C-3), 142.0 (C-5), 119.2 (C-6), 111.3 (C-4), 95.2 (C-1), 86.2 (C-7), 83.3 (C-8), 62.8 (C-11), 55.0 (OCH_3), 53.7 (CHNHAc), 52.7 (C-9), 45.2, 44.8 ($\text{COCH}_2 \times 2$), 41.9 (C-10), 38.3 (SCH_2), 27.7, 27.6 ($\text{CH}(\text{CH}_3)_2 \times 2$), 23.6, 23.5, 23.4, 23.4, 23.2 ($\text{CH}(\text{CH}_3)_2 \times 2$, NHAc), 21.6 (OAc). FAB-MS m/z : 622 (M + Na), FAB-HRMS m/z : calcd for $\text{C}_{28}\text{H}_{40}\text{NO}_{11}\text{SNa}$: 622.2298, found: 622.2275.

16. Gagnaire, D.; Vincendon, M. *Chem. Commun.* **1977**, 509.

17. MT-4 cells were suspended at a density of 5×10^5 cells/mL of RPMI medium 1640 supplemented with 10% fetal bovine serum. The cells were infected with HIV-1 strain NL43 for 2 h at 37 $^{\circ}\text{C}$ and washed twice, then 100 μL of infected cell suspension (4×10^5 cells/mL) was added to each well of microtiter trays containing 100 μL of appropriate dilution of the test compound. After incubation for 3 days at 37 $^{\circ}\text{C}$, the production of HIV-p24 antigen in the supernatants was evaluated by using a commercially available HIV-antigen kit (ZeptoMetrix).