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Tellimagrandin I, HCV invasion inhibitor from Rosae Rugosae Flos

Satoru Tamura [a], Gang-Ming Yang [a], Natsuko Yasueda [a], Yoshiharu Matsuura [b], Yasumasa Komoda [b], Nobutoshi Murakami [a,*]

[a] Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka Suita, Osaka 565-0871, Japan
[b] Research Center for Emerging Infectious Diseases and Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka Suita, Osaka 565-0871, Japan

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ABSTRACT

By use of the model virus, expressing the HCV envelope proteins E1 and E2, bioassay guided separation of the MeOH extract from *Rosa rugosa* Thunb. disclosed tellimagrandin I (**1**) together with eugeniin (**2**) and casuarictin (**3**) as the potent HCV invasion inhibitors. Furthermore, structure–activity relationship analysis of some relative tannins including the synthesized analogs elucidated the partial structures crucial for potent activity of **1**.

Hepatitis C virus (HCV) is the most important causative agent of posttransfusion and sporadic non-A, non-B hepatitis, infecting more than 170 million people worldwide.[1] HCV infection becomes chronic in most cases and may eventually result in hepatitis, liver cirrhosis, hepatic failure and hepatocellular carcinoma.[2] However, the current therapy based on combination of pegylated interferon with ribavirin is far from ideal, it is effective in about 50% of patients, dependent on the virus genotype, and has several serious side effects.[3] Thus, it has been urgently demanded to search for promising anti-HCV candidates. However, no establishment for an efficient in vitro culture of HCV prevented exploration for new anti-HCV seed principles by cell-based biochemical assay for a long period. Therefore, virus derived protease and RNA-polymerase from sub-genomic replicon system were considered as the most prominent targets up to recently.[4] In this context, several researchers tried to develop surrogate models to examine HCV entry. Recently, Matsuura et al. have established the model virus (E1E2 virus) encoding two envelope glycoproteins responsible for binding to receptors in virus invasion.[5] In addition, deficiency of either the E1- or E2-envelope protein was revealed to result in significant reduction of invasion efficacy into hepatocytes. Based on these biological outcomes, the envelope proteins, E1 and E2, were clarified to be essential for invasion of HCV. In spite of establishment of the HCV model virus, search for anti-HCV principles, interrupting the virus invasion process, have never been investigated by use of the model E1E2 virus. This circumstance prompted us to be engaged in exploring HCV invasion inhibitors from medicinal plants. Here, we deal with the HCV invasion inhibitor, tellimagrandin I (**1**) along with its structural requirement for biological potency of **1** from structure–activity relationship in the natural and synthesized relatives.

The assay to search for HCV invasion inhibitory principles was carried out by use of the E1E2 virus expressing not only the envelope proteins E1 and E2 but secretory alkaline phosphatase (SEAP).[6] After the virus invaded into the hepatocytes, SEAP generated in the host cells was secreted extracellularly. Thus, activity of the secreted SEAP is recognized as indication of HCV invasion. However, the samples, inhibiting directly SEAP and the process of protein production, must be regarded as effective in the assay using only the E1E2 virus. To eliminate the pseudo-positive samples, we also utilized the SEAP transformed G* virus possessing the G envelope instead of the E1 and E2. Namely, the samples, giving rise to low SEAP activity by only infection of the E1E2 virus, are considered to inhibit HCV invasion genuinely.

As a result of screening the extracts from about 400 medicinal plants in combination with both viruses, the MeOH extract from Rosae Rugosae Flos (flower buds of *Rosa rugosa* Thunb.) was disclosed as a promising candidate. The extract inhibit 77.2% of E1E2 and 24.6% of G* invasion at the concentration of 10 μg/mL, respectively. After this extract was successively partitioned between EtOAc and H_2O, *n*-BuOH and H_2O, the resulting EtOAc extract exhibited the strongest efficacy among the three extracts.

* Corresponding author at present address: Kyoto Pharmaceutical University, Japan. Tel.: +81 75 595 4634; fax: +81 75 595 4768.
E-mail addresses: murakami@phs.osaka-u.ac.jp, murakami@poppy.kyoto-phu.ac.jp (N. Murakami).

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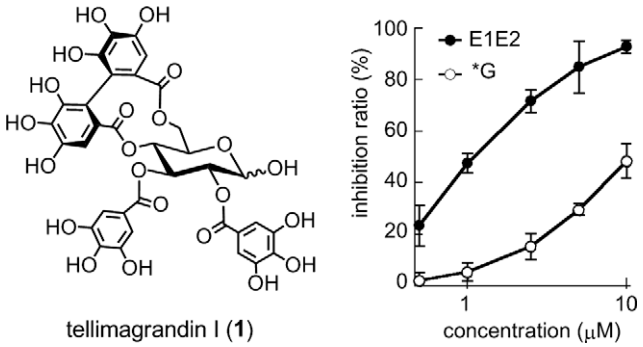
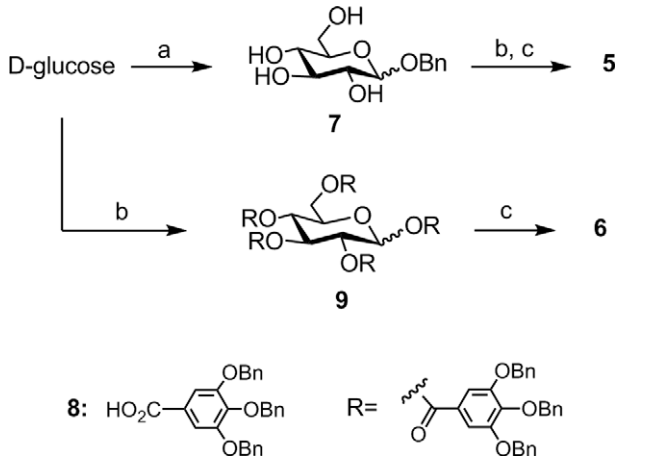


Figure 1. Inhibitory activity of tellimagrandin I (**1**) for invasion of HCV model virus.

Bioassay guide separation of the EtOAc extract by successive ODS column chromatography and reversed phase HPLC led to isolation of tellimagrandin I (**1**, 0.036% from the crude drug) as a responsible principle.

The IR spectrum of **1** showed the absorption bands due to the hydroxyl (3189 cm^{-1}) and ester carbonyl groups (1722, 1718 cm^{-1}) along with the aromatic rings (1611 cm^{-1}). The positive-ion FAB-MS gave the pseudomolecular ion peak at *m/z* 787 $[M+H]^+$, while the FABHRMS revealed the molecular formula of **1** to be $C_{34}H_{26}O_{22}$. The H–H correlation spectroscopy (COSY) spectrum indicated the presence of glucose residue of which 2-, 3-, 4-, and 6-hydroxyl groups were esterified. Furthermore, detailed analysis of the ^1H, ^{13}C NMR, and heteronuclear multiple quantum coherence (HMQC) spectra clarified the presence of one hexahydroxydiphenyl (HHDP) and two galloyl groups. In the CD spectrum, the negative split Cotton ascribable to the diphenyl functionality was observed around 250 nm [$\Delta\varepsilon$: −4.3 (265 nm), +10.1 (238 nm)], indicating *S* configuration of the HHDP moiety.[7] Taking these physicochemical properties into account, the active principle was unambiguously identified as tellimagrandin I (**1**) in comparison with the previously reported spectroscopic data.[8,9] Tellimagrandin I (**1**) inhibited invasion of the E1E2 virus with IC_{50} of 1.7 μM in a concentration dependent manner as illustrated in Figure 1. In addition, **1** showed approximate 10-fold selectivity against the G* virus from the IC_{50} values together with a little cytotoxicity, 15.6% of growth inhibition, against the host HepG2 cells at the concentration of 10 μM.

Extensive search for active principles from the MeOH extract of *Rosa rugosa* Thunb. afforded other two active principles showing nearly similar potency to tellimagrandin I (**1**). Analysis of the ^1H and ^{13}C NMR data indicated the two principles (**2** and **3**) to be the congeners of **1**. The ^1H and ^{13}C NMR spectra of **2** showed the presence of three galloyl and one HHDP groups. On the other hand,



Scheme 1. Preparation for relatives of tellimagrandin I without HHDP function. Reagents and conditions: (a) HCl gas, *n*BuOH, 82%; (b) **8**, EDCI·HCl, DMAP, DMF, 80% for **9**; (c) Pd–C, H_2, EtOAc–MeOH, two steps 78% for **5**, 99% for **6**.

the signals due to one galloyl and two HHDP groups appeared in the ^1H and ^{13}C NMR spectra of **3**. Intensive analysis of their CD and IR spectra, FAB-MS, HR FAB-MS as well as their ^1H and ^{13}C NMR data revealed them to be eugeniin (**2**, 0.078%)[10,11] and casuarictin (**3**, 0.069%),[8,12] respectively (Fig. 2).

Since the disclosed active principles (**1**–**3**) contained the HHDP and galloyl groups, participation of these functionalities in HCV invasion inhibitory activity was examined. Thus, the hydrolysable tannin, pedunculagin (**4**), was isolated from *Juglans regia*[13] and the two galloyl glucose analogs (**5** and **6**) were prepared (Fig. 2). The syntheses of the two analogs (**5** and **6**) were conducted as depicted in Scheme 1. Treatment of D-glucose with HCl gas in benzyl alcohol afforded 1-*O*-benzyl ether **7** in 82% yield. Condensation of **7** with 3,4,5-*O*-tribenzylgallic acid (**8**) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI·HCl) and 4-dimethylaminopyridine (DMAP) gave the corresponding ester. The ester was submitted to debenzylation by using Pd–C under a hydrogen atmosphere to provide 2,3,4,6-*O*-tetragalloyl-D-glucose (**5**)[14] as a mixture of α- and β-anomer in a ratio of 2:1 in 78% yield for two steps. On the other hand, coupling between D-glucose and **8** by EDCI·HCl and DMAP afforded pentaester **9** in 80% yield. Removal of the benzyl groups in **9** by Pd–C under a hydrogen atmosphere furnished 1,2,3,4,6-*O*-pentagalloyl-β-D-glucose (**6**)[8,14] in 99% yield (Scheme 1).

Inhibitory activity against HCV invasion of tellimagrandin I (**1**) and its relatives is summarized in Table 1. In respective compari-

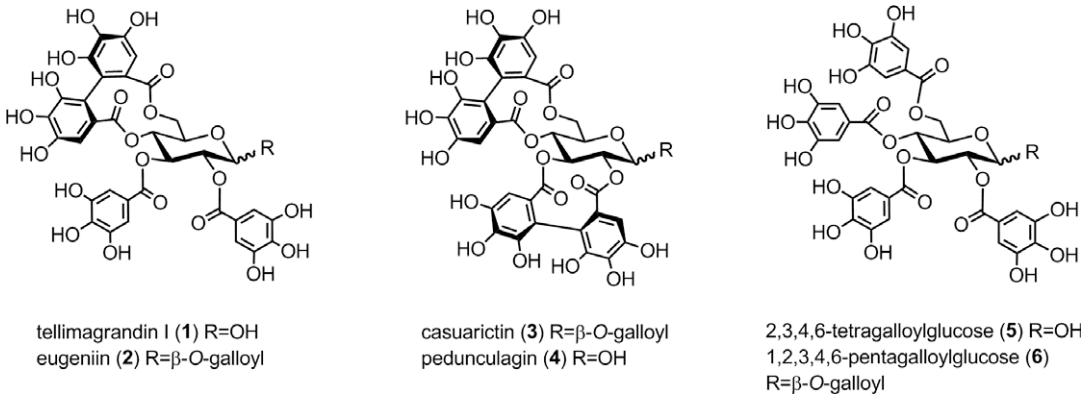


Figure 2. Tellimagrandin I and relatives evaluated for inhibition for HCV invasion.

Table 1
Inhibitory activity for HCV invasion of tellimagrandin I (**1**) and relatives (**2**–**6**)

	E1E2 virus[a]			G* virus[a]		
	10 μM	3 μM	1 μM	10 μM	3 μM	1 μM
Tellimagrandin I (**1**)	93.8 ± 6.7	74.3 ± 8.2	28.3 ± 9.8	45.6 ± 4.3	25.4 ± 8.3	6.4 ± 6.0
Eugeniin (**2**)	92.8 ± 1.3	51.3 ± 8.3	18.4 ± 7.3	35.7 ± 4.1	21.2 ± 4.2	17.2 ± 8.4
Casuarictin (**3**)	87.4 ± 6.3	49.6 ± 2.3	12.5 ± 3.7	45.1 ± 4.8	28.0 ± 10.2	15.5 ± 7.0
Pedunculagin (**4**)	81.1 ± 4.3	58.8 ± 4.4	14.6 ± 2.8	30.9 ± 4.1	14.8 ± 5.8	9.4 ± 2.2
Tetragalloylglucose (**5**)	43.7 ± 8.3	20.8 ± 2.1	9.1 ± 2.2	51.2 ± 9.3	40.7 ± 10.3	16.0 ± 3.1
Pentagalloylglucose (**6**)	72.3 ± 11.1	28.9 ± 6.3	4.2 ± 4.7	40.7 ± 1.8	22.8 ± 4.5	5.0 ± 4.7

[a] All values are expressed as mean ± SD of three experiments.

son of inhibitory activity between **1** and **5**, **2** and **6**, the HHDP groups located between C-4 and C-6 with *S*-configuration were shown to enhance the activity. Respective comparison of inhibitory potency between **1** and **4**, **2** and **3**, the galloyl groups on C-2 and C-3 instead of the HHDP groups were revealed to be crucial for the bioactivity. In the case of the galloyl esters (**5** and **6**) without the HHDP group, the penta-*O*-galloyl ester **6** exhibited more potent activity than the tetraester **5**.

In conclusion, we disclosed tellimagrandin I (**1**) along with eugeniin (**2**) and casuarictin (**3**) as the HCV invasion inhibitors using the model virus, expressing the HCV envelope proteins E1 and E2, through bioassay guided separation of the MeOH extract from the medicinal plant, Rosae Rugosae Flos. Additionally, structure–activity relationship analysis of the hydrolysable tannins including the natural and synthesized relatives clarified that the HHDP group bridged between C-4 and C-6 and the galloyl groups on C-2 and C-3 enhanced inhibitory activity for HCV invasion. Up to date, only iridoid monoterpenes, quinolylamines, and tricyclic diphenylamines have been found out as the HCV invasion inhibitors by using other model systems for HCV invasion.[15–17] It should be noteworthy for the active principles in the present investigation to possess a considerably different structural feature from the known inhibitors.

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6. The coding region of G protein gene in the full-length cDNA clone of Vesicular stomatitis virus (VSV) genome was replaced with the coding region of the SEAP gene, clipped from the plasmid, pNiFty2-56 K-SEAP (Nacalai). This plasmid was designated as pVSVΔG*. The G* virus was prepared by transfection of pVSVΔG* and the plasmid of G protein into CHO cells according to Takada's protocol.[18] The E1E2 virus was provided by infection of the G* virus to CHO cells expressing chimeric E1 and E2 proteins agreeably to Matsuura's procedure.[5] The resulting suspensions of both viruses were diluted to moderate titer: inoculation of 10 μL of suspension in the following culture exhibited about 20-fold SEAP activity.
   In the 96-well microculture plates, HepG2 cells (9.0×10^4 cells/mL) were cultured in 90 μL of Dulbecco's Modified Eagle medium (Nissui Pharmaceutical) containing 10% fetal bovine serum (Wako) at 37 °C under a 5% CO_2 atmosphere for 24 h. The test samples were dissolved in DMSO and diluted to appropriate concentrations using the medium, then 10 μL of each sample solution was inoculated. The final concentration of DMSO in the culture is 1.0%. After incubation at 37 °C under a 5% CO_2 atmosphere for 1 h, the E1E2 virus or G* virus suspension (10 μL) was inoculated. The cells were further incubated for 24 h, then SEAP activities in supernatants were evaluated by use of Great EscAPe SEAP Fluorescent Detection kit (BD Biosciences). Lactoferrin was used as the positive control for the assay and showed about 45% inhibition at the concentration of 30 μg/mL.
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