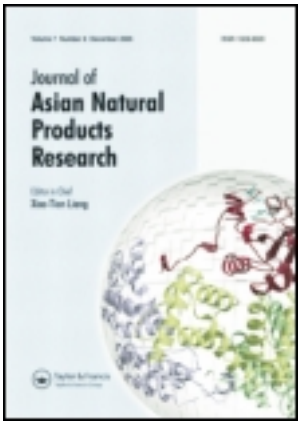

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Notoginsenoside ST-4 inhibits virus penetration of herpes simplex virus *in vitro*

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Further study on steam-treated notoginseng, the roots of *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae), which is a famous traditional Chinese medicine that is used both in raw and treated forms for a long time, led to the isolation of a new dammarane-type saponin, namely notoginsenoside ST-4. Its structure was elucidated to be 3β,12β,20(*S*)-tri-hydroxydammar-24-ene-3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 2)-β-D-glu-copyranoside, based on the detailed analyses of the 1D and 2D NMR spectral data and acidic hydrolysis. Notoginsenoside ST-4 was investigated for its antiviral activity on herpes simplex type 1 (HSV-1) and type 2 (HSV-2) *in vitro*. The 50% effective concentration (EC_{50}) values, determined by plaque reduction assay, were 16.47 ± 0.67 and 19.44 ± 1.16 μM for HSV-1 and HSV-2, respectively, whereas the 50% cytotoxic concentration (CC_{50}) determined by the XTT test on Vero cells was 510.64 ± 4.56 μM. As analyzed by attachment assay and penetration assay based on plaque reduction assay, the antiviral activity of notoginsenoside ST-4 was principally due to the penetration inhibition effects, which was confirmed by fluorescence microscopy observation that notoginsenoside ST-4 blocked the penetration of virus. Therefore, notoginsenoside ST-4 might be a promising agent for herpes simplex virus infection.

Keywords: notoginsenoside ST-4; herpes simplex virus; penetration; antiviral activity

1. Introduction

Notoginseng, the root of *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae) mainly cultivated in Yunnan Province of China, is a famous traditional Chinese medicinal herb used both in raw and processed forms. The raw form is mainly used for injuries from falls and for removing blood stasis, whereas the processed one is used as a tonic to promote blood circulation [1]. Our previous study on steam-treated notoginseng led to the isolation of four new dammarane-type triterpenoid saponins, namely, notoginsenosides ST-1–ST-3 and ST-5, together with 23 known ones [2]. In this study, a new dammarane-type saponin, named notoginsenoside ST-4, was further isolated from steam-treated notoginseng. Its structure was determined on the basis of detailed analyses of 1D and 2D NMR spectral data and acidic hydrolysis.

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The anti-herpes simplex (HSV) activity and the underlying mechanisms of action for notoginsenoside ST-4 against HSV-1 were also investigated.

Herpesviridae are classified into three subfamilies: alpha-herpesviruses (including HSV-1, HSV-2, and varicella-zoster virus), beta-herpesviruses and gamma-herpesviruses [3]. HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections. An infectious herpesvirus consists of four structures: a linear DNA-genome core, a capsid, a tegument, and an outer envelope [3]. During its life cycle, HSV-1 is first absorbed into the cell membrane. After penetration of absorbed virus, the incoming nucleocapsids are transported to the nuclear pore [4,5]. Acyclovir (ACV) has been used for the treatment of HSV infection and is associated diseases [6]. However, the efficacy of ACV is limited by the increase in the resistance of virus [7].

2. Results and discussion

2.1 Structural elucidation of the new compound

The new compound was obtained as a pale amorphous powder. Its molecular formula $C_{47}H_{80}O_{17}$ was elucidated by the negative HR-ESI-MS (*m/z* 915.5323 $[M - H]^-$). The fragment ion peaks at *m/z* 784 $[M\text{-}132(\text{pentosyl})]^-$ and 622 $[M\text{-}132\text{-}162(\text{hexosyl})]^-$ in the FAB-MS, together with the anomeric proton and carbon signals at δ_H 4.92 (d, $J = 8.0$ Hz), 5.51 (d, $J = 7.5$ Hz), and 5.39 (d, $J = 6.5$ Hz), and δ_C 104.8, 103.2, and 106.5, respectively, in the ^{1}H and ^{13}C NMR spectra, the existence of β-pentosyl and β-hexosyl units. Acidic hydrolysis of this compound gave D-glucose and D-xylose as the sugar residue. The ^{1}H and ^{13}C NMR spectral data of this compound were closely related to those of notoginsenoside Ft_1 [8], except for the chemical shifts of C-17 (δ_C 54.8), C-21 (δ_C 27.1), and C-22 (δ_C 35.9). These observations suggested that this compound was the C-20(*S*) epimer of notoginsenoside Ft_1, a protopanaxadiol monodesmoside [9]. Furthermore, the HMBC correlations of H-1′ at δ_H 4.92 with C-3 at δ_C 88.9, H-1″ at δ_H 5.51 with C-2′ at δ_C 83.0, and H-1‴ at δ_H 5.39 with C-2″ at δ_C 84.6 confirmed the sugar sequence and location in molecule, which were the same as those of nogoginsenoside Ft_1. Accordingly, the new compound was determined to be 3β,12β,20(*S*)-tri-hydroxydammar-24-ene-3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside, and named notoginsenoside ST-4 (Figure 1).

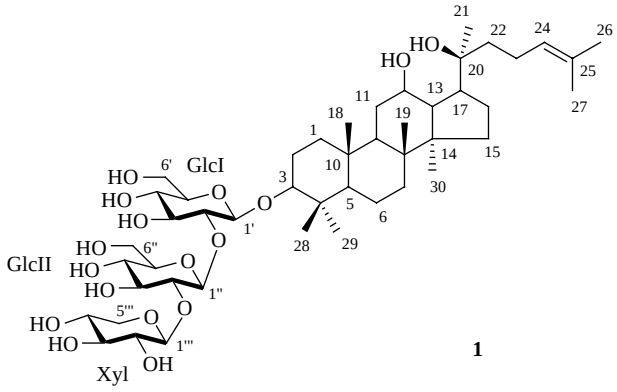

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Figure 1. Structure of notoginsenoside ST-4 isolated from the steam-treated notoginseng.

2.2 Assessment of cytotoxicity and anti-HSV activity of notoginsenoside ST-4

The CC_{50} of notoginsenoside ST-4 was 510.64 ± 4.56 μM and the CC_{50} of ACV was 2926.11 ± 26.38 μM. Notoginsenoside ST-4 inhibited HSV-1 with an EC_{50} of 16.47 ± 0.67 μM and inhibited HSV-2 with an EC_{50} of 19.44 ± 1.16 μM. The EC_{50} for ACV was comparable to previous studies for HSV-1, with EC_{50} of 0.98 ± 0.24 μM.

2.3 Effect of notoginsenoside ST-4-treatment on HSV-1 penetration

The cell and viruses were pre-treated with notoginsenoside ST-4 in two ways as described below. Notoginsenoside ST-4 inhibited HSV-1 absorption with an EC_{50} of 100,000.00 ± 0.00 μM and inhibited HSV-1 penetration with an EC_{50} of 16.46 ± 0.43 μM. The effect of notoginsenoside ST-4 was investigated and was found to be ineffective on absorption, but effective on penetration. To verify the results of penetration assay, we performed the immunofluorescence assay and found that at 4 h post-infection, most viruses (exemplified by HSV-1 capsid protein VP5, red fluorescence) have already penetrated into the cell, so that no viruses (red fluorescence) were detected on the cell membrane of infection control (Figure 2(A)). On the contrary, in the presence of notoginsenoside ST-4, punctate red fluorescence signals of viruses (arrow) were distributed over the cell membrane, which could not penetrate into the cell (Figure 2(B)). At 12 h post-infection, massive vp5 proteins (red fluorescene) were synthesized in the nucleus of infection control (Figure 2(C)). However, there was no vp5 protein synthesizing in the nucleus of the treatment group (Figure 2(D)). All the results

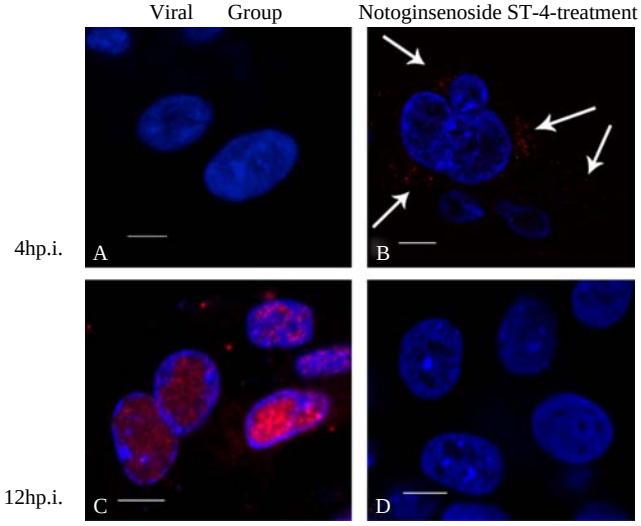

Figure 2. Notoginsenoside ST-4 inhibited HSV-1 penetration and viral protein (vp5) synthesis. Infected cells were incubated with notoginsenoside ST-4 (10 μM) treatment at 2 h post-infection and detected at 4 h post-infection and 12 h post-infection (B,D), or without notoginsenoside ST-4 treatment as viral control and detected at 4 h post-infection and 12 h post-infection (A,C). Cells were stained with antibody to vp5 (red fluorescene) and chromatin was stained with DAPI (blue fluorescene). At 4 h post-infection, most viruses have already penetrated into the cell (A). At 12 h post-infection, massive vp5 proteins (red fluorescene) were synthesized in the nucleus of infection control (C). In the presence of notoginsenoside ST-4, HSV-1 attached to the membrane, but the penetration of HSV-1 was blocked on the cell membrane (B, arrow). And at 12 h post-infection, there was no vp5 protein synthesizing in the nucleus (D). Scale bars are 9 μm.

indicated that penetration of HSV-1 was inhibited by notoginsenoside ST-4 treatment. Further studies will be required to explore the detailed antiviral mechanism of notoginsenoside ST-4. In conclusion, this study described that notoginsenoside ST-4 possessed an anti-HSV activity, which inhibited viral penetration to cells.

3. Experimental

3.1 General experimental procedures

Melting points (mp) were determind using *XRC-1* melting point apparatus, produced by Sichuan University, China (uncorrected). Optical rotation was performed on a P-1020 Polarimeter (JASCO, Tokyo, Japan). IR spectrum was measured on a Bruker Tensor 27 spectrometer with KBr pellets. ^{1}H and ^{13}C NMR, HMQC, and HMBC spectra were recorded in C_5D_5N with Bruker AM-400 and DRX-500 spectrometers. Coupling constants were expressed in Hertz, and chemical shifts were given on a δ (ppm) scale with TMS as an internal standard. FAB-MS was recorded on an AutoSpe-3000 spectrometer (VG, Manchester, UK) with glycerol as the matrix, in *m/z*. HR-ESI-MS was recorded on API Qstar Pulsa LC/TOF spectrometer.

Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical and Industrial Factory, Qingdao, China), MCI-gel CHP20P (75–100 μm; Mitsubishi Chemical Co., Ltd., Tokyo, Japan), and Rp-8 or Rp-18 gel (40–60 μm, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on silica gel H precoated plates (Qingdao Marine Chemical and Industrial Factory) with $CHCl_3$–MeOH–H_2O (75:25:3 or 80:20:2 or 85:15:1 or 90:10:1 v/v) and $CHCl_3$–MeOH (90:10 to 25:1), and Rp-8 or Rp-18 precoated plates (Merck) with MeOH–H_2O (60:40 to 100:0). Spots were detected by spraying with 10% H_2SO_4 in EtOH followed by heating.

3.2 Plant material

Air-dried notoginsengs were obtained from Wenshan County, Yunnan Province of China in May 2006. The raw materials were crushed into small grains, and then steamed at 120°C for 12 h, to give the steamed notoginseng, which were used for extraction and isolation in this study.

3.3 Extraction and isolation

As described in our previous work [2], the EtOH extract (778 g) of air-dried steamed notoginseng was dissolved in H_2O and passed through a macroporous adsorption resin (D101) column, eluting with H_2O and MeOH, successively. The MeOH eluates were combined and concentrated under reduced pressure to give a residue (440 g), which was further subjected to silica gel column eluted with $CHCl_3$:MeOH:H_2O (85:15:1) to afford nine fractions (frs 1–9). Further purification of Fr. 7 (38 g) by repeated CC over silica gel ($CHCl_3$–MeOH–H_2O, 80:20:2 to 70:30:5), Rp-8 (MeOH–H_2O, 1:1 to 4:1), Rp-18 (MeOH–H_2O, 1:1 to 4:1), and MCI-gel CHP20P (MeOH–H_2O, 3:7 to 0:1) afforded a new compound (31 mg, yield: 0.00066%), together with five known ones, which were determined as ginsenosides Rg_1 (151 mg), Rg_2 (23 mg), Rf (71 mg), notoginsenoside R2 (20 mg), and 20(*R/S*)-ginsenoside Rg_3 (238 mg), respectively, as described in the previous report [2].

3.3.1 Notoginsenoside ST-4 (1)

A pale amorphous powder: mp 187–191°C; $[\alpha]_D^{26}$ +3.5 (c = 0.209, MeOH); IR (KBr) ν_{max} (cm^{-1}) 3422, 2942, 2878, 1637, 1452, 1376, 1307, 1077, 1043, and 896; ^{1}H and ^{13}C NMR spectral data (Table 1). FAB-MS (negative ion mode): *m/z* 916 $[M]^-$, 784 [M-132(xylosyl)]$^-$, 622 [M-132(xylosyl)-162(glucosyl)]$^-$; HR-ESI-MS (negative ion mode): *m/z* 915.5323 $[M - H]^-$ (calcd for $C_{47}H_{79}O_{17}$, 915.5317).

Table 1. ^{13}C and ^{1}H NMR spectroscopic data of compound **1** (δ in ppm, J in Hz, in C_5D_5N).

No.	δ_C	δ_H	No.	δ_C	δ_H
1	39.2	1.49 m	23	23.1	2.62 m
		0.75 m			2.31 m
2	26.8	2.18 m	24	126.4	5.30 t (7.0)
		1.81 m	25	130.8	
3	88.9	3.29 dd (4.5, 11.5)	26	25.9	1.66 s
4	39.8		27	17.8	1.63 s
5	56.4	0.69 m	28	28.2	1.29 s
6	18.5	1.51 m	29	16.7	1.11 s
		1.38 m	30	17.1	0.96 s
7	35.2	1.47 m	GlcI-1′	104.8	4.92 d (8.0)
		1.24 m	2′	83.0	4.11 m
8	40.0		3′	78.4	4.35 m
9	50.4	1.39 m	4′	71.1	4.13 m
10	37.0		5′	78.7	3.95 m
11	32.1	2.03 m	6′	62.9	4.57 br d (7.5)
		1.53 m			4.35 m
12	71.2	3.95 m	GlcII-1″	103.2	5.51 d (7.5)
13	48.6	2.02 m	2″	84.6	4.19 m
14	51.8		3″	77.8	4.28 m
15	31.4	1.60 m	4″	71.8	4.20 m
		1.06 m	5″	78.0	3.87 m
16	26.9	1.91 m	6″	63.0	4.49 br d
		1.35 m	Xyl-1‴	106.5	5.39 d (6.5)
17	54.8	2.35 m	2‴	76.0	4.11 m
18	16.4	0.78 s	3‴	77.8	4.31 m
19	15.9	0.96 s	4‴	70.8	4.12 m
20	73.0		5‴	67.5	4.33 m
21	27.1	1.43 s			3.68 m
22	35.9	2.03 m			
		1.71 m			

3.4 Acid hydrolysis of compound 1

The compound (8 mg) was hydrolyzed with 2 M HCl–dioxane (1:1, 4 ml) under reflux for 8 h, respectively. The reaction mixture was partitioned between H_2O and $CHCl_3$ (2 ml × 3). The aqueous layer was neutralized with 2 M NaOH, and then dried to give a monosaccharide mixture. A solution of the sugar mixture in pyridine (2 ml) was added to L-cysteine methyl ester hydrochloride (about 1.5 mg) and kept at 60°C for 1 h. Then, trimethylsilylimidazole (about 1.5 ml) was added to the reaction mixture and kept at 60°C for 30 min. The mixture was detected by gas chromatography (GC) analysis. The GC analysis was run on Agilent Technologies HP5890 GC equipped with an H_2 flame ionization detector. The column was 30QC2/AC-5 quartz capillary column (30 m × 0.32 mm) with the following conditions: column temperature: 180°C/280°C, programed increase: 3°C/min, carrier gas: N_2 (1 ml/min), injector and detector temperature: 250°C, injection volume: 4 μl, and split ratio: 1/50. The configuration of the sugar moiety was determined by comparing the retention time with the derivatives of the authentic samples. The retention times of D-/L-glucose and D-/L-xylose were 19.715/20.159 and 14.606/15.256 min, respectively. The configurations of the sugar moieties from the new compound were D-glucose and D-xylose.

3.5 Cell and virus

African green monkey kidney cells (Vero; ATCC CCL81) were propagated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). HSV-1 strain F (ATCC VR733) and HSV-2 strain 333 were propagated in Vero cells and stored at −80°C until use. Virus titers were obtained by plaque assay.

3.6 Cytotoxic assay, antiviral assay, and statistical analysis

The cytotoxicity in Vero cells was determined by the XTT (tetrazolium salt (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)) assay [10]. Briefly, cells were seeded in a 96-well plate. After 24 h of incubation, cells were treated with or without various concentrations of notoginsenoside ST-4 and were then incubated for 72 h. Later, the XTT solution and phenazine methosulphate were added. The optical densities were measured with enzyme immunoassay reader (BIO-RAD 550) at a test wavelength of 480 nm and a reference wavelength of 630 nm. CC_{50} was calculated [11].

Antiviral activity was assessed with plaque reduction assay. Vero cells were infected with HSV-1 or HSV-2 (30 pfu/well) in the presence or absence of notoginsenoside ST-4 for 2 h and then overlaid with medium containing 1% of methylcellulose. The plate was incubated for 3 days before being fixed with formalin and then stained with crystal violet. EC_{50} was calculated by regression analysis of the dose–response curves generated from plaque reduction assay [11]. Each experiment was performed at least three times. Results were expressed as mean ± SD.

3.7 Attachment assay

The attachment assay was conducted according to the published procedure [11] with minor modifications. Briefly, the Vero cell monolayer was seeded in a 24-well culture plate and then prechilled at 4°C for 1 h. The cell monolayer was infected with HSV-1 at a multiplicity of infection of 10 in the absence or the presence of various concentrations of notoginsenoside ST-4. After further incubation at 4°C for 3 h, the medium was aspirated and the cell monolayer was overlaid with methylcellulose medium. After 3 days of incubation, the plaque reduction assay and statistical analysis were performed as described above.

3.8 Penetration assay

The penetration assay was performed according to the published procedure [11] with minor modifications. The Vero cell monolayer was prechilled at 4°C for 1 h. The cell monolayer was infected with HSV-1 at a multiplicity of infection of 10 and incubated at 4°C for 3 h. And then the infected cell monolayer was incubated at 37°C with or without notoginsenoside ST-4 for 10 min. After 10 min intervals, the infected cell monolayer was treated with acidic phosphate buffered saline (PBS, pH 3) for 1 min to inactivate nonpenetrating virus. Immediately after the addition of PBS (pH 11) to neutralize acidic PBS (pH 3), the neutral PBS was removed. The cell monolayer was washed with PBS (pH 7) and overlaid with methylcellulose medium. After 3 days of incubation, the plaque reduction assay and statistical analysis were performed as described above.

3.9 Indirect immunofluorescence and confocal microscopy

Cells were grown on coverslips and infected with HSV-1 at a multiplicity of infection of 10 for 2 h for viral adsorption on ice. Cells were washed with PBS (pH 7) and transferred into medium with or without notoginsenoside ST-4 (10 μM) and incubated for another 2 h at 37°C. Then the cells were fixed for 15 min with

4% paraformaldehyde (PFA), blocked with PBS containing 1% BSA for 1 h and subsequently incubated with anti-vp5 antibody (Santa Cruz; CA, USA) at dilutions of 1:500, followed by an Alexa Flour 488 secondary antibody (Molecular Probes, Invitrogen; Carlsbad, CA, USA) for 1 h. After each step, the slides were washed repeatedly with PBS, and finally they were preserved with PBS. The additional nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes) was performed for 20 min. Fluorescence was recorded with a confocal laser scan microscope (LSM 510 meta; Zeiss, Germany).

Cells were grown on coverslips and infected with HSV-1 at a multiplicity of infection of 10 for 2 h on ice. After absorption, cells were washed three times with PBS (pH 7) and transferred into the medium with or without notoginsenoside ST-4 (10 μM) and incubated for another 10 h at 37°C. Then, the cells were fixed for 15 min with 4% PFA and permeabilized with 0.02% Triton X-100 for 2 h. And then the cells were blocked and stained as described above.

Acknowledgements

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