Sulfated derivatives of 20(S)-ginsenoside Rh2 and their inhibitory effects on LPS-induced inflammatory cytokines and mediators

ABSTRACT

Ginsenoside Rh2 is one of the most important ginsenosides in ginseng with antitumor, antidiabetic, antiallergic, and anti-inflammatory effects. However, the extremely poor oral bioavailability induced by its low water solubility greatly limits the potency of Rh2 in clinical use. Therefore, in this study we sulfated 20(S)-ginsenoside Rh2 with chlorosulfonic acid and pyridine method, and got two new sulfated derivatives, Rh2-B1 and Rh2-B2, with higher water solubility. Their chemical structures were characterized by spectroscopic methods (IR, MS and NMR). Additionally, Rh2-B1 and Rh2-B2 had the greater anti-inflammatory effects than Rh2 through inhibiting inflammatory cytokines and mediators in LPS-induced mouse RAW264.7 macrophages cells. These results suggested that the sulfated modification of Rh2 improved its water solubility and the sulfated derivatives could be more potential candidates for developing as anti-inflammatory agents.

1. Introduction

Ginseng (the root of Panax ginseng C.A. Meyer) has been used for a long time as a traditional medicine in Asian countries. It has been used commonly among the populace for benefiting Qi, promoting the production of body fluid, calming the nerves, etc. The major active components of ginseng are ginsenosides [1]. Currently, the bioactivities of ginsenoside Rh2 have attracted more and more attention because of its various biological activities including antitumor, antidiabetic, antiallergic, and anti-inflammatory effects. It has been reported to have a growth suppressive effect on various cancer cells such as lung adenocarcinoma, breast cancer, and hepatocarcinoma cells [2–4]. In addition, Rh2 reduces the blood plasma glucose in STZ-diabetic rats [5]. Kim et al. reported that Rh2 inhibited the allergy, improved atopic and contact dermatitis, and protected ischemia–reperfusion brain injury by regulating expression of inflammatory cytokines [6–8]. However, the pharmacokinetic studies indicated that Rh2 has very poor oral bioavailability, which had been attributed to poor oral absorption. The extremely poor oral bioavailability of Rh2 was consistent with its low solubility and permeability, which greatly impedes our ability to demonstrate the potency of Rh2 in vivo [9–11]. Thus, it is essential to design and optimize the chemical structure of Rh2 by structural modification to improve its bioavailability by enhancing its water solubility.

It is reported that the introduction of a sulfate group on hydroxyl groups of the glucan chain could obviously improve its water solubility and enhance the pharmaceutical activities [12]. Up to now, many different biological activities of sulfated polysaccharides were obviously improved, such as
anticoagulation, antiviral, anticancer and anti-inflammatory effects [13–17]. Ginsenoside Rh2 is a steroidal saponin belonging to protopanaxadiol type, which has an aglycone of dammarane skeleton. The glycosyl of Rh2 was a pyran type sugar, similar to the monosaccharide of sulfated polysaccharides. Therefore, we think it is possible to sulfate Rh2 with the same method for sulfating polysaccharides.

Taken together, we hypothesized that ginsenoside Rh2 can be sulfated and its derivates have much better biological activities. Therefore, in this study we sulfated 20(S)-ginsenoside Rh2 with chlorosulfonic acid and pyridine method, and got two new sulfated derivates, Rh2-B1 and Rh2-B2, with higher water solubility. Additionally, their anti-inflammatory effects were evaluated on LPS-induced mouse RAW264.7 macrophage cells.

2. Experimental

2.1. Chemicals and reagents

20(S)-ginsenoside Rh2 (purity>98%), Rh2-B1, white crystals (MeOH), and Rh2-B2, white amorphous powder, (purity>98%), are the sulfated derivates of 20(S)-ginsenoside Rh2. Chlorosulfonic acid (purity>99.5%). Pyridine (purity>99.5%) was obtained from Shanghai Chemical Reagent Ltd. Silica gel (100–200 mesh) and TLC plate (GF254), ODS-A (50 μm, 1×25 cm), LPS (Escherichia coli 055:B5). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin for cell culture use. TNF-α, IL-6, IL-1β, IL-10, NO and PGE2 ELISA kits. The RAW 264.7 mouse macrophage cell line.

2.2. Sulfation of ginsenoside Rh2 with chlorosulfonic acid and pyridine

The sulfating agent was prepared by adding the desired amount of chlorosulfonic acid dropwise into 3 mL of pyridine in an ice bath with stirring. 200 mg of Rh2 dissolved in pyridine (the mole ratio of Rh2 to chlorosulfonic acid = 1:4) was added to the sulfating agent to carry out sulfating reaction under stirring for 2 h at room temperature. Then, NaOH (1 mol/L) was added to adjust the pH to 7.5. This solution was filtrated and evaporated under vacuum to obtain the crude sulfated Rh2, which contained 3 fractions (fractions A, B, and C) analyzed by silica gel TLC examination. The crude sulfated Rh2 was applied to silica gel column chromatography eluting with gradient solution of CHCl3-MeOH to afford fraction B. Fraction B was subjected to reversed-phase silica gel column chromatography and eluted with 80% MeOH to afford fractions B1 (Rh2-B1, 40 mg) and B2 (Rh2-B2, 46 mg) (shown in Supplementary Fig. 1).

2.3. Characterization of sulfated ginsenoside Rh2

The infrared (IR) spectra were recorded on a VERTEX 70 spectrometer (Bruker, Switzerland) from 4000 to 400 cm\(^{-1}\) using the KBr-disk method. The mass spectra (MS) were performed with QTRAPTM hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, U.S.A.). \(^{1}H\) and \(^{13}C\) NMR spectra were measured on a Bruker AV 400 NMR spectrometer (Bruker, Switzerland) in d-pyridine using TMS as an internal standard.

2.4. Bioassay for inflammatory cytokines and mediators

The anti-inflammatory activities of Rh2-B1 and -B2 were evaluated using LPS-induced RAW 264.7 cells. Briefly, the cells were grown in DMEM supplemented with 10% inactivated FBS, 3 mM glucose, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C and 5% CO\(_2\). RAW 264.7 cells were plated onto 24-well plates (4×10\(^{5}\) cells/mL) and allowed to acclimate for 24 h. The cells were cultured with or without 1 mg/L of LPS in the absence or presence of test compounds for desired time. MTT assay indicated that test compounds had no cytotoxicity to RAW264.7 cells at their designed concentrations (data not shown).

The cell supernatants were collected to measure the concentrations of TNF-α, IL-6, IL-1β, and IL-10 after incubation for 12 h, as well as to determine NO and PGE2 production for 24 h, using ELISA kits according to the manufacturer’s instructions. The experiments were done in triplicate and data are shown as mean ± SEM. Differences between mean values of normally distributed data were assessed by the one-way ANOVA (Dunnett’s t-test) and the Student’s t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

3. Results and discussion

The chemical structures of Rh2-B1 and -B2 were characterized by spectroscopic methods (IR, MS and NMR). Compared with IR spectrum of Rh2 [18], two new absorption bands appear in the IR spectrum of Rh2-B1 (Fig. 1) and -B2 (same as B1, data not shown), one at 1230 cm\(^{-1}\) describing an asymmetrical S=O stretching vibration and the other at 810 cm\(^{-1}\) representing a symmetrical C–O–S vibration associated with a C–O–SO\(_3\) group, indicating incorporation of the sulfating group. The molecular formula of Rh2-B2 was analyzed as C\(_{36}\)H\(_{61}\)O\(_{11}\)S\(_{1}\)Na\(_{1}\) by MS at m/z 702 [M+H]\(^{+}\) and

![Fig. 1. IR spectra of Rh2 and Rh2-B1.](image-url)
m/z 97 [M+H]+, and Rh2-B2 as C36H61O11S1Na1 by MS at m/z 701 [M]+. The 1H and 13C NMR data of Rh2-B1 and -B2 were similar to those of Rh2 [19,20], except for the obvious differences due to the presence of one additional sulfating signal (Supplementary Figs. 2–4 and Table 1). Two downfield shift signals δc 80.6 in Rh2-B1 and δc 68.6 in Rh2-B2, comparing with δc 71.1 (C-12) and δc 63.2 (G-C-6) in Rh2 were observed in the 13C NMR spectra. The observation indicated that two sulfate groups combined to the hydroxyl group at C-12 in Rh2-B1 and G-C-6 in Rh2-B2, respectively, because of the downfield shift of a carbon atom linked by a sulfate group [21]. In addition, these sulfated substitutions changed the original chemical surroundings of C-11, C-13, and G-C-5 in Rh2 (Table 1).

Therefore, Rh2-B1 was elucidated as sodium (8R, 10R, 14R)-17-((S)-2-hydroxy-6-methylhept-5-en-2-yl)-4, 4, 8, 10, 14-pentamethylhexadecahydro-3, 4, 5-trihydroxy-6-(hydroxymethyl) tetrahydoro-2H-pyran-2-yl) methyl sulfate (Fig. 2). The solubility of Rh2-B1 and Rh2-B2 in water was enhanced greatly because their polarity was increased by the sulfate group, similar to the increased water solubility of sulfated Bletilla striata polysaccharide [22].

Macrophages play a key role in the specific and non-specific immune responses during the inflammation process. After macrophages are activated by LPS, numerous inflammatory cytokines and large amounts of the inflammatory mediators will be released [23]. In order to explore the anti-inflammatory activities of Rh2-B1 and Rh2-B2, LPS-induced inflammatory cytokines and mediators in mouse RAW264.7 macrophages were examined. In this study, we found that Rh2 (5 μg/mL) did not affect the production of TNF-α, IL-6, IL-1β, IL-10, NO and PGE2 induced by LPS. These results are contradictory to the report that Rh2 inhibited the synthesis of PGE2 in LPS-stimulated RAW264.7 cells. The discrepancy may be due to the different assay methods of PGE2 they used and the different PGE2 expression levels in RAW264.7 cells among laboratories [8]. On the contrast, Rh2-B1 and Rh2-B2 significantly inhibited these inflammatory cytokines and mediators, and up-regulated the anti-inflammatory cytokine (IL-10), in a dose-dependent manner (Figs. 3–4). These results indicated that the anti-inflammatory effects of Rh2 were dramatically improved after being sulfated. Additional studies are ongoing to explore their exact mechanisms of anti-inflammation.

In summary, two new sulfated derivatives of 20(S)-ginsenoside Rh2, Rh2-B1 and Rh2-B2, have been isolated. Their structures were elucidated based on the analysis of IR, MS, and NMR spectra. After the sulfation, the water solubility of Rh2-B1 and Rh2-B2 was enhanced greatly. And they possess the greater anti-inflammatory effects than Rh2 through inhibiting inflammatory cytokines and mediators. This study provided evidence for the development of new anti-inflammatory agents from the sulfated ginsenosides.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2012.12.021.
Fig. 3. Effects of Rh2-B1 and Rh2-B2 on the secretions of TNF-α, IL-6, IL-1β and IL-10 in LPS-induced RAW264.7 cells. The cells were treated with LPS (1 mg/L) alone or LPS plus different concentrations (0.2, 1 and 5 mg/L) of Rh2-B1, Rh2-B2 or Rh2 for 12 h. The values represent mean±SEM of three independent experiments. **P<0.01 vs. control, ***P<0.01 vs. LPS group.
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References


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