Identification of potential inflammatory inhibitors from Aster tataricus

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MAPKs

A B S T R A C T

Aster tataricus L.f. is a traditional Eastern Asian herbal medicine used for the relief of cough-related illnesses. In this study, 32 known compounds and two novel monoterpene glycosides were isolated from the roots of A. tataricus. With the aid of reported data, elucidation of the root-extract components was carried out using a multitude of spectroscopic techniques. All isolates were investigated for their ability to inhibit nitric oxide (NO) secretion in lipopolysaccharide-activated RAW264.7 cells. Compound 7 remarkably suppressed NO production with an IC50 value of 8.5 µM. In addition, compound 7 exhibited significant inhibitory activity against the production of inflammatory cytokines (prostaglandin E2, interleukin-6, and interleukin-1 beta) and the expression of inflammatory enzymes (inducible nitric oxide synthase and cyclooxygenase-2) via inhibition of nuclear factor-kappa B activation. Moreover, compound 7 effectively prevented the downstream activation of the mitogen-activated protein kinase signaling pathway by inhibiting phosphorylation of c-Jun N-terminal kinases, extracellular signal-regulated kinases, and p38. These results outline compound 7 as a potential inhibitor for the broad treatment of inflammatory diseases, such as atopic dermatitis, asthma, and various allergies.

1. Introduction

Phlegm and recurrent coughing are two representative clinical symptoms of airway mucus hypersecretion, which are attributed to chronic inflammatory airway illnesses [1]. As an underlying self-defense mechanism, inflammation is a complex physiological process involving both the immune/vascular systems and molecular mediators, all of which are involved in sequestering inflammatory agents such as bacteria, viruses, and damaged cells. Activated macrophages mediate the inflammation process by secreting diverse pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) [2]. NO is a cell-derived signaling molecule produced by inducible nitric oxide synthase (iNOS) [3]. Excessive expression of NO leads to the overproduction of reactive oxygen species, which in turn damage DNA, leading to apoptosis [4]. Nuclear factor-kappa B (NF-κB) is a common transcription factor that expresses inflammatory cytokines and mediator molecules in mammals [2]. Activation of NF-κB is initiated when phosphorylation-induced (carried out by inhibitor of κB kinase) degradation of inhibitor of κB (IκB) occurs, which results in NF-κB entering the nucleus and activating pro-inflammatory gene expression [5,6]. Mitogen-activated protein kinases (MAPKs) are a group of protein kinases involved in a series of cellular responses, including inflammation and apoptosis [7]. The activation of MAPKs (c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38) by MAPK kinases (MEKs) has a significant effect on the process of inflammation [5,8].

Aster tataricus L.f. (family Asteraceae) is a perennial plant that possesses small, colorful flowers. It is found in many regions in Eastern Asia, including mainland China, South Korea, and Japan [9]. Its rhizomes and roots have been used as an herbal material to assist in the treatments of cough, asthma, pharyngitis, and dysuria [10]. A variety of chemical constituents have been obtained from A. tataricus, including tetracyclic triterpene ketones, triterpenoid saponins, monoterpensoids, peptides, caffeoylquinic acid derivatives, and flavonoids [10–13]. A study outlining the use of an ethanol extract of A. tataricus for the treatment of diabetic mice showed that anti-inflammatory activity was achieved by suppression of pro-inflammatory cytokines and activation of the NF-κB signaling pathway [14]. In addition, 4-hydroxyxypheylacetic acid isolated from A. tataricus showed anti-inflammatory effects by attenuating hypertonic and hypoxia-inducible factor 1-alpha production in seawater aspiration-induced lung injury in rats [15].

The goal of this study was to discover compounds with anti-inflammatory activity by evaluating the 40 known compounds and two novel monoterpene glycosides isolated from the roots of A. tataricus.
inflammatory properties isolated from *A. tataricus*, which could provide valuable evidence supporting its traditional use in the treatment of coughing and related illnesses. Our phytochemical study led to the isolation of two novel monoterpene glycosides (1 and 2) and 32 known compounds (3–34). Extensive chromatographical techniques were used to isolate the compounds from a crude methanol extract of the roots and rhizomes of *A. tataricus*. Consequently, all isolates were evaluated for their inhibitory activity against lipopolysaccharide (LPS)-induced NO production in RAW264.7 cells.

2. Experimental

2.1. General experimental procedures

Optical rotations were determined on a JASCO P-2000 polarimeter (Tokyo, Japan) at 25 °C. Column chromatography (CC) was performed by using silica gel (SiO2; 70–230, 230–400 µm particles size; Fuji Silysia Chemical Ltd., Kasugai, Japan) at 25 °C. Column chromatography (CC) was performed by using pre-coated silica gel 60 F 254 and reversed phase thin-layer chromatography (TLC) was performed by using pre-coated silica gel 60 F 254 (Merck, Darmstadt, Germany). The NMR spectra was recorded by using a JEOL ECA 600 spectrometer (1H, 600 MHz; 13C, 150 MHz; JEOL Ltd, Tokyo, Japan), δ in ppm relative to Me6Si as an internal standard and J in Hz. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 accurate-mass quantitation-time of flight-liquid Chromatography/mass spectrometry (Q-TOF LC/MS). The FT-IR spectra were conducted by using Nicolet 380 FT-IR spectrometer; KBr pellets; ṽ in cm−1.

2.2. Plant material

Dried rhizomes and roots of *A. tataricus* were provided by Korean Medicine Application Center, Korea Institute of Oriental Medicine, and taxonomically identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 18115) was deposited at Herbarium of the College of Pharmacy, Chungnam National University.

2.3. Compounds

From the methanolic extract of *A. tataricus*, thirty-four compounds (1–34) were isolated and structurally elucidated. Compounds dissolved in DMSO were diluted to the final concentration in fresh media before each experiment. To not affect the cell growth, the final DMSO concentration did not exceed 0.5% in all experiments.

2.4. Extraction and isolation

Dried crushed rhizomes and roots (3.3 kg) of *A. tataricus* were extracted with MeOH (8.0 L × 4 times) under reflux. The MeOH extract (1.6 kg) was suspended in water and partitioned with n-hexane (16.0 g), EtOAc (25.0 g), and n-ButOH (100.0 g) fractions. The n-hexane layer was subjected to silica gel (4.0 × 30 cm) column chromatography with a gradient of n-hexane-acetone (10:1, 5:1, 1:1, 2.0 L for each steps) to give 2 fractions (Fr. 1A and 1B). Fraction 1A (4.3 g) was isolated using YMC RP-18 (3.0 × 80 cm) column chromatography with an MeOH–H2O (4:1, 5:1, 1:1, 2.0 L for each steps) elution solvent to obtain compounds 4 (300.0 mg), 5 (140.0 mg), 6 (500.0 mg), 7 (20.0 mg), 9 (20.0 mg), 10 (10.0 mg), 11 (100.0 mg), 12 (8.0 mg), and 13 (2.0 mg). Fraction 1B (1.0 g) was separated using YMC RP-18 (1.5 × 80 cm) column chromatography with an MeOH–H2O (1:1, 2:1; 2.0 L for each steps) elution solvent to obtain compounds 3 (40.0 mg) and 8 (8.0 mg). The EtOAc layer (25.0 g) was subjected to silica gel (4.0 × 15 cm) column chromatography with a gradient of CHCl3–MeOH–H2O (10:1:0, 7:1:0:1, 5:1:0:1, 3:1:0:1, 1:1:0:1, 1:0:1:0, 2:0:1:0, 10:1:0, 5:1:0:1, 2:1:0:1; 2.0 L for each steps) to obtain 12 sub-fractions (Fr. 2B1–2B12). Fraction 2B2 (670.0 mg) was isolated using YMC RP-18 (1.0 × 60 cm) column chromatography with an MeOH–H2O (1.5, 1:4, 1:2; 900 mL for each steps) elution solvent to give 22 (30.0 mg) and 28 (70.0 mg). Fraction 2B4 (1.3 g) was purified using YMC RP-18 (2.0 × 80 cm) column chromatography with an MeOH–H2O (1.2, 1:1.5, 1.5 L for each steps) elution solvent to give 16 (7.0 mg), 19 (70.0 mg), 29 (6.5 mg), and 30 (40.0 mg). Fraction 2B7 (946.3 mg) was subjected to YMC RP-18 (1.0 × 60 cm) column chromatography with an MeOH–H2O (1:1, 1:2, 1:1, 1.5 L for each steps) elution solvent to give 10 (1.0 mg), 14 (5.5 mg), 15 (4.0 mg), 18 (1.5 mg), 23 (92.2 mg), and 27 (16.0 mg). Fraction 2C (6.6 g) was isolated using YMC RP-18 (3.0 × 80 cm) column chromatography with an MeOH–H2O (1:5, 1:2, 1:2, 1:2; 2L for each steps) elution solvent to give 14B2 (2.0 mg) and 14 (2.0 mg).

2.5. Acid hydrolysis of compounds 1 and 2

Compounds 1 and 2 (3.0 mg) were heated in 3 mL 1 N HCl (dioxane–H2O) at 80 °C for 3 h. The residue was partitioned between EtOAc and H2O to give the yield the sugar fractions. The aqueous layer to dioxane–H2O) at 80 °C for 3 h. The residue was partitioned between EtOAc and H2O to give the yield the sugar fractions. The aqueous layer was filtered and analyzed by gas chromatography. Retention times of the persilylated monosaccharide derivatives were as follows: 2-p-anisopropyl (tR 9.29 min) and 2-p-glucose (tR, 14.11 min) were confirmed by comparison with those of authentic standards.

2.6. Measurements of NO production and cell viability

The nitric oxide (NO) content secreted from the macrophages was...
1H and 13C NMR spectroscopic data (δ ppm) for 1 and 2.

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Assignments were done by HSQC, HMBC, COSY, and ROESY experiments.

1 H spectra were measured in methanol-d₄ at 600.

2 13C spectra were measured at 150 MHz.

3 Overlapped signals. Coupling constants (in parentheses) are in Hz.

The results were presented as means ± SEM. One-way ANOVA followed by Dunnett’s test was performed using Prism 5 software (GraphPad software, San Diego, CA, USA) to determine significant differences between the treatment groups and the LPS alone group. Data were considered statistically significant at p < 0.05.

3. Results and discussion

3.1. Identification of compounds 1–34

As part of ongoing research for novel anti-inflammatory agents from medicinal plants, purification of a methanol extract of A. tataricus yielded 34 compounds, including two novel monoterpene glycosides (1 and 2) and 32 known compounds: three monoterpenoids (3–5), five fatty acids (6–10), four triterpenes (11–14), one diterpene (15), one sesquiterpene (16), eleven caffeoylquinic acid derivatives (17–27), three lignans (28–30), and four flavonoids (31–34). Among all isolates, compounds 17, 18, and 20–30 were isolated from A. tataricus for the first time, and compounds 18, 22–25, and 29 were isolated from the Asteraceae family for the first time (Fig. 1).

Compound 1 was isolated as colorless needles ([α] = −57.7, c 0.1, MeOH). High-resolution electro-spray ionization mass spectrometry (HRESIMS) showed a pseudo-molecular ion peak at m/z 487.2147 [M + Na]+ (calked for 487.2150), which was consistent with a molecular formula of C₂₉H₃₉O₁₁. The infrared (IR) spectrum showed typical absorption bands for hydroxy groups (3400 cm⁻¹) and an ether group (1233 cm⁻¹) for hydroxyethers.

3.2. Determination of PGE2, IL-1β, IL-6, and TNF-α

RAW264.7 cells (2 × 10⁶ cells/well) were cultured in a 6-well plate for 24 h. Mixture of the cell media and Griess reagent (each 100 μL) were mixed and the supernatants were quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer’s protocol.
and the reversed correlations between H-2 ($\delta_H$ 1.81), H-4 ($\delta_H$ 1.78), H-7 ($\delta_H$ 1.58), and C-6 ($\delta_C$ 68.6). In the rotating frame Overhauser effect spectroscopy (ROESY) spectrum (Fig. 3), spatial proximities were observed between H-2/H-4 and H-7/H-10 (exo), indicating an α-orientation of H-2. Consequently, ROE correlations (Fig. 3) were observed between H-6 with H-8/H-9 (endo), but no correlations were observed between H-4 ($\delta_H$ 1.78) and H-7 ($\delta_H$ 1.58), revealing a β-orientation of H-6.

Additionally, the $^1H$ NMR spectrum (Table 1) of compound 1 showed signals for two anomic protons at $\delta_H$ 4.26 (1H, d, $J = 7.8$ Hz, H-1′) and 5.06 (1H, d, $J = 2.5$ Hz, H-1″), which gave HSQC correlations with two anomic carbon signals at $\delta_C$ 104.6 and 111.2. The analysis of chemical shifts and interpretation of the $^1H$–$^1H$ COSY, HSQC, and HMBC data revealed the existence of one β-glucopyranosyl and one β-apiofuranosyl moiety. The β-configurations of glucopyranosyl and apiofuranosyl units were indicated by the relatively large coupling constants of the anomic protons. Acid hydrolysis of compound 1 with 1N HCl afforded α-glucose and α-apiose. The HMBC correlations (Fig. 2) between H-8 ($\delta_H$ 3.52/3.91) and C-1′ ($\delta_C$ 104.6), with the reversed correlation between H-1′ ($\delta_H$ 4.26) and C-8 ($\delta_C$ 68.8), confirmed that the sugar chain was linked to C-8 of the aglycone. This was further confirmed by ROE correlations between H-1′ ($\delta_H$ 4.26) and H-8 ($\delta_H$ 3.52/3.91). Furthermore, the linkage of the β-apiofuranosyl unit at C-6′ of the β-glucopyranosyl was supported by a HMBC correlation (Fig. 2) between H-1″ ($\delta_H$ 5.06) and C-6′ ($\delta_C$ 68.9), and a reversed correlation between H-6′ ($\delta_H$ 3.65/4.02) and $\delta_C$ 111.2 (C-1″). This was further strengthened by the observed downfield shift by ~5 ppm of C-6′ ($\delta_C$ 68.9) and the ROESY correlations between H-6′ ($\delta_H$ 3.65/4.02) and H-1″ ($\delta_H$ 5.06). Therefore, the structure of compound 1 was characterized as (2-endo, 6-exo)-6-hydroxy-3,3-dimethylbicyclo[2.2.1]hept-2-yl)methyl-8-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside, namely shiosinoside A$_1$.

Compound 2 was obtained as pale yellowish needles ([α] = −53.7, c 0.1, MeOH), and its molecular formula was C$_{21}$H$_{34}$O$_{11}$, determined by HRESIMS with a sodium adduct molecular ion peak at m/z 485.1997 [M + Na]$^+$ (calcd for 485.1993). Its IR spectrum showed characteristic absorption bands for hydroxy groups (3385 cm$^{-1}$), a carbonyl group (1750 cm$^{-1}$), and an ether group (1400 cm$^{-1}$). The $^1H$ NMR spectrum (Table 1) of compound 2 showed two tertiary methyls at $\delta_H$ 1.09 (3H, s, H-9) and $\delta_H$ 1.18 (3H, s, H-10), and two anomic protons at $\delta_H$ 5.42 (1H,
Comparison with reported data confirmed the chemical structures of compounds 3–34 as the following known compounds: shionoside A (3) [11], shionoside B (4) [11], (3,3-dimethylbicyclo[2.2.1]hept-2-yl)methyl-O-β-D-glucopyranoside (5) [11], lachnophyllol (6) [19], lachnophyllol acetate (7) [20], cetylic acid (8) [21], α-linolenic acid (9) [22], α-linoleic acid (10) [23], bungeolic acid (11) [24], (+)-isobauerol (12) [25], epifriedelinol (13) [26], shionon (14) [27], (4a)-17-(acetyloxy)kauran-18-oic acid (15) [28], (+)-spathulenol (16) [29], benzyl-O-β-D-glucopyranoside (17) [30], jacobitic acid (18) [31], caffeic acid (19) [32], methyl chlorogenate (20) [33], methyl 4-caffeoylquinic acid (21) [33], 4-O-feruloylquinic acid methyl ester (22) [34], 3,5-O-dicaffeoyl-1-O-methylquinic acid methyl ester (23) [35], arilltan B (24) [36], pariposide F (25) [37], helonioside A (26) [37], helonioside B (27) [37], lariciresinol 9-O-β-D-glucopyranoside (28) [38], pinoresinol O-β-D-glucopyranoside (29) [39], isolariciresinol 9-O-β-D-glucopyranoside (30) [40], kaempferol (31) [41], astragalin (32) [42], quercetin (33) [43], and isoorientin (34) [43].

The overproduction of NO in cells is associated with inflammatory diseases and carcinogenesis. Various reports have monitored NO levels within cells when subjected to the anti-inflammatory properties of phytochemicals derived from natural products [44–46]. Thus, the anti-inflammatory inhibitory effects of the A. tataricus isolates (1–34) on NO production were evaluated in LPS-activated RAW264.7 macrophages. Ten compounds, fatty acids (6–10), terpenoids (11–15), and flavonoids (31 and 33), displayed potent inhibitory effects, with half-maximal inhibitory concentration (IC50) values of 6.9–47.2 µM (Table 2). Of
these bioactive isolates, lachnophyllol acetate (7, IC_{50}: 8.5 µM), exhibited a higher inhibitory activity than lachnophyllol (6, IC_{50}: 26.3 µM), which may be due to the presence of an acetyl group at the acetylenic alcohol in compound 7. Inflammation symptoms such as edema and pain can be attributed to PGE2 stimulation in response to inflammatory stimuli, such as tissue injury and exogenous microorganisms; it is well known that nonsteroidal anti-inflammatory drugs suppress PGE2 synthesis [47]. Pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, are key mediators of inflammation processes that lead to the pathogenesis of acute or chronic inflammatory diseases; thus, their blockade has been studied for the development of anti-inflammatory drugs [48]. To investigate whether these pro-inflammatory mediators were regulated by compound 7, we assessed the PGE2, TNF-α, IL-6, and IL-1β levels from supernatants in LPS-treated RAW264.7 cells. Compound 7 showed significant inhibitory activity against the release of PGE2 and pro-inflammatory cytokines IL-6 and IL-1β, but not TNF-α, suggesting that anti-inflammatory activity of compound 7 was due to the suppression of PGE2, IL-6, and IL-1β, as well as NO (Fig. 4).

Chronic expression of NO and PGE2, catalyzed and synthesized by iNOS and cyclooxygenase-2 (COX-2), respectively, contributes to the mediation of inflammatory processes [49]. To evaluate the inhibitory effects of compound 7 on NO and PGE2 levels, as well as iNOS and COX-2 expression, we performed Western blot analysis (Fig. 5). Protein expression of iNOS and COX-2 were reduced by compound 7 in a dose-dependent manner. Therefore, inhibitory activity of compound 7 on NO and PGE2 levels was significantly correlated with a decrease in both

<table>
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<th>IC_{50} (µM)</th>
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Results are expressed as the IC_{50} values (mean ± SEM) of three independent experiments (n = 3).

* Macrophages cytotoxicity was not observed at the IC_{50}.

Fig. 4. Effect of compound 7 on LPS-induced pro-inflammatory cytokine production in RAW264.7 cells. PGE2, TNF-α, IL-6, and IL-1β levels were measured using enzyme-linked immunosorbent assay, with dexamethasone (Dexa) as a positive control. Results are presented as the means ± SEM of duplicate experiments. *p < 0.05 compared to the LPS-alone treatment group.
Fig. 5. Effect of compound 7 on LPS-stimulated iNOS and COX-2 expression in RAW264.7 cells. iNOS and COX-2 protein levels were measured by Western blot analysis with β-actin serving as a loading control and dexamethasone (Dexa) as a positive control. The band intensity of proteins was quantified using ImageJ software. Data are expressed as the means ± SEM. * p < 0.05 compared to the LPS-alone treatment group.

Fig. 6. Effect of compound 7 on LPS-induced IκB and NF-κB activation in RAW264.7 cells. For measurement of IκB and NF-κB activity, Western blot analysis was performed with cytosolic and nuclear extracts using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). β-Actin and Lamin B served as loading controls to normalize levels of IκB and NF-κB respectively, with dexamethasone (Dexa) used as a positive control. Band intensities of the IκB/β-actin and NF-κB/Lamin B ratios were quantitated using ImageJ software. Data are expressed as the means ± SEM. * p < 0.05 compared to the LPS-alone treatment group.
iNOS and COX-2 expression.

The expression of pro-inflammatory mediators or genes, such as iNOS and COX-2, are mainly regulated by transcription factor NF-κB when activated by LPS or other inflammatory cytokines [50]. Phosphorylation and subsequent degradation of IκB activates the inflammatory process via translocation of the liberated NF-κB to the nucleus [49]. Thus, we examined whether compound 7 inhibited the phosphorylation and degradation of IκB and translocation of NF-κB to the nucleus. Compound 7 significantly suppressed the phosphorylation and degradation of IκB in the cytosol, and decreased translocation of p65, a subunit of the heterodimer of NF-κB, into the nucleus (Fig. 6).

These data suggest that the inhibitory activity of compound 7 on LPS-stimulated pro-inflammatory mediators may be involved in reduction of NF-κB activity via IκB phosphorylation and degradation.

Three major MAPKs, JNK, ERK, and p38 MAPK, regulate physiological homeostasis in response to a variety of extracellular stress signals, including mitosis, osmosis, ultraviolet radiation, and heat shock [51]. The MAPK signal cascade is involved in inflammation processes and includes phosphorylation of JNK, ERK, and p38 MAPK by MEKs [52]; therefore, the MAPK signaling pathway is desirable as a target for the treatment of inflammatory disorders. To examine whether phosphorylation of MAPKs was suppressed by compound 7, we evaluated its inhibitory activity on LPS-stimulated p-JNK, p-ERK, and p-p38 MAPK via Western blot analysis. Phosphorylation of JNK, ERK, and p38 MAPK was significantly inhibited by compound 7 (Fig. 7). Interestingly, when comparing the band intensity ratios of p-JNK/JNK, p-ERK/ERK, and p-p38/p38, compound 7 had a greater impact on phosphorylation levels of p38 MAPK than JNK and ERK (Fig. 7). Several studies have reported that iNOS expression is regulated by activation of p38 MAPK and the NF-κB signaling pathway in LPS-stimulated macrophages [52,53]. Thus, these data suggest that the anti-inflammatory activity of compound 7 is due to inhibition of the p38 MAPK signaling cascades.

4. Conclusion

Previous studies have reported the anti-inflammatory activity of extracts from A. tataricus [54,55]. However, the specific chemical component(s) responsible for the observed inhibitory activity have not been elucidated. In the present study, two new monoterpenoid glycosides (1 and 2), along with known derivatives 3–34, were isolated from A. tataricus, and their chemical structures identified by HRESIMS and NMR spectroscopic analyses. All isolates (1–34) were evaluated for their inhibitory effects on LPS-stimulated NO release in RAW264.7 cells. Based on their IC50 values, lachnophyllol acetate (7) exhibited the most potent anti-inflammatory activity, possibly due in part to the presence of an acetyl group at the terminal lachnophyllol residue. Furthermore, compound 7 inhibited iNOS and COX-2 protein expression, as well as the inflammatory mediators NO, PGE2, IL-1β, and IL-6. Its anti-inflammatory activity may be a result of inhibition of NF-κB and MAPK activation. Therefore, our findings suggest that compound 7 could be a candidate drug for the treatment of inflammatory diseases mediated via the NF-κB and MAPK signaling pathways.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.
Acknowledgments

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Appendix A. Supplementary material

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References