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ISSN: 1478-6419 (Print) 1478-6427 (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

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To cite this article: Qinghu Wang, Ying Xin, Biligetu Pa, Xiang He & Wenqiang Bao (2019): Synthesis and antitumor activity of sacroflavonoside, Natural Product Research, DOI: 10.1080/14786419.2019.1645660

To link to this article: https://doi.org/10.1080/14786419.2019.1645660

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2019.1645660

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SHORT COMMUNICATION

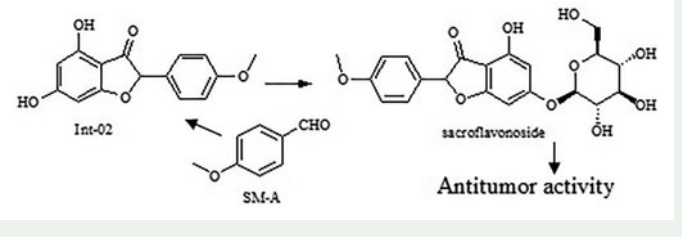
Synthesis and antitumor activity of sacroflavonoside

Qinghu Wang, Ying Xin*, Biligetu Pa, Xiang He and Wenqiang Bao

College of Traditional Mongolian Medicine, Inner Mongolia University for Nationalities, Tongliao, Inner Mongolia, China

ABSTRACT

Sacroflavonoside, a new derivative of diphenylethene, was isolated from *Artemisia sacrorum*, which have been found to possess the inhibitory effect on the proliferation of gastric carcinoma cells (MKN-45) *in vitro* in our previous studies. With anisaldehyde (SM-A) as starting material, the sacroflavonoside was synthesized by nucleophilic addition, electrophilic substitution and dehydration cyclization. The structure of sacroflavonoside was established by 1 D (^{1}H NMR and ^{13}C NMR) and 2 D-NMR (HSQC and HMBC) spectral analysis. The antitumor activity and potential mechanism against MKN-45 cells of sacroflavonoside were evaluated *in vitro*. The results showed that sacroflavonoside could significantly induce MKN-45 cells apoptosis and autophagy by increasing the expression of Bax, Caspase-3, Beclin1 and LC3-II proteins and decreasing the expression of Bcl-2 protein at low micromole level. This investigation provided a valuable lead structure for the development of antitumor drugs.



ARTICLE HISTORY
Received 24 May 2019
Accepted 14 July 2019

KEYWORDS
Sacroflavonoside; Diphenylethene; Synthesis; Antitumor activity

1. Introduction

Although the treatment of cancer had made significant progress, it remains the leading cause of death worldwide. It was reported that annual cancer cases will rise to 22 million in the next two decades (Song 2015). The treatments of cancer mainly include surgery, chemotherapy and radiotherapy. However, surgery is usually the treatments of choice for early stage cancers, for patients with unrespectable disease, local control

CONTACT Qinghu Wang wqh196812@163.com College of Traditional Mongolian Medicine, Inner Mongolia University for Nationalities, Tongliao 028000, Inner Mongolia, China
*The author contributed equally to this work

Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2019.1645660.

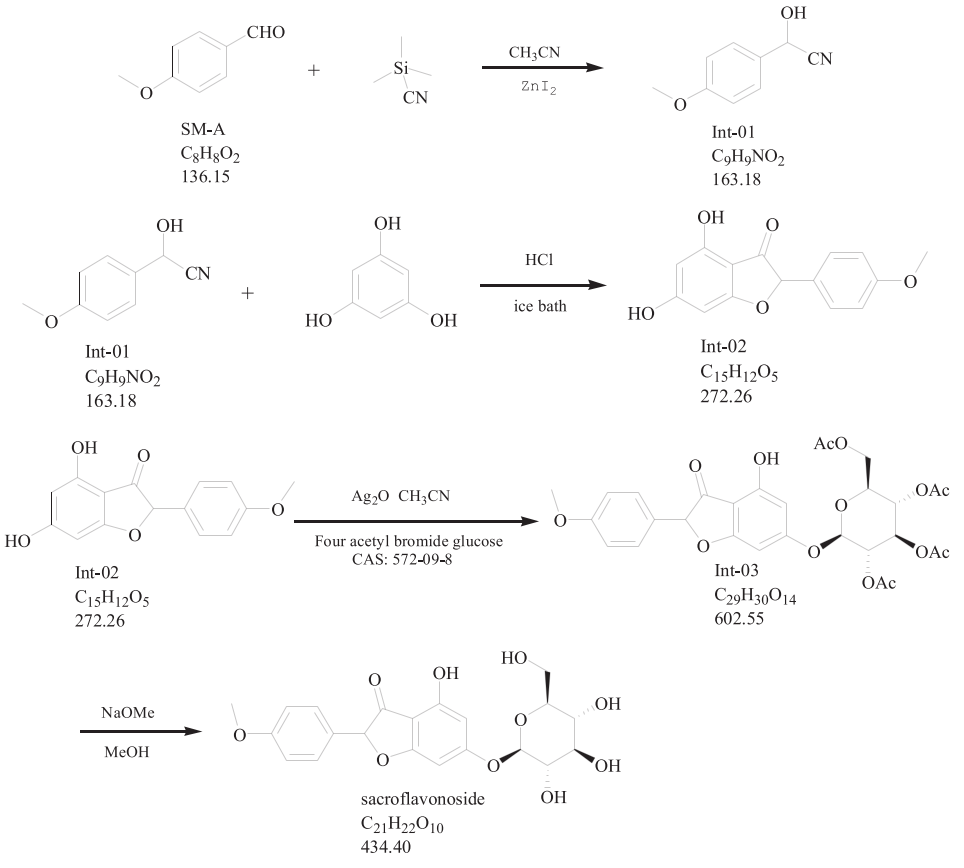


Figure 1. Synthesis route of sacroflavonoside.

can be achieved with chemotherapy combined with radiotherapy or some combination (Torre et al. 2015; Li et al. 2015). At present, chemotherapy is still the main type of cancer treatment, and phytochemicals have been a crucial part of anti-cancer drugs. Indeed, more than half of the approved anticancer drugs are either natural products or developed on the basis of knowledge gained from natural products. In addition, about 75% of plant-derived drugs used today in the clinic come from traditional medicines (Li-Weber 2009).

The aerial parts of *Artemisia sacrorum* Ledeb. (family Composite), also named Hareshabake in Mongolian, is one of the most popular and multi-purpose herbs used in China. In the traditional Mongolian medicines, *A. sacrorum* are widely used for clinical treatment of insecticidal, analgesic, convergence of pus and yellow water, detumescence, breaking the lump and so on (Wang et al. 2015). Breaking the lump is a classification type of Mongolian medicine efficacy, equivalent to the antitumor effect of modern medicine (Wang et al. 2015). It was found that the extract of dichloromethane and ethyl acetate of *A. sacrorum* had certain toxicity on breast cancer cells (MCF-7) and gastric cancer cells (MKN45) *in vitro* in our previous studies (Yu et al. 2016). The chemical constituents of *A. sacrorum* reported mainly contained terpenoids

(Wu et al. 1994; Zhang 1998), coumarins (Zhang and Li 1993) and flavonoids (Zhang et al. 1990).

Sacroflavonoside, a new derivative of diphenylethene, is isolated from *A. sacrorum*. Diphenylethens are the secondary metabolites with a C_6-C_2-C_6 skeleton and possesses a variety of biological activities including antitumor, antihypertensive, antioxidant, and so on (Busquets et al. 2007; Csiszar et al. 2009). It has been showed that sacroflavonoside had inhibitory effect on the proliferation of MNK45 cells *in vitro* in our previous studies, with relatively low IC_{50} value of 5.03 μM (Yu et al. 2016). However, it is difficult to get enough sacroflavonoside by isolation and purification due to the low content in *A. sacrorum*.

In the present study, we carried out the synthesis of sacroflavonoside by using nucleophilic addition, electrophilic substitution and dehydration cyclization. The structure of sacroflavonoside was established by 1 D (^{1}H NMR and ^{13}C NMR) and 2 D-NMR (HSQC, HMBC and HR-MS) spectral analysis. To further investigate the potential use of sacroflavonoside as antitumor compound, the mechanism of antitumor effects of sacroflavonoside was studied.

2. Results and discussion

2.1. Synthesis *of* sacroflavonoside

Scroflavonoside was synthesized by nucleophilic addition, electrophilic substitution and dehydration cyclization. A synthetic route to the compound is illustrated in Figure 1. When intermediate-01 (Int-01) was synthesized, trimethylcyanosilane was used as reactant instead of potassium cyanide in this experiment. It was first carried out in dichloromethane, and the yield was only 35%. When the solvent was changed to acetonitrile, the yield increased to 59%. During the synthesis of intermediate-02 (Int-02), yellow viscous liquid was easily obtained at room temperature, which reduced the yield. The reaction was controlled in ice bath and satisfactory results are obtained. Under the experimental conditions, the yield of scroflavonoside was 55%. The purity of scroflavonoside was 97.0% after separation and purification by silica gel column chromatography. In conclusion, a facile synthetic route of scroflavonoside was found by studying the reaction temperature and solvent conditions.

2.2. Structure elucidation of sacroflavonoside

The structure of sacroflavonoside was lucidated on the basis of spectroscopic methods including IR, 1 D NMR, 2 D NMR and HR-ESI-MS. In the ^{1}H NMR spectrum, a signal was observed as a singlet at 5.10 ppm. This proton signal provided correlation with the carbon at 87.9 ppm in 2 D HSQC, leading to its assignment as one oxidated methylene proton (Figure 2a). The proton showed strong HMBC correlations with C-4 (δ_C 104.1 ppm), C-3 (δ_C 183.7 ppm) and C-2′,6′ (δ_C 122.4 ppm) attached to C-2 at 87.9 ppm (Figure 2b), indicating the formation of furanone ring. Moreover, the anomeric proton at C-1″ position (5.05 ppm, $J = 7.5$ Hz) appeared as a doublet (Figure S1a) indicating it was a β-D-glucopyranose based on the spectral data with those reported in the literature (Tanaka et al. 2007) and the coupling constant of the anomeric proton. The

HMBC correlations (Figure S1b) from this proton to C-7 (δ_C 163.2 ppm) indicated that the β-D-glucopyranose was attached to C-7. All the proton carbon correlations were studied to deduce the structure of this compound and it was named as sacroflavonoside. The IR analysis ascertained the presence of carbonyl (1677 cm^{-1}). Further, it was confirmed by high resolution mass which was in close agreement with the calculated mass ($[M-H]^-$ for $C_{21}H_{22}O_{10}$, calculated: *m/z* 433.1135, found: *m/z* 433.1144).

2.3. Influence of sacroflavonoside on apoptosis and autophagy

In our previous study, we have demonstrated that sacroflavonoside can inhibit the growth of different digestive tumor cells, and MKN45 was the most sensitive one among the gastric cancer cell lines tested with relatively low IC_{50} value of 5.03μM (Yu et al. 2016). With a view to determine whether sacroflavonoside inhibit the proliferation of MKN45 cells by activating apoptotic pathway and another autophagic pathway, we observed the expression of Bax, Bcl-2, Caspase-3, Beclin1 and LC3B by Western blot analysis. The results showed that the expression of Bax, Caspase-3, Beclin1 and LC3-II proteins increased and the expression of Bcl-2 protein decreased in MKN45 cells treated with sacroflavonoside (Figure S2).

Caspase-3, as an important member of Caspases family that initiates and executes endogenous and exogenous apoptosis induced by various inducing factors, is a key factor in the interaction of endogenous and exogenous pathways of apoptosis (Islam et al. 2000). Activation of Caspase-3 can cause DNA repair and apoptosis by hydrolyzing PARP in the nucleus (Li et al. 2004). The activity of caspase-3 in MKN45 cells was increased 24 h after sacroflavonoside was added. Bcl-2 family proteins (including Bad, Bax, Bid, Bcl-2, and Bcl-xL) play a key role in mitochondrial-mediated apoptosis. Bad and Bax induce apoptosis by promoting mitochondrial release of cytochrome c and Smac. Bcl-2 and BclxL are known as anti-apoptotic proteins because they inhibit mitochondrial-mediated apoptosis by blocking mitochondrial release of cytochrome c or Smac. A shift in the balance between anti-apoptotic and pro-apoptotic proteins in the Bcl-2 family could trigger mitochondrial-dependent caspase activation and further induce cell apoptosis (Danial and Korsmeyer 2004; Green and Kroemer 2004). Sacroflavonoside can affect the expression of the Bcl-2 family of proteins by up-regulating Bax and down-regulating Bcl-2. Thus, it is possible that sacroflavonoside induces mitochondrial release of cytochrome-C and Smac by affecting the expression of the bcl-2 family of proteins.

On the other hand, there was increase in Beclin-1, which was identified as regulatory factors of autophagy by its existence in pro-autophagic complexes (Kang et al. 2011). LC3 is a recognized autophagic marker that can be detected in the initiation of autophagy. LC3-II is a truncated form of LC-3, and is necessary to combine with the seclusion membrane for wrapping around cytoplasm components fundamentally at random in the primary stage of autophagy (Takeuchi et al. 2005). These results suggest that treatment with sacroflavonoside induces activation of two types of cell death pathway, not only apoptosis but also autophagy.

3. Experimental

3.1. General experimental procedures

Anisaldehyde, trimethylsilyl cyanide, phloroglucin and tetraacetyl-α-D- bromide glucose was purchased from Aladdin Company, China. All the other reagents and chemicals were purchased from commercial suppliers and used as received unless otherwise stated. When needed, the reactions were carried out in oven dried glassware under a positive pressure of dry nitrogen. Melting points were determined on a STUART-SMP3 melting point apparatus without correction. IR spectra were recorded on PE-2000 spectrometer in KBr pellets and were reported in cm^{-1}. All NMR spectra were recorded on a Bruker AV-II 500 MHz NMR spectrometer with $CDCl_3$ or DMSO-d_6 as solvent and tetramethylsilane (TMS) as the internal standard, operating at 500 MHz for ^1H, and 125 MHz for ^{13}C, respectively. All chemical shift values were reported in units of δ (ppm). The following abbreviations were used to indicate the peak multiplicity: s = singlet; d = doublet; t = triplet; m = multiplet; br = broad. MS spectra were recorded on a Waters Xevo Q-TOF HRMS instrument. Reactions and chromatography fractions were performed on silica gel (200-300mesh) using the indicated eluents and thin-layer chromatography was carried out on silica gel plates with a layer thickness of 0.25 mm.

3.2. Synthesis of sacroflavonoside

To a solution of anisaldehyde (1.02 g, 7.5 mmol) in acetonitrile (36 mL) was added trimethylsilyl cyanide 1.18 mL (0.878 g, 8.8 mmol) at room temperature and zinc iodide (0.0306 g) in the environment of nitrogen protection successively. The reaction mixture was refluxed for 1.5 h and then filtered and concentrated. The residue of p-methyl benzol cyanohydrin (1.63 g, 1.0 mmol) was dissolved in absolute ether (90 mL). Phloroglucin (1.38 g, 10.9 mmol) dried by vacuum in 110 °C for 3 hours is added to the above solution. The reaction mixture was cooled immediately in ice bath and then poured into dry hydrochloric acid gas for 1 h, and was placed in the refrigerator for 3 days and then filtered, washed with ether and removed aether. The product was added to 150 mL aqueous solution in hydrochloric acid (0.1 mmol/L), refluxed for 3 h, filtered and washed with water, dried by vacuum, and got coarse powder called Int-02.

To a solution of Int-02 (136 mg, 0.5 mmol) in acetonitrile (10 mL), was added tetraacetyl-α-D- bromide glucose (369 mg, 0.9 mmol) and then was stirred evenly. To the reaction mixture was added silver oxide (173 mg, 0.75 mmol) and then stirred evenly for 5 h in the environment of nitrogen protection successively. The reaction liquid was filtered, concentrated and purified by silica gel chromatography with petroleum ether/acetone (4/1, *v*/*v*) to afford the pure product as a yellow needle.

Sacroflavonoside: Yellow needle. UV (MeOH) λ_{max} nm (log ε): 281 (3.92); IR (neat) ν (cm^{-1}): 3452, 1668, 1517; ^1H-NMR(500 MHz, DMSO-d_6) δ: 5.10 (1H, s, H-2), 6. 47 (1H, d, J = 2.0 Hz, H-5), 6.72 (1H, d, J = 2.0 Hz, H-7), 7.36 (2H, d, J = 9.0 Hz, H-2′, 6′), 7.08 (2H, d, J = 9.0 Hz, H-3′, 5′), 3.89 (3H, s, OCH_3), 5.05 (1H, d, J = 7.5 Hz, H-1″). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 87.9 (C-2), 183.7 (C-3), 161.5 (C-4), 100.5 (C-5) , 163.2 (C-6), 95.2 (C-7), 154.9 (C-8), 104.1 (C-9), 144.8 (C-1′), 122.4 (C-2′), 115. 9 (C-3′), 158.3 (C-4′), 115.9 (C-5′),

122.4 (C-6′), 56.5 (OCH_3), 100.3 (C-1′′), 76.9 (C-2′′), 73.5 (C-3′′), 70.0 (C-4′′), 77.6 (C-5′′), 61.0 (C-6′′). HR-ESI-MS: *m/z* 433.1144 $[M\text{-}H]^-$ (calcd for $C_{21}H_{22}O_{10}$, 433.1135).

3.3. Cell lines and culture conditions

Human gastric cancer cell line MKN-45 was purchased from American Type Culture Collection (ATCC), and was cultivated in RPMI 1640 medium according to the manufacturer's instructions. Medium was supplemented with 10% FBS, and all tumor cells were incubated at 37 °C and 5% CO_2 incubator.

3.4. Western blot analysis

Cells (1×10^6cells/mL) grown in six-well plates were incubated at 37 °C for 24 h with furanoside of Artemisia sacrorum treatment at various concentrations (2.5 μmol, 5 μmol). Proteins from cell lysates were extracted using a Tissue or Cell Total Protein Extraction Kit containing protease and phosphatase inhibitor cocktails. Cell lysates were centrifuged at 20,000 g for 10 min at 4°C, and protein concentration in supernatant was measured by using MicroBCA Protein Assay Reagent Kit. Protein lysates were heated in 99 °C for 10 min before being mixed evenly and centrifuged slightly. The proteins were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes followed by blocking for 2 h with 5% nonfat milk dissolved in water. The membranes were incubated with primary antibodies (cleaved GAPDH, Bax, Bcl-2, Caspase-3, Beclin1 and LC3B) overnight at 4 °C. All antibodies for Western blot were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. GAPDH was tested in the same gel as internal control for the loading sample. The membranes were incubated with fuorescent antibodies at room temperature for 2 h. After being washed, the bound antibodies were detected by the ECL Western blot detection system. Western blot analyses were repeated 3 times.

4. Conclusion

In conclusion, the present study demonstrated that sacroflavonoside could induce apoptosis and autophagy of the human gastric cancer cell line MKN45. The apoptotic pathway triggered by sacroflavonoside in MKN45 cells was dependent on mitochondrial and caspase-3 pathways, also controlled by the ratio of Bcl-2/Bax. The autophagy pathway activated by sacroflavonoside in MKN45 cells was dependent on Beclin-1. Sacroflavonoside may be useful as a novel effective reagent in treating gastric cancers, which generally have poor outcomes. We plan to investigate the induction mechanism of tumour cell death in detail, especially how sacroflavonoside turns the cell death programme on, and continue research for in vivo models of antitumour therapy.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81660698). Jazan University for providing grant to conduct this study through Ethnopharmacology Research Group.

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