Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

Article

Identification and Characterization of Two New Forced Degradation Products of Saikosaponin a under the Stress of Acid Hydrolytic Condition

Jun Li, Qiang Xu and Hua Jiang *

College of Chemistry and Pharmaceutical Engineering, Henan University of Science and Technology, Luoyang 471023, China; huomanlee@126.com (J.L.); shrib@126.com (Q.X.)

* Correspondence: jhltl2003@126.com; Tel.: +86-379-6423-1914

Abstract: Saikosaponin (SS) a is a compound with various pharmacological properties and easily degraded into SSb_1 and SSg in acid condition. In present work another two new degradation products of SSa formed under acid hydrolytic condition were detected and isolated by analytical and semi-preparative liquid chromatography technology, furthermore their structures were characterized as hydroxyl-saikosaponin a and SSb_2 by spectral analysis, which is not only essential in stability-indicating method development and validation but also could be used as a worst case to assess the analytical method performance of SSa. Moreover their structural transformation pathways were also proposed.

Keywords: Acid hydrolysis; Degradation product; Saikosaponin a

1. Introduction

SSa (Figure 1) is one major bioactive compound in the herbal medicine of radix bupleuri and exhibits various pharmacological properties such as anticonvulsant, antiepileptic and anti-inflammatory[1-3]. Recent researches paid attention to its anti-inflammatory effects in different physiological processes [3-5]. Studies have proposed several signaling pathways in inflammatory response after the treatment of SSa, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), transcription factor nuclear factor kappa B (NF-κB), toll-like receptor 4 (TLR4), and mitogen-activated protein kinase (MAPK) [4-6]. So SSa possesses the potential to be a drug candidate originated from natural product to treat inflammatory clinically.

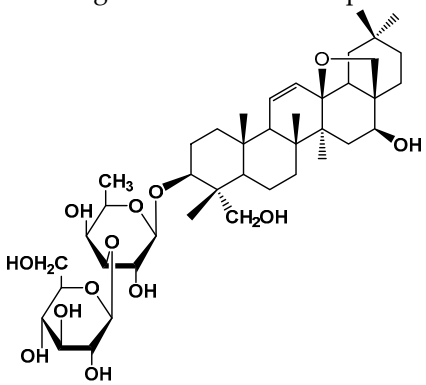

Figure 1. Structural formula of SSa

During drug product development the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines mention the necessity of stress testing to elucidate the inherent stability of the drug substance and the characterization of all degradation products formed in drug products. The information obtained may help to the establishment of the degradation patterns and a better quality control method [7, 8].

Previous research indicated that among all the stress conditions mentioned in the ICH guidelines including heating in water, dry heating, oxidation, exposure of alkali solutions and solid

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at *Molecules* **2016**, *21*, 1232; doi:10.3390/molecules21091232

power to light, SSa was relatively stable except for the stress of acid hydrolytic condition, in which the H^+ serves as an effective accelerator in the degradation process. A thorough literature survey revealed that the compounds such as SSb$_1$ and SSg had been well documented as the degradation products of SSa under the acid condition [9-13]. However in our detailed studies on the forced degradation of SSa in acid hydrolytic condition another two new degradation products were found, which needed to be characterized in accordance with ICH regulatory guidelines.

The present manuscript describes the structure characterization of the two new degradation products of SSa in acid hydrolytic condition and its structural transformation pathways.

2. Results and Discussion

2.1. Identification of new acid degradation products

The HPLC method was employed to achieve the best separation of the acid hydrolysis sample of SSa. During the optimization process, preliminary studies were carried out on Hypersil C18 column (4.6 mm×250 mm, 5 mm) using methanol: H_2O (70:30, v/v) as a mobile phase[14]. However the peaks corresponding to degradation products were not resolved completely. The peak 1 (Figure 2) was mixed with the solvent peaks under 210 nm, and the peak 3 and 4 (Figure 2) were badly overlapped under 254 nm. To get acceptable separation between SSa and its degradation products, the flow rate and composition of the mobile phase were systematically varied to optimize the method[15]. Finally, adequate separation of peaks with good resolution was obtained using methanol (A) and H_2O (B) in a gradient mode. The solvent program was set as follows: (*T*min/A:B *T*0/ 60:40; *T*8.0/60:40; *T*10/50:50; *T*20/60:40; *T*26/60:40). The flow rate of 1.0 mL·min^{-1}, column temperature at 30 °C, injection volume of 20 μL and wavelength of 210 and 254 nm were found to be suitable to achieve the separation (Figure 2).

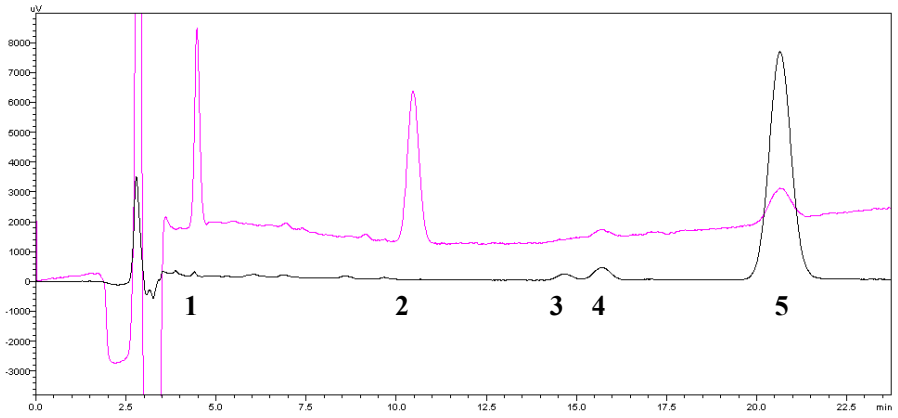

Figure 2. HPLC chromatography of SSa (2) and its acid degradation products (1,3,4,5) under the detection wavelength of 210 nm (pink line) and 254 nm (black line)

Under the optimized HPLC conditions, four degradation compound peaks were clearly presented (Figure 2). However the literature search revealed that under acid condition there were only two degradation products reported for SSa, which were SSb$_1$ and SSg [9-13]. Therefore there were another two new degradation products of SSa, which had not been clarified. By retention time comparation experiment with the authentic substances the peaks with the retention time of 21.50 min and 16.00 min were identified as SSb$_1$ and SSg respectively. Then the peaks with the retention time of 4.50 min and 14.75 min were recognized as other two new unknown degradation products of SSa (Table 1).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

Table 1 Degradation products of saikosaponin a formed in acid forced degradation experiment

No.	Peak lable	Reation time (min)	About the peak
1	Peak-1	4.50	Unknown impurity
2	Peak-2	11.50	Saikosaponin a
3	Peak-3	14.75	Unknown impurity
4	Peak-4	16.00	Saikosaponin g
5	Peak-5	21.50	Saikosaponin b_1

2.2 Characterization of the two new degradation compounds

The two new degradation compounds, arising from SSa under the tress of acid hydrolyses process, led to the interest of its structure evaluation, which not only would be useful to the stability-indicating method establishment for SSa as a potential drug candidate but also could give more clues for the new understanding of the structure transformation mechanism. The isolation was performed on a semi-preparative HPLC system, and the NMR spectral analyses were used for the structure evaluation.

2.2.1 Degradation compound of peak 1

Peak 1 was isolated as white amorphous powder, positive Molish reaction, which molecular formula was determined to be $C_{42}H_{70}O_{14}$ by the pseudomolecular ion peak at m/z 821.4546 $[M+Na]^+$ in the positive HRESI-MS experiment. In the UV spectrum, there is no absorption peak, except a slope curve started from 220 nm to 200 nm (Supplementary Materials Figure S2), which indicates the absence of conjugated double bonds. Its ^{13}C NMR spectrum was similar to that of SSb_3 (Table 2). However, the signal of a methoxyl group was detected in SSb_3, but not in peak 1. By comparing the ^{13}C NMR chemical shifts of peak 1 with those of SSb_3, the C-11 signal of the aglycone shifted upfield by 9.1 ppm and neighbouring C-9 and C-12 signals shifted downfield by 1.7 and 5.6 ppm (Table 2), respectively. These spectral data suggested that peak 1 possesses a hydroxyl group at C-11 of the aglycone moiety, but not a methoxyl group in SSb_3. So its structure was determined as hydroxyl-saikosaponin a (Figure 3). Its NMR data were in agreement with the reported literature [16].

2.2.2 Degradation compound of peak 3

Peak 3 was obtained as white amorphous powder, positive Molish reaction, and exhibited a pseudomolecular ion peak in its positive-ion HRESI-MS at m/z 803.4559 $[M+Na]^+$, corresponding to the molecular formula of $C_{42}H_{68}O_{13}$. The UV spectrum shows characteristic heteroannular diene absorption (λ_{max} 242, 254, 263 nm) (Supplementary Materials Figure S6). The ^{13}C NMR spectroscopic data of peak 3 showed close resemblance to those of SSb_1 (Table 2). Their main difference was the signal at C16, which shifted upfield by 8.6 ppm in peak 3. The spectral data indicated that an α-OH was located at C16 of the aglycone in peak 3, while a β-OH was linked at C16 in SSb_1. Then the structure of peak 3 was decided as SSb_2 (Figure 3). The NMR data were identical to the reported literature [16].

Table 2 ^{13}C NMR spectral data comparation of peak 1 with SSb_3 and peak 3 with SSb_1 (C_5D_5N, δppm)

No.	**Peak1**	**SSb_3**	**Peak3**	**SSb_1**	No.	**Peak1**	**SSb_3**	**Peak3**	**SSb_1**
1	41.5	40.1	38.4	38.4	23	64.3	64.2	64.7	64.0
2	26.8	26.5	26.1	26.1	24	13.7	13.6	13.1	13.1
3	81.8	81.7	81.6	81.5	25	18.3	18.2	18.4	18.9
4	43.9	43.9	43.8	43.7	26	18.3	18.5	17.4	17.0
5	47.8	47.6	47.3	47.3	27	26.8	26.5	21.8	22.0

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

6	17.8	17.9	18.9	18.2	28	68.7	68.5	64.7	64.0
7	33.3	33.3	32.5	32.6	29	33.5	33.3	25.1	24.9
8	41.0	41.0	41.2	40.5	30	24.0	24.0	32.3	32.4
9	55.7	54.0	54.0	54.5	MeO		52.1		
10	36.9	36.8	36.7	36.5	glc-1	106.8	106.7	106.7	106.7
11	66.8	75.9	126.3	127.0	2	75.9	75.9	75.9	75.8
12	128.1	122.5	126.3	125.7	3	78.9	78.8	78.7	78.8
13	145.2	148.3	136.2	136.4	4	72.2	72.2	72.2	72.2
14	43.9	43.9	42.0	44.2	5	78.5	78.5	78.5	78.8
15	38.2	37.0	32.6	35.0	6	62.8	62.7	62.9	62.7
16	66.4	66.2	67.9	76.5	fuc-1	106.1	106.0	106.0	106.0
17	43.9	44.0	45.4	44.4	2	71.6	71.8	71.8	71.7
18	43.9	44.0	133.1	133.3	3	85.2	85.3	85.5	85.4
19	46.5	47.0	39.1	38.4	4	71.9	71.9	71.9	71.7
20	31.0	31.1	32.7	32.6	5	71.1	71.0	71.1	71.1
21	34.2	34.2	35.6	35.1	6	17.4	17.2	17.2	17.2
22	26.0	26.2	24.6	30.0					

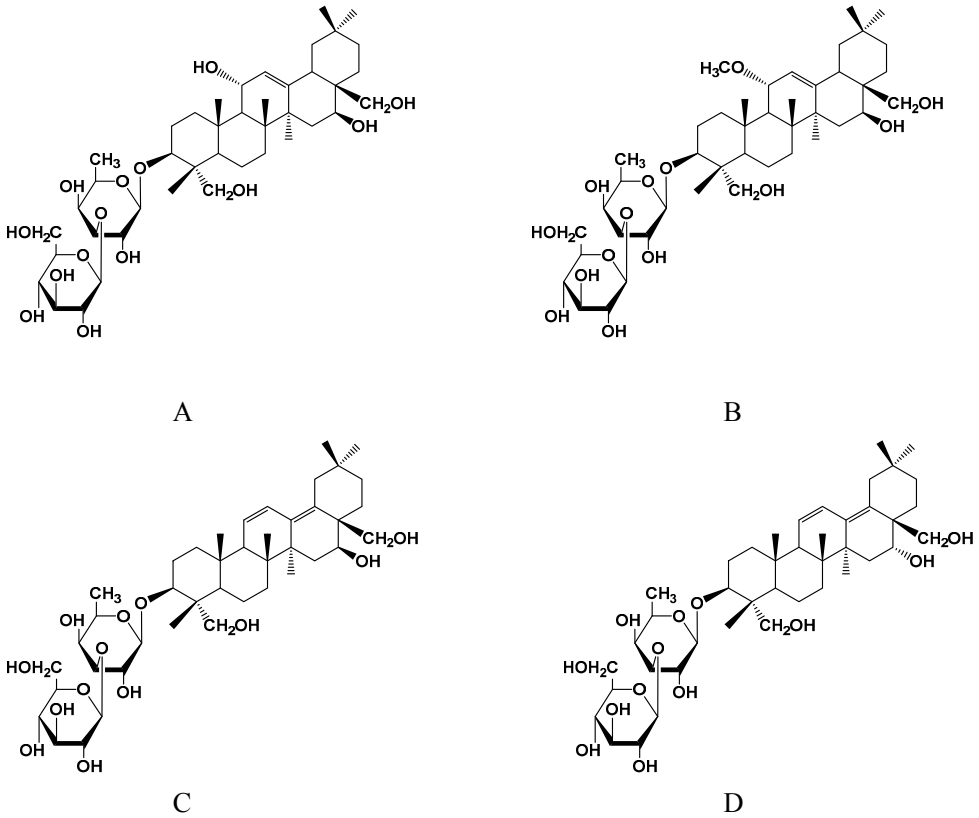

Figure 3. Structural formulas of hydroxyl-saikosaponin a (A) SSb_3 (B) SSb_1 (C) and SSb_2(D)

2.3 Structure transformation mechanism of hydroxyl-saikosaponin a

SSb_1 had been reported as the main degradation product of SSa, however there are few literatures recognizing hydroxyl-saikosaponin a as a degradation product derived from SSa in acid

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at *Molecules* **2016**, *21*, 1232; doi:10.3390/molecules21091232

hydrolysis process. Figure 4 portrayed the proposed structure transformation route of hydroxyl-saikosaponin a. Firstly a proton transfers from hydronium ion (H_3O^+) to the O atom of the 13β-28 epoxy ring in SSa forming an alkyloxonium ion, of which a dissociation is involved giving an allylic carbocation at C13. Because a vinyl group is an extremely effective electron-releasing substituent, a resonance interaction of the type shown permits the π electrons of the double bond at C11and C12 to be delocalized dispersing the positive charge, which means that the positive charge is shared by the two end carbons in the allylic unit. The positive charge on carbon and the vacant *p* orbital combine to make carbocation strongly electrophilic, which could be readily reacted with H_2O which serves as a nucleophile. And because of the steric hindrance H_2O would more likely react with the carbonation at C11 than C13. The bonds to the positively charged carbon are coplanar and define a plane of symmetry in the carbocation, which is achiral. Theoretically the H_2O would attack the carbocation at its two mirror-image faces yielding two alkyloxonium ions with different configuration. But taking the effect of steric hindrance into consideration the alkyloxonium ion with *α* relative configuration at C11 predominates. At last another H_2O acts as a base to remove a proton from the alkyloxonium ion to give the product of hydroxyl-saikosaponin a.

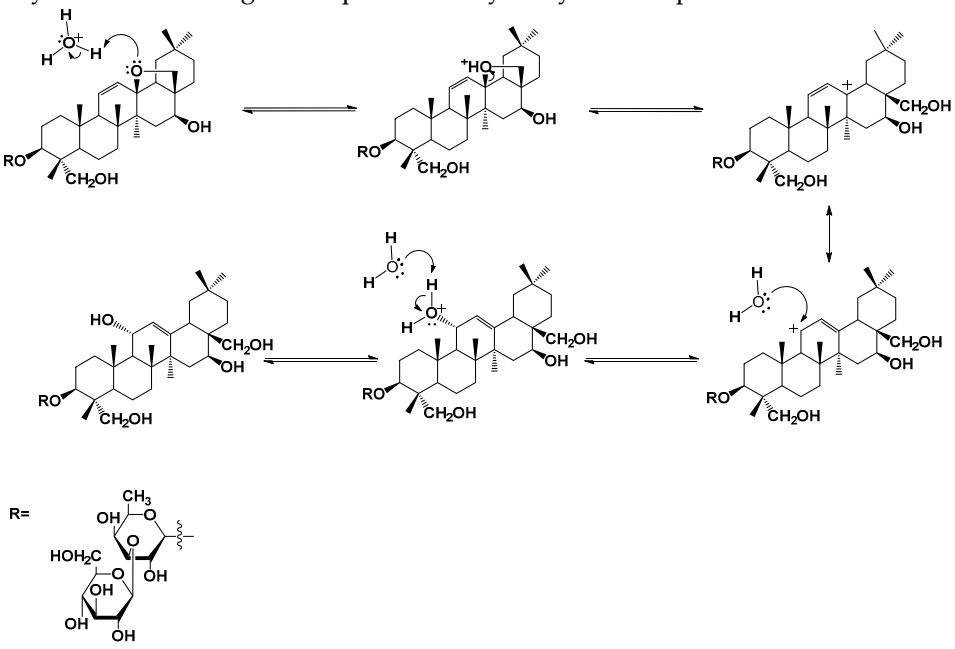

Figure 4. Proposed structure transformation pathway of hydroxyl-saikosaponin a

2.4 Structure transformation mechanism of SSb₂

SSb_2 had been known as the degradation product of SSd, an isomer of SSa with the only difference at the configuration of OH at C16. It was firstly detected in the acid hydrolysis system of SSa in our study. Based on the in-depth observation of the structure relationship SSb_2 was proposed to be derived from the compound of SSb_1. Being similar as hydroxyl-saikosaponin a, the formation of SSb_2 was described in Figure 5. Protonation of O atom of the hydroxyl group at C16 firstly takes place mediated by the hydronium ion (H_3O+) forming an alkyloxonium ion, which was followed by a dissociation giving a molecule of H_2O and a carbocation at C16. Then another H_2O acts as a nucleophile to capture the carbocation from the direction with a low level of steric hindrance, which permits the alkyloxonium ion at C16 with *α* relative congfigration to predominate. Then deprotonation of the alkyloxonium ion yields the product of SSb_2, during which H_2O acts as a Brønsted base.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

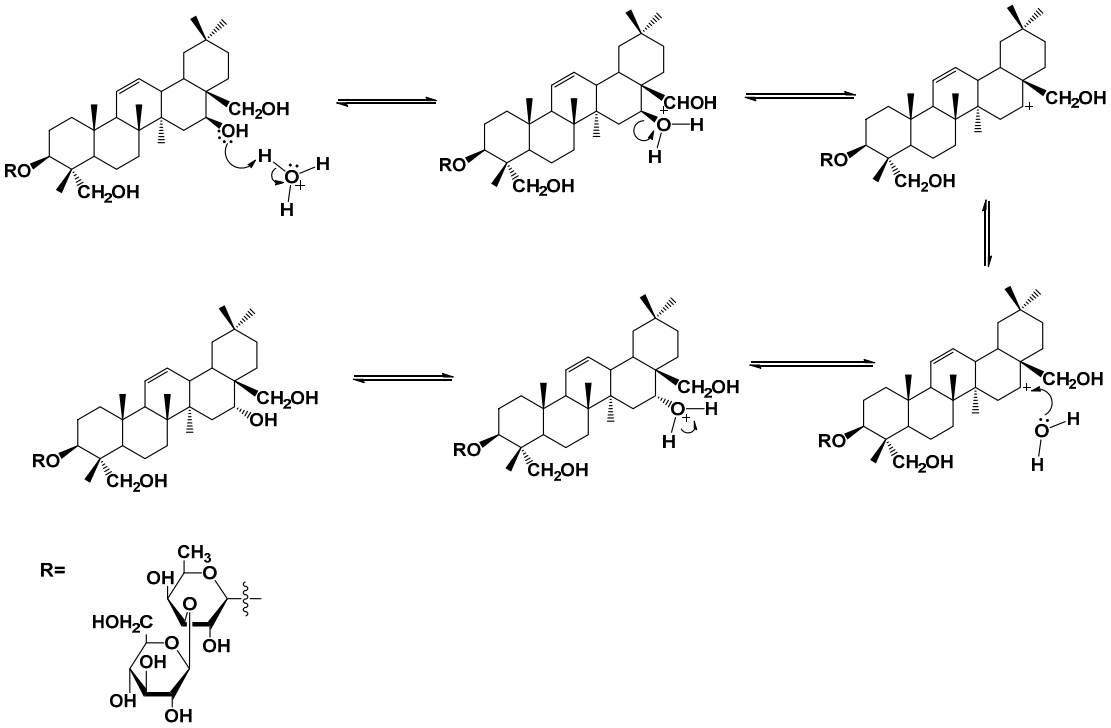

Figure 5. Proposed structure transformation pathway of saikosaponin b2

2.5 Structure transformation path of SSa in acid condition

In the acid condition SSa could not only decomposed to the known degradation compounds SSb1 and SSg but also to SSb2 and hydrol-saikosaponin a which were firstly detected as another two new degradation compounds. It is clear that SSb2 SSb1 and SSg are the isomers of SSa and hydrol-saikosaponin a is derived from the hydration reaction of SSa with H_2O. The structure transformation path of SSa in acid condition was displayed in Figure 6.

Moreover, in acidic condition SSa could also be hydrolyzed into prosaikogenin F A H and saikogenin F A H, during which the hydrolysis reaction takes place on the glycosidic bond of the sugar chain. But in our study the above mentioned aglycones and monofucoside of SSa were not detected, which could be explained that it is difficult for the hydrolyses reaction to take place on the sugar side chain under the reaction conditons used in this research.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

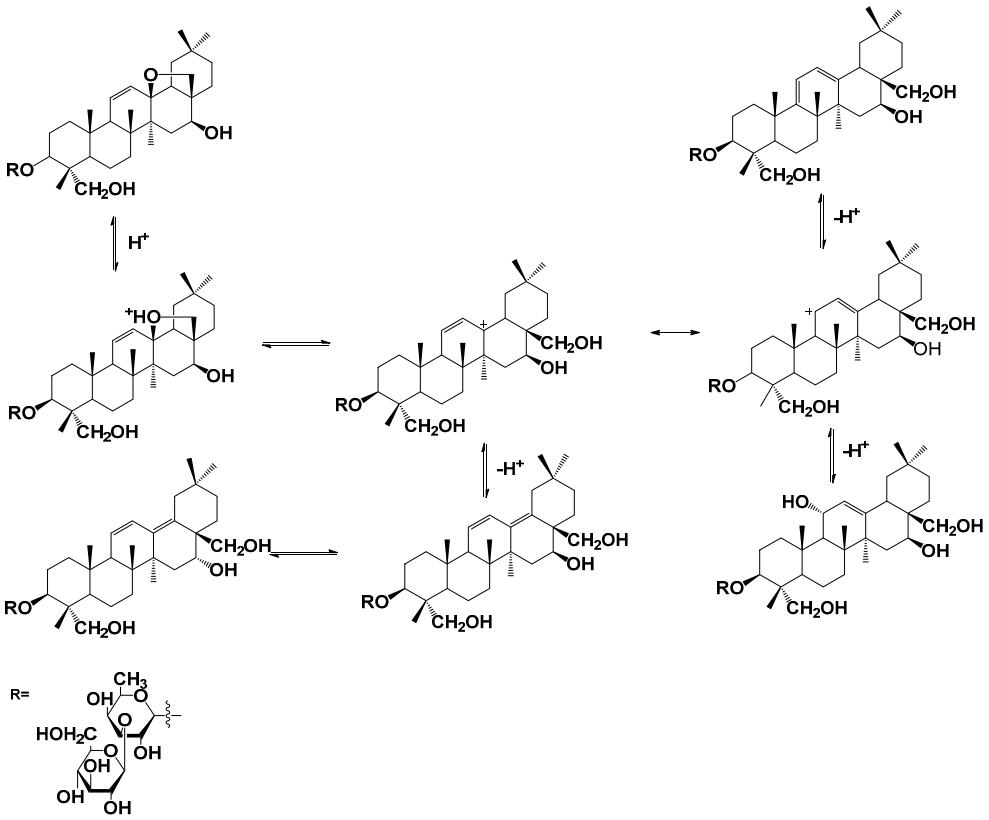

Figure 6. Structure transformation map of saikosaponin a under the stress of aicd hydrolytic condition

3. Materials and methods

3.1. Chemicals and Reagents

SSa SSb1 and SSg was supplied by Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Methanol (HPLC grade) were purchased from Dikma (Tianjin, China) and used without purification. Analytical reagent grade hydrochloric acid and sodium hydroxide pellets were purchased from Wulian Chemical Factory (Shanghai, China). Ultrapure water was obtained from Milli-Q water (18M) (Millipore, USA). All other chemicals were of AR grade.

3.2 Sample preparation

SSa was dissolved in water under the aid of heat and sonication. The stock was diluted 50:50 (v/v) with 0.2N HCl, which was heated at 80 °C for 10 h. After cooling, the solution was neutralized by NaOH solution and applied onto a glass column (20 mm × 300 mm, i.d.) packed with AB-8 macroporous resin (Chemical Plant of Nankai University, Tianjin, China). Deioned water was used to remove the NaCl and 70% aqueous ethanol was used to elute the target compouds. After eluting, solvent was removed using a rotary evaporator under vacuum, and the residue was lyophilized. Three milligrams of the lyophilized sample was redissolved in 10 ml of methanol and filtered through 0.45 μm membrane filters for HPLC analysis. And 100 mg sample was used for the further purification of the degradation compounds on a semi-preparative HPLC.

3.3 HPLC analysis

The chromatographic separation of compounds was performed on a Shimadzu Prominence LC-20A System (Shimadzu, Japan) equipped with a degasser DGU-20A5, a binary gradient pump LC-20AB and a diode array detection system SPD-M20A. The column used was a 5μm Hypersil ODS C18 column (4.6 mm×250mm i.d.), which was operated at a temperature of 30 °C. The methanol (A)-H_2O (B) was used as the mobile phase. The LC gradient program was set as (*T*min

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

/A:B *T*0/60:40; *T*8.0/60:40; *T*10/50:50; *T*20/60:40; *T*26/60:40). The flow rate was 1.0 mL·min^{-1}. The injection volume was 20 μL and the wavelengths were set at 210 nm and 254 nm.

3.4 Isolation by semi-preparative HPLC

Purified degradation compounds of SSa for structure evaluation by spectra analysis were isolated from the extract by means of repeated semi-preparative HPLC (Chuangxin Tongheng Co. Ltd, China). The column used was a YMC-Pack ODS-A Column (5μm, 20mm×250mm, i.d.). The mobile phase was (*T*min/A:B *T*0/70:30; *T*20/60:40; *T*40/65:35; *T*50/70:30) with the detection wavelength of 210 and 254 nm. The flow rate was set at 5.0 mL·min^{-1}, and the injection volume was 500 μL. The purity of the compounds was examined by analytical HPLC–DAD.

3.5 NMR spectra

NMR spectra were recorded on a Bruker AV-400 spectrometers using C_5D_5N as solvent. Tetramethylsilane (TMS) was used as internal standard. The Chemical shifts (δ) are in ppm and the coupling constants (*J*) are in Hz. The sample for NMR analysis was obtained by semi-preparative HPLC.

4. Concluding remarks

According to the ICH guideline on impurities, it is required for the identification of the degradation products formed under a variety of stress conditions, and outlining of degradation pathways and mechanisms. The two new degradation products formed during forced degradation study on SSa under the stress of acid hydrolysis condition were identified as SSb_2 and hydrol-saikosaponin a, which is not only essential in stability-indicating method development and validation but also could be used as a worst case to assess the analytical method performance for SSa. Moreover it can also help identify conditions under which additional controls should be employed in manufacturing and storage and help to understand the reactive chemistry of SSa, which is very valuable information with respect to the QbD knowledge space and the establishment of mass balance at early stage during forced degradation studies prevents surprises later during formal stability studies.

Supplementary Materials: The following are available online at www.mdpi.com/link.

Acknowledgements: The authors gratefully acknowledge the financial support from National Natural Science Foundation of China (81001615) and The doctoral scientific research foundation of Henan University of Science and Technology (13480058).

Author Contributions: Jun Li and Hua Jiang conceived and designed the experiments; Jun Li and Qiang Xu contributed to the practical aspects of the research work; Qiang Xu contributed reagents and materials; Hua Jiang and Jun Li analyzed the data ; Jun Li and Qiang Xu wrote the paper and Hua Jiang critically reviewed the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors approved the manuscript and declare no conflict of interest.

References

1. Yu, Y. H.; Xie, W.; Bao, Y.; Li, H. M.; Hu, S. J.; Xing, J. L. Saikosaponin a mediates the anticonvulsant properties in the HNC models of AE and SE by inhibiting NMDA receptor current and persistent sodium current. *PLoS One* **2012**, 11, e50694.
2. Xie, W.; Bao, Y. Dynamic observation of the effect of saikosaponin a on mice with experimental seizure. *J. Guangdong Pharm. Univ*. **2006**, 22, 65-67.
3. Lu, C.N.; Yuan, Z. G.; Zhang, X. L.; Yan, R.; Zhao, Y. Q.; Liao, M.; Chen, J. X. Saikosaponin a and its epimer saikosaponin d exhibit anti-inflammatory activity by suppressing activation of NF-kB signaling pathway. *Int. Immunopharmacol* **2012**, 14, 121-126.
4. Fu, Y.; Hu, X.; Cao, Y.; Zhang, Z.; Zhang, N. Saikosaponin a inhibits lipopolysaccharide-oxidative stress and inflammation in Human umbilical vein endothelial cells via preventing TLR4 translocation into lipid rafts. *Free Rddical Bio. Med.* **2015**, 89, 777-785.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 9 August 2016 doi:10.20944/preprints201608.0086.v1

Peer-reviewed version available at Molecules 2016, 21, 1232; doi:10.3390/molecules21091232

5. Kim, S. O.; Park, J. Y.; Jeon, S. Y.; Yang, C. H.; Kim, M. R. Saikosaponin a, an active compound of Radix Bupleuri, attenuates inflammation in hypertrophied 3T3-L1 adipocytes via ERK/NF-kB signaling pathways. *Int. J. Mol. Med*. **2015**, 35, 1126-1132.
6. Zhu, J.; Luo, C.; Wang, P.; He, Q.; Zhou, J.; Peng, H. Saikosaponin A mediates the inflammatory response by inhibiting the MAPK and NF-kB pathways in LPS-stimulated RAW 264.7 cells. *Exp. Ther. Med.* **2013**, 5, 1345-1350.
7. Ravi, T. K.; Gandhimathi, M.; Suganthi, A.; Sarovar, S. Forced-degradation study of valdecoxib as bulk drug and in tablet formulation by HPTLC. *J. Sep. Sci.* **2006**, 11, 1647-1652.
8. Shabir, G. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *J. Chromatogr. A* **2003**, 987, 57-66.
9. Li, X. Q.; Gao, Q. T.; Chen, X. H.; Bi, K. S. High Performance Liquid Chromatographic Assay of Saikosaponins from Radix Bupleuri in China. *Biol. Pharm. Bull* **2005**, 28, 1736-1742.
10. Shimizu, K.; Amagaya, S.; Ogihara, Y. Structural transformation of saikosaponins by gastric juice and intestinal flora. *J. Pharmacobiodyn* **1985**, 8, 718-725.
11. Li, J.; Jiang, H.; Zhang, Y. P.; Wang, X.S; Li, X. M. Determination of Saikosaponin b_1 and b_2 in Chai-Huang Tablet by HPLC. *Chin. Pharmacol. J.* **2011**, 46, 61-63.
12. Ohtake, N.; Nakai, Y.; Yamamoto, M.; Sakakibara, I. Separation and isolation methods for analysis of the active principles of Sho-saiko-to (SST) oriental medicine. *J. Chromatogr. B* **2004**, 812, 135-148.
13. Sumino, M.; Saito, Y.,; Ikegami, F.; Hirasaki, Y.; Namiki, T. Extraction efficiency of shosaikoto (xiaochaihu tang) and investigation of the major constituents in the residual crude drugs. *Evid Based Complement Alternat Med.* **2012**, Article ID 890524.
14. Zhao, L. C; Li, W.; He, Y. F.; Li, X. H.; Wang, Z.; Liu W. C.; Zheng ,Y. N.; Liang. J. Fast and Sensitive LC-DAD-ESI/MS Method for Analysis of Saikosaponins c, a, and d from the Roots of Bupleurum Falcatum (Sandaochaihu). *Molecules* **2011**, 16, 1533-1543.
15. Zhang, G. S.; Hu, P. Y.; Li, D. X.; He, M. Z.; Rao, X. ; Luo, X. J.; Wang, Y. S.; Wang, Y. R. Formulations, Hemolytic and Pharmacokinetic Studies on Saikosaponin a and Saikosaponin d Compound Liposomes. *Molecules* **2015**, 20, 5889-5907
16. Li, J.; Jiang, H.; Zhang, Y. P.; Zhang, Q.; Xu, Y. Structure of secondary saikosaponins in saiko decoction. *Chin. J. Chin. Mater. Med.* **2012**, 37, 3078-3082.

Sample Availability: Samples of the compounds are available from the authors.

© 2016 by the authors; licensee *Preprints*, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).