Stability and Structure Studies on Alisol A 24-Acetate

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Alisol A 24-acetate is one of the main active triterpenoid compounds isolated from *Rhizoma Alismatis*, which is a famous Traditional Chinese Medicine, and has been determined for the quality control of this crude drug. In this study, alisol A 24-acetate was found to be unstable in solvents and its stability in different solvents was investigated in detail. The results showed that alisol A 24-acetate and 23-acetate inter-transformed in solvents and the transformation rate was more rapid in protic solvents than in aprotic solvents. Moreover, both alisol A 24-acetate and 23-acetate were deacetylated to yield alisol A when kept in methanol for a long time. This is the first report on the structural transformation between alisol A 24-acetate, alisol A 23-acetate and alisol A. In addition, the single crystal X-ray structure of alisol A 24-acetate and the NMR data of alisol A 23-acetate were also reported for the first time.

Key words alisol A 24-acetate; alisol A 23-acetate; stability; *Rhizoma Alismatis*; *Alisma orientalis*

Rhizoma Alismatis, the dried rhizome of *Alisma orientalis* (SAM.) JUZEP, is a famous Traditional Chinese Medicine (TCM) which has been widely used for diuretic, hypolipidemic, anti-inflammatory and anti-diabetic purposes in China for more than a thousand years. Protostane-type triterpenes are the principal active constituents of *Rhizoma Alismatis* and more than 50 unique protostane-type triterpenes, including alisols A, B and their monoacetates, have been isolated from this herbal drug.[1—3] As the bioactive "marker compounds" of *Rhizoma Alismatis*, protostane-type triterpenes, including alisol A 24-acetate, which is one of the main active triterpenoid compounds isolated from *Rhizoma Alismatis*, have been determined for the quality control of *Rhizoma Alismatis*.[4,5] However, during the course of our research on the quantification of alisol A 24-acetate, it was found that alisol A 24-acetate was unstable and could be transformed into other compounds in methanol.

In this paper, the stability of alisol A 24-acetate in different solvents was described in detail. Furthermore, by using LC-MS, NMR, and single crystal X-ray diffraction techniques, the structures of the compounds transformed from alisol A 24-acetate and the single crystal X-ray structure of alisol A 24-acetate were elucidated for the first time.

Experimental

General Experimental Procedures A Shimadzu 10A HPLC system (Tokyo, Japan), equipped with a quaternary pump, a diode array spectrophotometric detector (DAD) and an Altech ELSD-2000ES evaporative light scattering detector (ELSD), was used for HPLC analysis. NMR spectra were recorded on a Bruker DRX-500 NMR spectrometer equipped with 5 mm probes using TMS as internal standard. LC-MS analysis was performed on an Agilent 1000 HPLC system (Palo Alto, CA, U.S.A.) equipped with a MDS SCIEX QSTAR mass spectrometer with an ESI source. A single crystal was mounted on a Japan MAC DIP-2030K area detector diffraction meter (Tokyo, Japan). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). HPLC-grade deionized water (H_2O) was purified by Milli-Q Water purification system (Millipore, MA, U.S.A.). MeOH, absolute ethanol (EtOH), 95% EtOH, ethyl acetate (EtOAc), acetone (Me_2CO), and chloroform ($CHCl_3$) for analysis were of analytic grade from Beijing Fine Chemical Company (Beijing, PR China). The aqueous organics except for 95% EtOH were mixed with the corresponding organics and HPLC-grade deionized H_2O. Absolute MeOH was obtained by refluxing of MeOH with sodium (Na) and then distilling.

Plant Material *Rhizoma Alismatis* was collected in August 2003 from Fujian Province, PR China, and identified by Prof. Hubiao Chen, School of Pharmaceutical Sciences, Peking University Health Science Center. A voucher specimen was deposited at the Herbarium of the School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing, PR China.

Reference Compounds Alisol A 24-acetate and alisol A were obtained from *Rhizoma Alismatis* by chromatography methods, and their structures were characterized by spectral methods, including MS, ^{1}H-, ^{13}C- and 2D-NMR spectra.

HPLC Analysis HPLC analysis was performed on a YMC analytical column (Tokyo, Japan) with 5 μm C18-reversed phase material (250×4.60 mm i.d.) using CH_3CN/H_2O (70% or 52%) as the mobile phase with the flow rate of 1.0 ml/min at room temperature. The injection volume was 20 μl. For UV detection, the detection wavelength were set at 210 nm, while for ELSD detection, the tube temperature was set at 90 °C, and air was used as the gas with the flow rate of 2.5 l/min.

LC-MS Analysis LC-MS analysis was performed on a YMC analytical column (Tokyo, Japan) with 5 μm C18-reversed phase material (250×4.60 mm i.d.) using CH_3CN/H_2O (52%, v/v) as the mobile phase. The flow rate was 1.0 ml/min and split 0.4 ml/min to mass spectrometer. The injection volume was 20 μl. Mass spectra were acquired using a MDS SCIEX QSTAR mass spectrometer equipped with an ESI source. All the mass spectra were acquired in negative or positive ion mode with ion source temperature at 400 °C, and detector voltage at 2200 V. The mass spectra were recorded in the range m/z 100—1000 amu.

Single Crystal X-Ray Diffraction Single crystals of alisol A 24-acetate were obtained from EtOAc solution of A-M2. A single crystal having dimensions of 0.15×0.30×0.50 mm was mounted on a Japan MAC DIP-2030K area detector diffractometer with graphite monochromated Mo$K\alpha$ radiation (λ=0.71073 Å). The crystal data are as follows: monoclinic, space group $C2$ (#5), a=34.329(2), b=7.550(1), c=27.653(2) Å, β=120.206(3)°, V=6194.1(23) Å^3, Z=8, D_X=1.143 g/cm^3. A total of 4720 independent reflections were collected at 23 °C by using the ω scanning mode ($2\theta \leq 50.0°$), of which 4662 intensity data with $|F|^2 \geq 2\sigma|F|^2$ were taken as observed reflections. The structure was solved by direct methods using SHELXS-97 software. The least-squares refinement converged with agreement factors of R_1=0.068 and wR_2=0.207.

Results and Discussion

Discovery of the Instability of Alisol A 24-Acetate Alisol A 24-acetate (Fig. 1) was isolated from 95% EtOH extraction of *Rhizoma Alismatis* through repeated column chromatography on silica gel with petrol ether/Me_2CO (7 : 3) as

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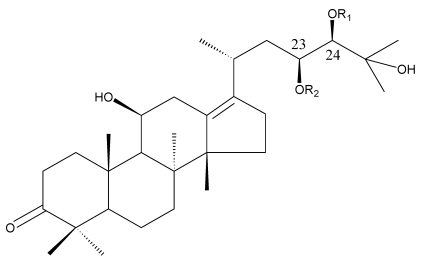
the mobile phase, and its structure was established by comparing its spectroscopic data, including MS, ^{1}H- and ^{13}C-NMR data, with those of published in cited references.[2] However, it was found that its purity was not good enough to be used as a reference compound for quantification. So this compound was further purified by semi-preparative HPLC eluting with 80% aqueous MeOH. It was interesting to find that in the HPLC two main peaks (peak 1 and peak 2) were revealed after purification by HPLC, among which the retention time of peak 1 (P_1) was same as that of alisol A 24-acetate, although there was only one main peak (P_1) before the HPLC preparation (Fig. 2). This phenomenon suggested that alisol A 24-acetate might be transformed to another compound (peak 2, P_2) during the procedure of HPLC purification of alisol A 24-acetate. In order to clarify the reasons causing the instability of alisol A 24-acetate, the sample consisting of two peaks, named A-M2, was subjected to semi-preparative HPLC again eluting with 80% MeOH and the fractions containing P_1 or P_2 were collected respectively. The collected fractions containing P_1 or P_2 were condensed properly under nitrogen stream at room temperature to avoid heating that involved during the above HPLC purification procedure and then analyzed by HPLC right away. The result showed that the fraction containing P_1 revealed one peak at the same retention time of P_1, while the fraction containing P_2 yielded one peak at the same retention time of P_2. However, as time went on, P_1 transformed to P_2 and P_2 transformed to P_1 respectively (Fig. 3). Based on the above evidences, it could be concluded that alisol A 24-acetate (P_1) and another compound (P_2) could be inter-transformed in 80% MeOH, and this transformation was not caused but could be accelerated by heating. Additionally, alisol A 24-acetate was not found to be transformed to other compounds during the course of purification by column chromatography on silica gel eluting with petrol ether/Me_2CO. So, the solvent used might be an important factor that caused its instability, and thus the stability of alisol A 24-acetate in different solvents was studied in detail.



alisol A	R_1 = H	R_2 = H
alisol A 23-acetate	R_1 = H	R_2 = $COCH_3$
alisol A 24-acetate	R_1 = $COCH_3$	R_2 = H

Fig. 1. Structures of Alisol A, Alisol A 23-Acetate and Alisol A 24-Acetate

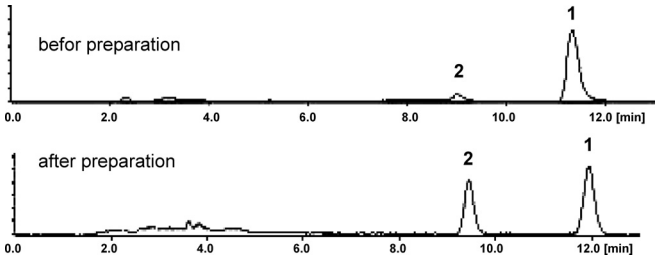


Fig. 2. HPLC-UV Chromatograms of Alisol A 24-Acetate before and after HPLC Preparation

Stability of Alisol A 24-Acetate in Different Solvents

Alisol A 24-acetate was divided into several portions and dissolved in different solvents, including absolute MeOH, MeOH, absolute EtOH, 95% EtOH, Me_2CO, CH_3CN, EtOAc, and $CHCl_3$, to prepare the solution of different solvents with the concentration of 0.5 mg/ml, respectively. Then, the samples were stored at room temperature and examined by HPLC-ELSD in turn at different times. The results showed that the transformation of P_1 (alisol A 24-acetate) to P_2 occurred in somewhat different degrees in all the above solvents, and the transformation rate in absolute MeOH, MeOH, 95% EtOH, especially in absolute MeOH, was more rapid than that in Me_2CO, CH_3CN, EtOAc, and $CHCl_3$ (Fig. 4). In addition, it was also found that the transformation rate of alisol A 24-acetate in absolute MeOH and absolute EtOH was much different from that in their corresponding solvent containing H_2O. Considering that H_2O was the most common agent, the effect of H_2O in organic solvents on this transformation was studied. The results indicated that except for in MeOH, H_2O in the other organic solvents studied accelerated the transformation of alisol A 24-acetate (Fig. 5). Thus it could be concluded that in protic solvents the transformation of alisol A 24-acetate was rapid, while in aprotic solvents the transformation was relatively slow. So we believed that protonation was involved in acetyl transformation and the transformation rate in different solvents depended on the protonation intensity of the solvents.

Additionally, it was also found that when alisol A 24-acetate was kept in absolute MeOH or MeOH for a long time at room temperature, *e.g.* over 14 d in MeOH, three peaks (P_1, P_2 and P_3) revealed in the HPLC chromatogram, and the re-

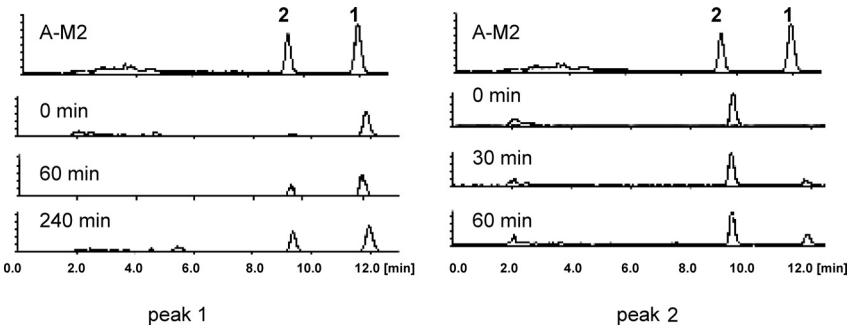


Fig. 3. HPLC-UV Chromatograms of Peak 1 and Peak 2 at Different Times

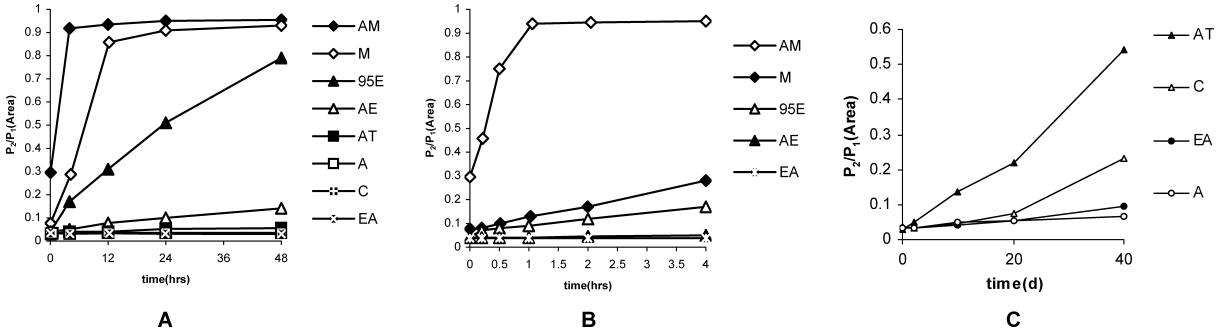


Fig. 4. Structure Transformation of Alisol A 24-Acetate in Different Solvents at Different Times (A) in 48 h; (B) in 4 h; (C) in 40 d

AM: absolute MeOH, M: MeOH, AE: absolute EtOH, 95E: 95% EtOH, A: Me_2CO, AT: CH_3CN, EA: EtOAc, C: $CHCl_3$.

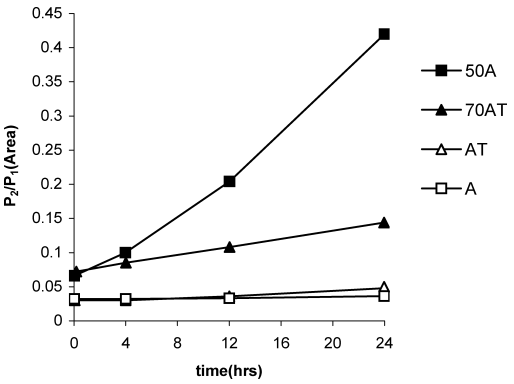


Fig. 5. Structure Transformation of Alisol A 24-Acetate in Solvents with and without H_2O

A: Me_2CO; 50A: 50% aqueous Me_2CO; AT: CH_3CN; 70AT: 70% aqueous CH_3CN.

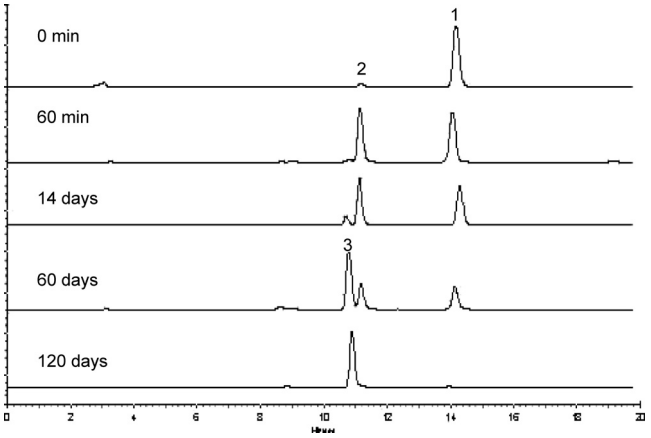


Fig. 6. Structure Transformation of Alisol A 24-Acetate in MeOH during Long Time

tention times of P_1, P_2 and P_3 were the same as those of alisol A 24-acetate, the transformed compound referred above and alisol A respectively. Moreover, when alisol A 24-acetate was kept in absolute MeOH or MeOH for a longer time, *e.g.* 120 d in MeOH, P_3 appeared as the main peak while P_1 and P_2 reduced to very little amount (Fig. 6). However, P_3 was not yet be detected when alisol A 24-acetate was kept in other solvents even for 40 d.

Structure Identification of the Transformed Compound P_2 From the above results of alisol A 24-acetate's stability in different solvents, we realized that alisol A 24-acetate was relatively stable in aprotic solvents. So A-M2 was subjected to silica-HPLC using hexane/EtOAc (75 : 25) as the eluent so as to try to obtain the pure P_1 and P_2 for further characterizing their structures. P_1 was obtained as a pure compound and elucidated as alisol A 24-acetate by comparing its retention time, ^{1}H- and ^{13}C-NMR data with those of standard of alisol A 24-acetate. Unfortunately, P_2 was obtained as a mixture of P_1 and P_2 and the intensity ratio of P_1 to P_2 was about 1 : 3 according to the ratio of their HPLC peak areas. Therefore the structure of P_2 had to be characterized through elucidating the structures of the mixture A-M2.

LC-MS (ESI-TOF) analysis of A-M2 showed that both P_1 and P_2 revealed the same quasi-molecular ion peaks at *m/z* 555 $[M+Na]^+$ and 571 $[M+K]^+$, the same major fragment ion peaks at *m/z* 515 $[M-H_2O+H]^+$ in the positive mode, and the same quasi-molecular ion peaks at *m/z* 531 $[M-H]^-$ in the negative mode, suggesting that both the molecular weights of P_1 and P_2 were 532, which were the same as that of alisol A 24-acetate. Therefore, P_1 was further proved to be alisol A 24-acetate and P_2 was deduced to be the isomer of alisol A 24-acetate. The ^{1}H- and ^{13}C-NMR spectra of A-M2 showed that it did comprise signals due to two compounds, and for the convenience of description later they were named as A-M2-1 and A-M2-2 respectively. The ^{1}H-NMR spectrum of A-M2 revealed 16 singlets and 2 doublets for methyl groups, among which the signals at δ 2.17 (3H, s) and 2.05 (3H, s) were the characteristic signals for acetyl group. Comparing the ^{1}H- and ^{13}C-NMR data of A-M2 with those of alisol A 24-acetate, it showed that the ^{1}H- and ^{13}C-NMR data due to A-M2-1 were identical with those of alisol A 24-acetate and the remnant ^{1}H- and ^{13}C-NMR signals due to A-M2-2 were also very similar with those of alisol A 24-acetate except for a few signals ascribed to the side chain. Then 2D-NMR, including ^{1}H–^{1}HCOSY, HMQC, HMBC, and NOESY, were recorded and the ^{1}H- and ^{13}C-NMR signals of A-M2-1 and A-M2-2 were assigned completely (Table 1). By comparing the ^{1}H- and ^{13}C-NMR data of A-M2-2 with those of alisol A 24-acetate, the H-23 signal of A-M2-2 shifted downfield to δ 4.91 (br t, 6.0) while the H-24 signal of A-M2-2 shifted upfield to δ 3.23 (s). The ^{1}H–^{1}HCOSY cross peaks of H-23 (δ 4.91) of A-M2-2 with δ 1.77, 1.67 (H-22) and 3.23 (H-24), and the HMBC correlations of H-23 (δ 4.91) of A-M2-2 with δ 170.91 ($COCH_3$), 37.90 (C-22), 28.72 (C-20), and 72.55 (C-25), indicated that A-M2-2 should be a 23-acetate which was produced through acetyl transformation of alisol A 24-acetate. As acetyl transformation is a popular reaction and the configuration of carbons does not change during the procedure. So we deduced that the configuration at C-24 and C-23 of A-M2-2 must keep unchanged. Thus the

Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Data of A-M2-1 and A-M2-2 in $CDCl_3$ (ppm, *J* in Hz)

No.	A-M2-1		A-M2-2		No.	A-M2-1		A-M2-2	
	^{1}H	^{13}C	^{1}H	^{13}C		^{1}H	^{13}C	^{1}H	^{13}C
1	2.22, 2.09[a)]	30.92	2.22, 2.09[a)]	30.92	17		135.12		135.06
2	2.33, 2.68[a)]	34.29	2.33, 2.68[a)]	34.17	18	1.12, s	23.12	1.14, s	22.92
3		220.49		220.32	19	1.05, s	25.53	1.05, s	25.61
4		46.93		46.93	20	2.81[a)]	27.83	2.59[a)]	28.72
5	2.06[a)]	48.50	2.06[a)]	48.44	21	0.97, d (4.0)	20.03	0.98, d (4.0)	20.09
6	1.44, 1.25[a)]	20.03	1.45, 1.28[a)]	20.09	22	1.24, 1.35[a)]	39.60	1.77, 1.67	37.90
7	1.23[a)]	33.73	1.22[a)]	33.68	23	3.88, m	68.86	4.91, br t (6.0)	71.76
8		40.37		40.71	24	4.58, s	78.61	3.23, s	78.25
9	1.74, d (11.0)	49.47	1.72, d (10.5)	49.78	25		73.86		72.55
10		36.90		36.90	26	1.14, s	27.31	1.19, s	25.48
11	3.85[a)]	69.96	3.83[a)]	70.13	27	1.28, s	26.61	1.20, s	26.55
12	2.80, 2.01[a)]	34.36	2.60, 1.95[a)]	34.53	28	1.05, s	29.52	1.05, s	29.66
13		138.08		137.36	29	1.06, s	19.96	1.06, s	20.03
14		56.97		56.97	30	0.98, s	24.09	0.97, s	23.86
15	1.33, 1.87[a)]	30.43	1.33, 1.87[a)]	30.60	$COCH_3$	2.18, s	20.77	2.05, s	21.40
16	2.13[a)]	29.02	2.18[a)]	29.37	$COCH_3$		170.79		170.91

a) Overlapped signals.

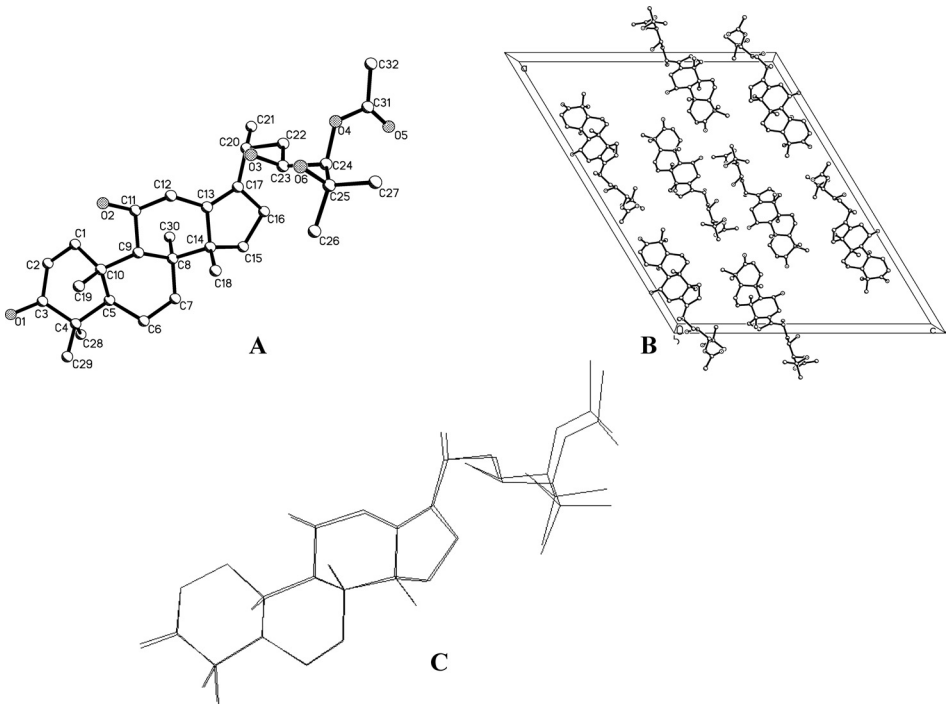


Fig. 7. The Single Crystal Structure of Alisol A 24-Acetate

(A) single molecule; (B) 8 molecules in one unit cell; (C) superimpose of two molecules in one asymmetric unit.

structure of A-M2-2 was established as alisol A 23-acetate (Fig. 1). By comparing the area ratio of P_1 to P_2 in the HPLC chromatogram and that of characteristic ^{1}H-NMR signals of A-M2-1 and A-M2-2, P_1 was deduced to be corresponded to A-M2-1 and P_2 to A-M2-2, which was consistent with the above HPLC results.

To further prove the correctness of structural elucidation of alisol A 24-acetate and 23-acetate, X-ray diffraction analysis was carried out using single crystals obtained from EtOAc solution of A-M2. From the X-ray structure here obtained, only the single crystal structure of alisol A 24-acetate was obtained and its stereochemistry was determined to be as that shown in Figs. 1 and 7 on the basis of the absolute configuration of 19-$\beta$$CH_3$ of steroids. Rings A and B were in twist boat conformation, ring C in chair conformation, ring D in envelop conformation, the configurations of A/B and B/C were linked in trans type, and the stereochemistry of the side chain was as that shown in Figs. 1 and 7. In addition, there existed two molecules with the same configuration but different conformation of the side chain in one asymmetric unit of the crystal cell because of the single bond rotation (Fig. 7). Crystallographic data for the structure of alisol A 24-acetate reported in this paper had been deposited with the Cambridge Crystallographic Data Centre (deposition num-

ber: 633142). The supplementary crystallographic data can be obtained free of charge *via* www.ccdc.cam.au.uk/conts/retrieving.html or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K.; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk.

A-M2 was proved to be a mixture of alisol A 24-acetate and 23-acetate on the basis of HPLC and NMR evidences, however, the single crystal X-ray diffraction experiment showed that the single crystal obtained from A-M2 consisted of alisol A 24-acetate only. The inconsistence of the results made us to the further analysis of the single crystal, which was used for single crystal X-ray diffraction, by HPLC. The single crystal was dissolved in EtOAc and analyzed by HPLC-ELSD right away. The HPLC chromatogram showed P_1 as the main peak while P_2 in very little amount. On the other hand, for the mother solution both P_1 and P_2 were revealed in the HPLC chromatogram (Fig. 8). So, it could be concluded that only the pure crystal of alisol A 24-acetate was obtained from the mixture of alisol A 24-acetate and 23-acetate

Alisol A 24-acetate was reported to be isolated from the genus *Alisma* in numerous reports, but the inter-transformation of alisol A 24-acetate and 23-acetate had not been reported previously. Alisol A 23-acetate was once reported to be obtained from *Alisma plantago-aquatica* L. var. *orientale* in 1968,[6] but revised to alisol A 24-acetate by themselves very soon.[7] Murata T. and the co-authors proposed that alisol A 24-acetate might be transformed to alisol A 23-acetate during the procedure of HIO_4 oxidation through acetyl transformation[7] and their proposal was in good agreement with our results in this paper.

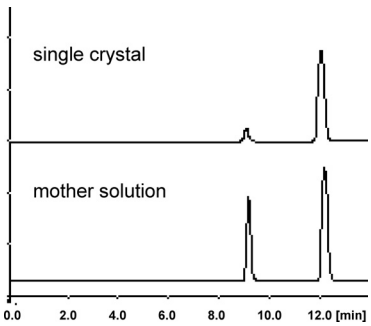


Fig. 8. HPLC-ELSD Chromatograms of A-M2

Structure Identification of the Transformed Compound P_3 As shown above that when alisol A 24-acetate was kept in absolute MeOH and MeOH for a long time, P_3 was produced, whose retention time was the same as that of alisol A. The LC-MS (in positive and negative ion mode) analysis of A-M3, *i.e.* the sample containing P_1, P_2 and P_3, proved that the rentention time and molecular weight of P_1, P_2 and P_3 were identical with that of alisol A 24-acetate, alisol A 23-acetate and alisol A, respectively. Furthermore, the LC-MS data of P_3 were the same as those of the reference compound of alisol A. Based on the above evidences, P_3 was elucidated as alisol A ultimately.

This is the first report on the instability of alisol A 24-acetate and structural transformation between alisol A 24-acetate, alisol A 23-acetate and alisol A. In addition, the single crystal X-ray structure of alisol A 24-acetate and the NMR data of alisol A 23-acetate were also reported for the first time.

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