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Isolation and identification of degradation products of salvianolic acid A by NMR and LC-MS

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ABSTRACT

Salvianolic acid A was subjected to thermal degradation condition in distilled water at 90 °C. Four degradation products, including two novel compounds (1, 2) and two known ones (5, 6), were isolated by reverse-phase semi-preparative liquid chromatography and identified on the basis of NMR and MS data. Other two degradation products (3, 4) were determined by LC-MS analysis.

1. Introduction

Salvianolic acid A (SalA, Fig. 1) is one of major components of *Salvia miltiorrhiza* (Danshen in Chinese) and its traditional Chinese medicinal preparations [1,2]. This caffeoyl derivative showed various bioactivities, such as protective effect against impairment of memory induced by cerebral ischemia-reperfusion [3], scavenger of oxygen radicals released by activated neutrophils [4], protective action on hepatocyte injured by peroxidation, inhibition of lipid peroxidation in mitochondrial membrane of hepatic cells [5] and apoptosis of human neuroblastoma SH-SY5Y cells [6], and protective effect on acute liver injury induced by CCl_4 in rats [7]. As a result, there is a great interest in SalA as a new drug in the pharmaceutical field. During our research about Danshen injection, a popular preparation of Danshen, SalA was found instable either in distilled water even at room temperature [8]. However, to our knowledge, no publication reporting the instability and its reaction products of SalA in aqueous solution was found. Undoubtedly, determination of degradation products of SalA is very important for efficacy and safety of potential drugs derived from SalA. The present paper described the isolation and identification of degradation products formed in aqueous solution.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile was chromatographic grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Deionized water was generated by a Mill-Q academic water purification system (Milford, MA, USA). Salvianolic acid A was isolated from the aqueous extract of Danshen by authors. It was identified by the MS and NMR technology and its purity (≥95%) was determined by HPLC analysis.

2.2. High-performance liquid chromatography (HPLC)

The analytical HPLC system HP 1100 series (Agilent Technologies, Waldbronn, Germany) equipped with the Chemstation Software (Agilent Technologies) and comprised

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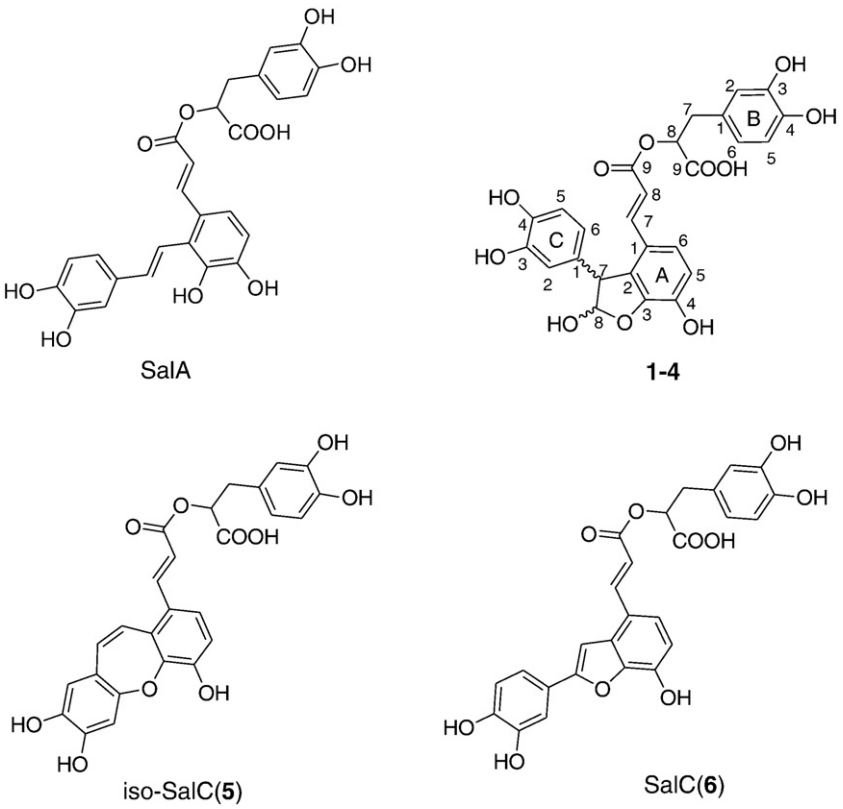

Fig. 1. Chemical structures of SalA and its oxidation products.

a quaternary pump, an online vacuum degasser, an auto-sampler, a thermostated column compartment, and a UV detector. The analysis was carried out on a Tigerkin C_{18} column (200 mm × 4.6 mm i.d., 5.0 μm particle size) from DALIAN SIPORE CO., LTD (Dalian, China) and the elution programme was as described in other report of our research group [8]. The detective wavelength was 280 nm.

The preparative HPLC system HP 1200 series (Agilent Technologies, Waldbronn, Germany) equipped with the Chemstation Software (Agilent Technologies) and comprised a quaternary pump and A DAD detector. Reaction products were semi-prepared by a Zorbax C_{18} column (9.4 mm × 25 cm) and mobile phases of 0.5% aqueous formic acid (A) and acetonitrile (B) with the elution program as follows: 0~28 min, A-B (82: 18, v/v); 28~60 min, A-B (82: 18~65: 35, v/v) with a linear gradient. The flow rate is 4 mL/min.

2.3. NMR spectroscopy

The 1D-NMR (^{1}H NMR, ^{13}C NMR and DEPT) and 2D-NMR (^{1}H-^{1}H COSY, HMQC and HMBC) were recorded on Bruker 500 MHz nuclear magnetic resonance spectrometer using CD_3OD as solvent.

2.4. LC-MS

HPLC method was followed as described in Section 2.2. Mass spectra were recorded on an LCQ deca XP^{plus} mass spectrometer (Thermo Finnigan, San Jose, USA) which equipped with an ESI interface at 350 °C. Detection of ions was performed with negative ion mode.

3. Results and discussion

3.1. Enrichment and isolation of degradation products

Purified SalA (about 200 mg) was dissolved in distilled water (10 mL) and then heated at 90 °C in water bath. The post-react solvent was analyzed by the analytical HPLC to ensure that SalA was entirely transformed. At last four degradation products were isolated and purified from the post-react solvent by semi-preparative HPLC.

3.2. Structural elucidation of degradation products

Compound 1 was obtained as yellow powder. The molecular formula of compound 1, $C_{26}H_{22}O_{11}$, was established from HR-ESI-MS (negative) at *m/z* 509.1053 $[M-H]^-$ (calcd. for $C_{26}H_{21}O_{11}$, 509.1078) and ^{13}C-NMR spectral data. Its molecular is more one oxygen atom than SalA, which indicated that compound 1 was oxidative product of SalA. The proton NMR spectrum of 1 showed the presence of eight aromatic proton signals and two olefinic proton signals, which were distinguished by their coupling constants, and five aliphatic proton signals (Table 1). Comparing with NMR data of SalA, two olefinic protons were absent while two aliphatic ones were

Table 1
NMR spectral data for 1 and 2 (CD_3OD).

Unit	Position	1	2
A	5	7.15, d, J = 8.0 Hz	7.16, d, J = 8.0 Hz
	6	6.82, d, J = 8.0 Hz	6.81, d, J = 8.0 Hz
	7	7.53, d, J = 16.0 Hz	7.50, d, J = 16.0 Hz
	8	6.20, d, J = 16.0 Hz	6.20, d, J = 16.0 Hz
B	2	6.70, bs	6.73, bs
	5	6.73, d, J = 8.0 Hz	6.71, d, J = 8.0 Hz
	6	6.49, dd, J = 8.0, 1.5 Hz	6.58, dd, J = 8.0, 1.5 Hz
	7α	3.05, dd, J = 14.5, 4.0 Hz	3.05, dd, J = 14.5, 4.0 Hz
	7β	2.94, dd, J = 14.5, 4.0 Hz	2.96, dd, J = 14.5, 4.0 Hz
	8	5.09, dd, J = 4.0 Hz	5.10, dd, J = 4.0 Hz
C	2	6.45, bs	6.45, bs
	5	6.87, d, J = 8.0 Hz	6.71, d, J = 8.0 Hz
	6	6.50, dd, J = 8.0, 1.5 Hz	6.48, dd, J = 8.0, 1.5 Hz
	7	4.38, bs	4.35, bs
	8	5.65, bs	5.66, bs

Table 2
^{13}C-NMR spectral data for 1 and 2 (CD_3OD, 125 MHz).

Position	1	2	Position	1	2
A1	122.8	122.8	B5	115.4	115.4
A2	130.7	130.8	B6	120.7	120.5
A3	144.0	143.8	B7	36.5	36.4
A4	146.0	145.9	B8	73.3	73.4
A5	121.5	121.2	B9	172.1	172.2
A6	116.1	116.1	C1	131.6	131.7
A7	142.8	142.7	C2	113.9	113.9
A 8	114.8	114.8	C3	145.4	145.4
A 9	166.8	166.9	C4	143.8	144.0
B1	127.9	127.9	C5	115.0	114.9
B2	116.0	116.1	C6	118.8	118.7
B3	144.7	144.7	C7	55.6	55.6
B4	144.2	144.2	C8	108.3	108.3

added, which indicated compound 1 may be degraded from SalA by oxidation of double bond. Through the analysis of 2D NMR data (^{1}H-^{1}H COSY, HMQC, and HMBC, Fig. 2), the structural subunit of A and B in compound 1 (Fig. 1), whose primary atoms were carboxylic carbon signals of δ_C 166.8 and δ_C 172.1, respectively, were firstly determined. These two subunits were identical with SalA. Among unassigned NMR proton signals, a broad singlet signal (δ 6.45), a doublet signal (δ 6.87, J = 8.0 Hz) and a doublet-doublet signal (δ 6.50, J = 8.0, 1.5 Hz) composed an ABX coupling system identical to SalA. The last two broad singlet signals (δ 5.65 and δ 4.38) were different points from the spectrum of SlaA and their directly connected carbon signals were assigned as δ_C 55.6 and δ_C 108.3, respectively, from HMQC. A hydroxyl proton signal (δ 7.47) was observed to correlate to proton signal δ 5.52 (δ 5.65 in CD_3OD) in ^{1}H-^{1}H COSY when NMR solvent is DMSO-d_6, which indicated a hemiacetal group. So far, the structural subunit C of 1 was elucidated. Finally the planar structure of compound 1 was determined as shown in Fig. 1 and proton and carbon signals had been totally assigned on the basis of 2D-NMR analysis (Tables 1 and 2).

Compound 2 was obtained as yellow powder. This compound had been baseline separated from compound 1 by HPLC. However this compound showed very similar MS

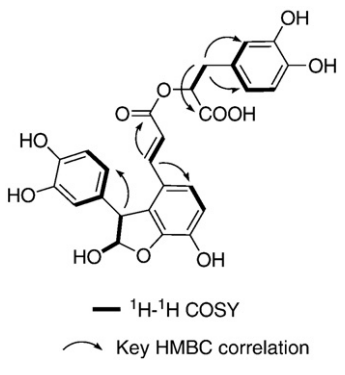

Fig. 2. Key ^{1} H-^{1} H COSY and HMBC correlations for 1 and 2.

(m/z 509, negative ion, Fig. 3) and NMR data as those of compound 1. The planar structure of 2 was evaluated as shown in Fig. 1 and its proton and carbon signals were assigned on the basis of 2D-NMR analysis and comparison with those of 1 (Tables 1 and 2). As to the stereochemistry of 1 and 2, the configuration of dihydrobenzofuran ring was determined as S/S or R/R according to the fact of $J_{C7\sim C8} \approx 0$ Hz. Interestingly, other two degradation compounds (3 and 4) were found in the thermal degradation sample of SalA (Fig. 4), which showed the same MS spectra as those of compounds 1 and 2 (Fig. 3). It is conjecturable that compounds 3 and 4 were epimers of 1 or 2 with the S/R or R/S configuration in dihydrobenzofuran ring. Compounds 5 and 6 were obtained as yellow powder. The ESI mass spectra (negative ions) of compounds 5 and 6 gave the same quasi-molecular ion peaks at m/z 491 amu ($[M\text{-}H]^-$) in ESIMS, which indicated that the two compounds have the same molecular weight of 492, less 2 Da than SalA. Comparison with the reported MS and NMR data [9–11], compounds 5 and 6 were identified as isosalvianolic acid C and salvianolic acid C (iso-SalC and SlaC, Fig. 1).

3.3. Formation mechanism of degradation products

According to their chemical structures, these degradation compounds 1–6 were oxidation products of SalA, in which 1–4 were the products of mono-oxygenation and 5–6 were the result of dehydrogenation. The proposed oxidation pathway is shown in Fig. 5, in which pinacol rearrangement and hemiacetal formation reaction will produce epimers. The latter is a stereo-selective step by Cram Rule and the major products are R/R and S/S configuration at the dihydrobenzofuran ring, which consisted with the result of LC-MS analysis (Fig. 4).

Acknowledgements

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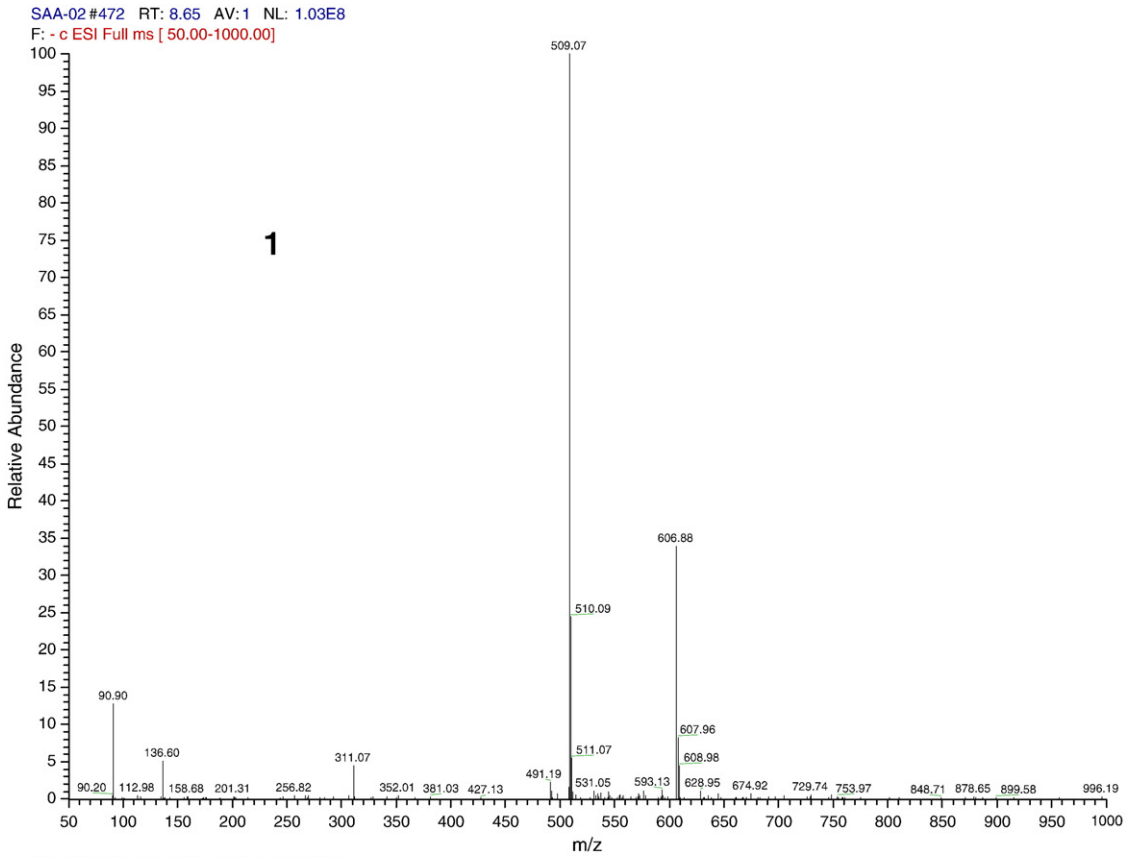
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510.09
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607.96
136.60
511.07
608.98
311.07
491.19
90.20
112.98
158.68
201.31
256.82
352.01
381.03
427.13
531.05
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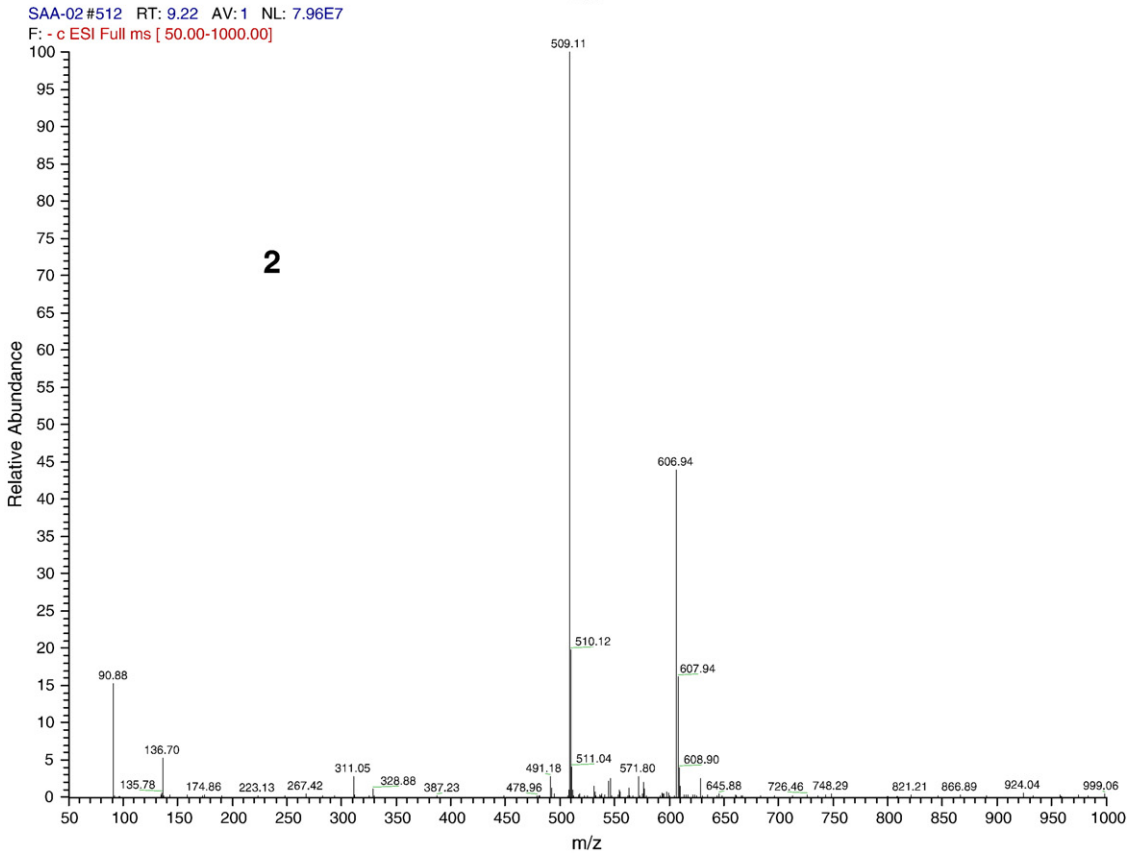
899.58
996.19
Relative Abundance
m/z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267.42
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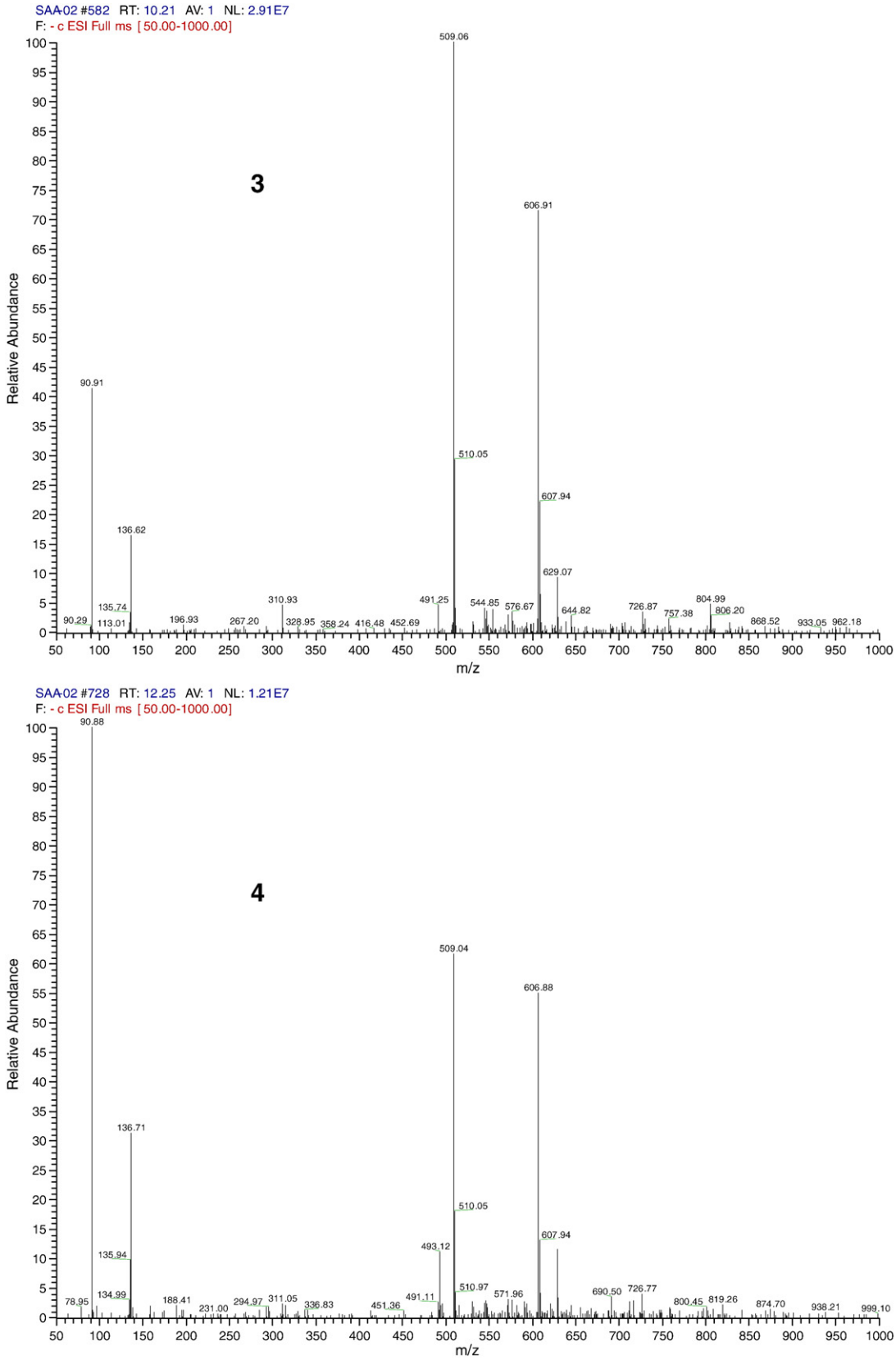

Fig. 3. MS spectra of compounds 1–4.

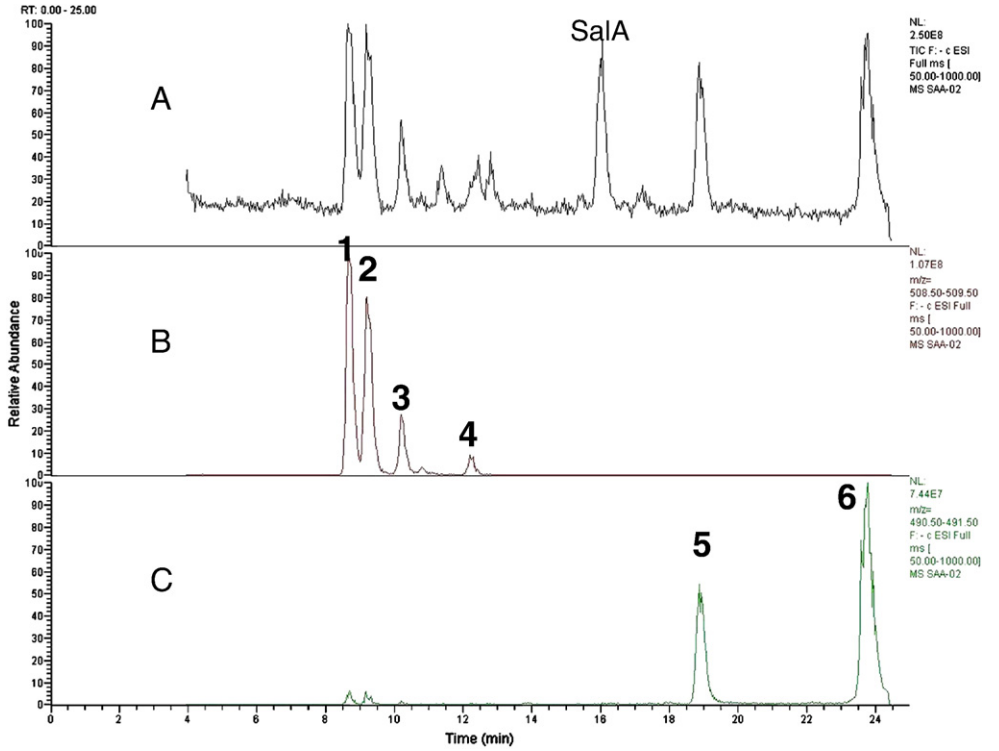

Fig. 4. LC-MS analysis of the thermal degradation sample of SalA (A: TIC, negative ion; B: *m/z* 509 ion extraction; C: *m/z* 491 ion extraction).

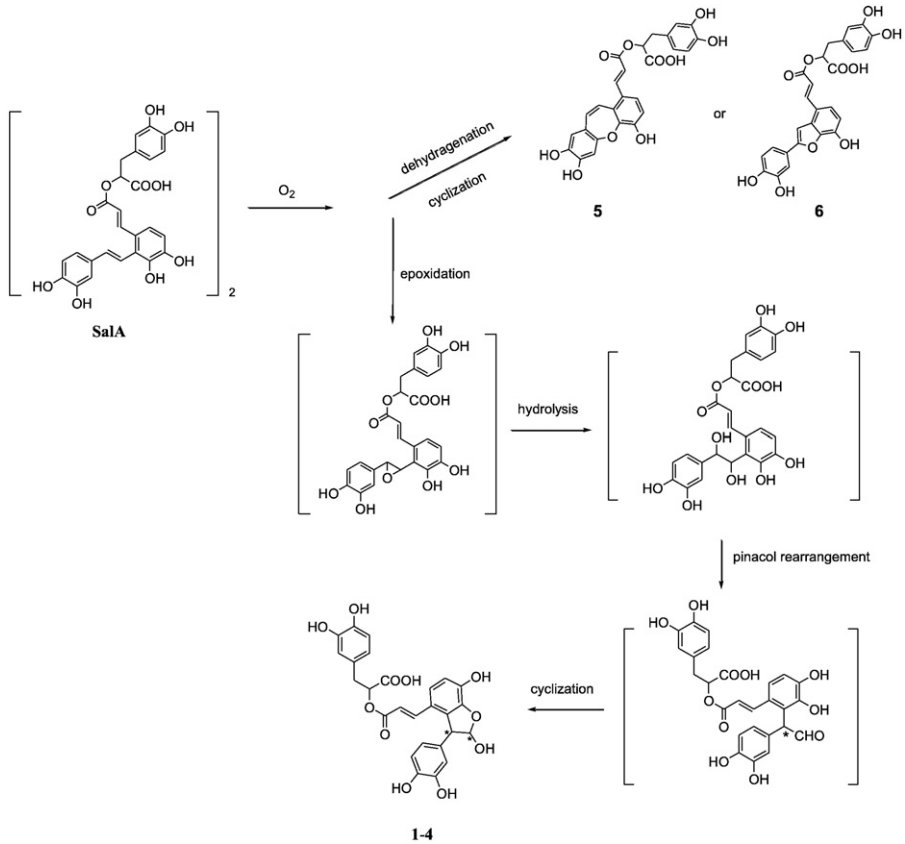

Fig. 5. Proposed forming pathway of degradation products.

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