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RESEARCH ARTICLE

Neutral Loss Scan - Based Strategy for Integrated Identification of Amorfrutin Derivatives, New Peroxisome Proliferator-Activated Receptor Gamma Agonists, from *Amorpha Fruticosa* by UPLC-QqQ-MS/MS and UPLC-Q-TOF-MS

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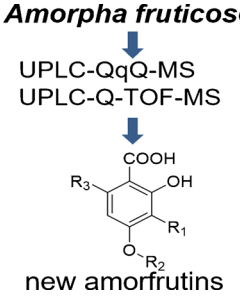

Abstract. Amorfrutins with a 2-hydroxybenzoic acid core structure are promising natural PPARγ agonists with potent antidiabetic activity. Owing to the complex matrix and low concentration in botanical material, the identification of unknown amorfrutins remains a challenge. In the present study, a combined application of UPLC-Q-TOF-MS and UPLC-QqQ-MS was developed to discover unknown amorfrutins from fruits of *Amorpha fruticosa*. First, reference compounds of amorfrutin A (AA), amorfrutin B (AB), and 2-carboxy-3,5-dihydroxy-4-geranylbibenzyl (AC) were analyzed using UPLC-Q-TOF-MS to reveal the characteristic fragment ions and the possible neutral loss. Second, the extract of *A. fruticosa* was separated and screened by UPLC-QqQ-MS using neutral loss scan to find out suspect compounds associated with the specified neutral fragment Δ*m*/*z* 44. Third, the extract was re-analyzed by UPLC-Q-TOF-MS to obtain the exact mass of quasi-molecular ion and fragment ions of each suspect compound, and to subsequently calculate their corresponding molecular formulas. Finally, according to the molecular formula of suspect compound and its fragment ions and comparing with literature data, structure elucidation of four unidentified amorfrutins was achieved. The results indicated that the combination of QqQ-MS neutral loss scan and Q-TOF-MS molecular formula calculation was proven to be a powerful tool for unknown natural product identification, and this strategy provides an effective solution to discover natural products or metabolites of trace content.

Keywords: Amorfrutins, PPARγ agonists, *Amorpha fruticosa*, UPLC-QqQ-MS/MS, UPLC-Q-TOF-MS, Neutral loss scan

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Introduction

As a nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ) plays a key role in glucose and lipid metabolism [1]. Thiazolidinediones such as rosiglitazone and pioglitazone are strong PPARγ agonists showing significant insulin-sensitivity activities and hence widely used as a potent antidiabetic in the clinic. Unfortunately, the application of synthetic PPARγ agonists has been restricted because of

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adverse effects such as fluid retention, weight gain, and cardiovascular complication [2]. Discovery of natural PPARγ agonists as lead compounds for the development of new antidiabetics are urgently needed in order to prevent and treat these globally spreading metabolic diseases.

Amorfrutins were initially characterized as antibacterial agents from fruits of *Amorpha fruticosa* L. (Fabaceae) in the 1980s [3], and interests in this class of compounds was rekindled by the discovery that amorfrutins could act as a new class of natural PPARγ agonists in recent years. The interaction pattern of amorfrutins and PPARγ is quite different from that of thiazolidinediones, resulting in the selective activation of a subset of genes under PPARγ control [4]. In vitro and in vivo tests indicated that amorfrutins are promising selective PPARγ agonists with potent antidiabetic activity and seemingly devoid of undesirable side effects typical of synthetic PPARγ drugs [5, 6].

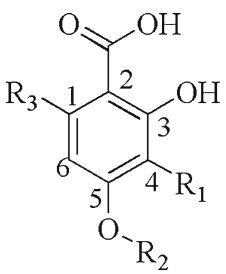
Amorfrutins feature a simple planar 2-hydroxybenzoic acid core structure decorated with a *para*-methoxy- or hydroxy group, a *meta*-isoprenoid side chain (R1), and an *ortho*-aromatic or alkyl side chain (R3; Figure 1) [4, 7]. Due to the difficulties in their isolation from complex matrix and low concentrations in botanical materials, only six amorfrutins, namely amorfrutin A, B, C, D, 2, and 3 have been identified till now [8, 9]. Therefore, identification of more new amorfrutins is desirable to facilitate illuminating the structure–activity relationship and further optimizing their druggability.

LC-MS technique is usually very effective for rapid identification of unknown compounds based on their LC retention behaviors and MS characteristics [10]. Owing to its high sensibility and rapid scanning ability, MS is regarded as a powerful tool for screening trace compounds from complex matrix such as botanical extracts and biological samples [11–13].

TOF-MS is able to determine the exact mass of target compounds and hence to afford corresponding molecular formula. Therefore, TOF-MS is often used for full scanning in qualitative analysis. When target compounds are located and identified, QqQ-MS is an excellent selection for the subsequent quantitative analysis [14, 15].

QqQ-MS can be performed in different scan modes, including precursor ion scan, product ion scan, neutral loss scan, and so on [16]. In practice product ion scan is the most frequently used mode for quantitative analysis. However, we noticed that the less used mode neutral loss scan would be potent in qualitative analysis for certain types of compounds, especially for which specific neutral fragments from their precursor ion [17, 18] can be produced. Under neutral loss scan mode, Q1 and Q3 parts of QqQ-MS are set for full scanning, while Q2 part is used as collision cell for producing fragments. Once the instant difference of *m*/*z* values between Q1 and Q3 parts conform to the set value, namely the molecular weight of neutral fragment, signals of precursor ion in the Q1 part will be recorded. The *m*/*z* comparison between two mass analyzers Q1 and Q3 would significantly filter out irrelevant signals and maximize the sensibility and selectivity of MS measurement, so that a clean chromatogram can be obtained from complex matrix for further analysis.

Compared with conventional methods utilized in natural product research, we believe that online UPLC-MS/MS analysis would provide a rapid, efficient, and convenient strategy for screening unknown compounds with a same core structure as complex matrix. In the present study, a combined application of UPLC-Q-TOF-MS and UPLC-QqQ-MS was developed in order to discover unidentified amorfrutins from fruits of *A. fruticosa*. First, reference compounds were analyzed using UPLC-Q-TOF-MS to reveal the mass fragmentation regularities of amorfrutins by characteristic fragment ions, and to subsequently find out the possible neutral fragments. Second, the extract of *A. fruticosa* was separated and screened by UPLC-QqQ-MS using neutral loss scan to find out suspect compounds associated with the specified neutral fragments, and to record the corresponding *m*/*z* value and retention time. Third, the extract of *A. fruticosa* was re-analyzed under the established chromatographic condition by UPLC-Q-TOF-MS to obtain the exact mass of quasi-molecular ion and fragment ions of each suspect compound, and to subsequently calculate their corresponding molecular formulas. Finally, according to the molecular formula of suspect compound and its fragment ions and comparing with literature data, structure elucidation of the suspect compound could be achieved. Thus, unknown amorfrutins might be discovered from *A. fruticosa*. To our best known, the present study is the first to combine QqQ-MS neutral loss scan and Q-TOF-MS molecular formula calculation for unknown natural product identification, and we believe that this strategy provides an effective solution to discover natural products or metabolites of trace content.



	R_1	R_2	R_3
AA	prenyl	CH_3	phenylethyl
AB	geranyl	CH_3	phenylethyl
AC	geranyl	H	phenylethyl

Figure 1. The core structure of amorfrutins and the chemical structures of amorfrutin A (AA), amorfrutin B (AB), and 2-carboxy-3,5-dihydroxy-4-geranylbibenzyl (AC)

Experimental

Chemicals and Herb Materials

Fruits of *A. fruticosa* authenticated by Professor Hao Zhang (Sichuan University, China) were collected at Nantong, Jiangsu Province, China. Three authentic reference compounds, namely amorfrutin A (AA), amorfrutin B (AB), and 2-carboxy-3,5-dihydroxy-4-geranylbibenzyl (AC) were isolated and purified in-house from fruits of *A. fruticosa*. Their structures (Figure 1) were characterized by multiple spectroscopic analyses (UV, HR-ESI-MS, ^{1}H NMR, and ^{13}C NMR) and comparison with the literatures [3, 19]. The isolation process and structural identification of the reference compounds AA, AB, and AC are provided as Supplementary Materials. The purity of each compound was more than 98%, determined by UPLC-DAD analysis. Ultra-pure water prepared by Milli-Q water purification system (Millipore, Bedford, MA, USA), chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), and formic acid (TCI, Shanghai, China) were used for LC-MS analysis. Other chemicals and reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Preparation of A. fruticosa Extract

Air-dried and powdered fruits of *A. fruticosa* (20 g) were extracted with 80% aqueous ethanol (100 mL × 3 times) by ultrasonic (30 min for each time) at room temperature. The combined extract was concentrated in vacuum at 45 °C. The residue was suspended in 40% aqueous ethanol (20 mL), and then partitioned with *n*-hexane. The *n*-hexane layer was evaporated to yield a dark brown semi-solid (937 mg). The *n*-hexane-soluble extract was stored at –4 °C before analysis. The concentrated *n*-hexane extract was dissolved in methanol and filtered through 0.2 μm membrane filter before UPLC analysis.

UPLC-QqQ-MS Analysis

UPLC analysis was carried out on an Agilent 1290 UPLC system (Agilent, CA, USA) equipped with a binary pump, an autosampler, a photodiode array detector, and a column heater. *A. fruticosa* extract was separated on a Waters BEH C18 analytical column (100 × 2.1 mm i.d., 1.7 μm). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid, using a gradient elution of 60% B at 0.0–0.5 min, 60%–70% at 0.5–6.5 min, 70%–90% at 6.5–10.0 min, 90% at 10.0–11.0 min, 90%–60% at 11.0–11.1 min, 60% at 11.1-15.0 min. The flow rate was 0.2 mL/min, and the column temperature was kept at 35 °C.

QqQ-MS measurements were performed on an Agilent 6460 triple quadrupole mass spectrometer (Agilent, Hanover, Germany) equipped with an electrospray ionization (ESI) source. The ESI-MS spectra were acquired in negative ion mode. The mass spectrometry detector (MSD) parameters were as follows: drying gas (N_2) flow rate, 11 L/min; drying gas temperature, 350 °C; nebulizer pressure, 40 psi; Delta EMV (–), 300; scan type, neutral loss; loss: 44; fragmentor, 150; collision energy, 20; accelerator voltage: 4; capillary voltage, 4000 V. Data collection and processing were conducted with MassHunter Workstation (ver. B 06.00, Agilent Technologies).

UPLC-Q-TOF-MS Analysis

The chromatographic separation was performed by the method described above. MS analysis was carried out on a 4600 Q-TOF mass spectrometer (AB Sciex, Concord, CA, USA) equipped with an electrospray ionization (ESI) source. Q-TOF-MS analysis was performed in negative mode. The conditions of the ESI source were as follows: ion source gas 1 (GS1) (N_2): 50 psi; curtain gas: 35 psi; temperature, 500 °C; ionspray voltage floating, -4500 V; for MS/MS experiments, the collision energy (CE) was set at –30 eV. The mass range was set at *m/z* 100–1000 for MS and *m/z* 50–1000 for MS/MS. The system was operated under Analyst 1.6 and Peak 2.0 (AB Sciex, Concord, CA, USA)

Results and Discussions

TOF-MS/MS Characterization of the Reference Compounds (AA, AB and AC)

Optimization of the MS Collision Energy The electrospray ionization source was operated in negative ion mode since amorfrutins have a core structure of 2-hydroxybenzoic acid. The main parameters of TOF-MS and TOF-MS/MS were set by convention except for the collision energy (CE). The CE value for TOF-MS/MS was optimized to maximum fragment information for structural characterization extraction. TOF-MS/MS measurements of compounds AA, AB, and AC were carried out across the rang from –10 eV to –60 eV rising every –5 eV with 80% acetonitrile/water solution (*v/v*, containing 0.1% formic acid). According to the available number and relative abundances of fragment ions, the optimized CE value was finally set at –30 eV.

Mass Spectrum Fragmentation Pathways of Amorfrutins Deduced from the TOF-MS/MS Spectra of Reference Compounds

The molecular formula of each fragment ion could be calculated according to its exact mass from TOF mass spectrum. As shown in Figure 2, a characteristic neutral loss of carbon dioxide (△*m/z* 44, CO_2) was obvious in each TOF-MS/MS spectrum of reference compound (AA, AB, or AC) by comparing the chemical formula between quasi-molecular ion and fragment ions. Taking compound AA as an example: the chemical formula of *m/z* 339.1614 $[M–H]^-$ (calcd. for $C_{21}H_{23}O_4^-$ *m/z* 339.1602) and *m/z* 295.1712 (calcd. for $C_{20}H_{23}O_2^-$ *m/z* 295.1704) were established by TOF-MS/MS, respectively. The product ion $C_{20}H_{23}O_2^-$ was formed from the precursor ion $C_{21}H_{23}O_4^-$ through a neutral loss of CO_2 unit

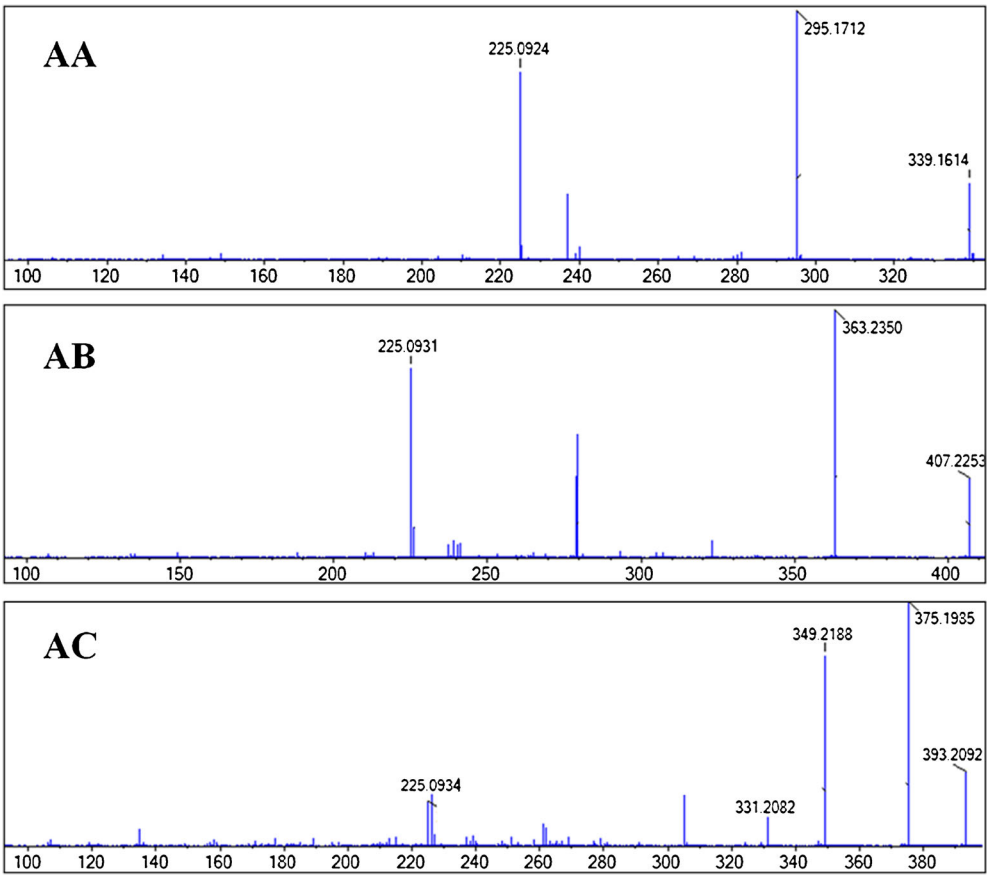


Figure 2. The TOF-MS/MS spectra of reference compounds (AA, AB, and AC)

(△*m/z* 43.9902, calcd. for CO_2*m/z* 43.9898). If the C-4 position of 2-hydroxybenzoic acid core structure was hydroxyl (–OH) rather than methoxy (–OCH_3), a very distinct neutral loss of H_2O appeared in the mass spectrum (for compound AC: *m/z* 393.2092 $[M–H]^-$ → *m/z* 375.1985 $[M–H–H_2O]^-$). In addition, *m/z* 225.0924, *m/z* 225.0931, and *m/z* 225.0934 (calcd. for $C_{15}H_{13}O_2^-$*m/z* 225.0921) were characteristic fragment ions in TOF-MS/MS spectra of compound AA, AB, and AC, respectively. After a detailed analysis of fragment ions, the fragment pathways of compound AA, AB, and AC are summarized in Figure 3.

Detection of 'Unknown Compounds' Using Neutral Loss Scan Mode by UPLC-QqQ-MS

Optimization of UPLC Chromatographic Conditions The mobile phase was optimized through comparisons of different ratio and gradient profile of methanol/acetonitrile. An acidified mobile phase could minimize peak tailing and improve symmetry factor and theoretical plate number [20]. Thus, a linear gradient of acetonitrile and water containing 0.1% formic acid was chosen as mobile phase for UPLC separation. Given that types of analytical column with different end-capping, surface area, and carbon load have a critical influence on the LC separation, the separation effect on three other chromatographic columns was also investigated, including UHP Innoval C18 (50 × 2.1 mm i.d., 1.9 μm), ACQUITY UPLC HSS T3 C18 (100 × 2.1 mm i.d., 1.8 μm), and Agilent Extend-C 18 (50 × 2.1 mm i.d., 1.8 μm). The result revealed that the best separation was achieved on ACQUITY UPLC BEH C18 analytical column (100 × 2.1 mm i.d., 1.7 μm). The column temperature and flow rate were not decisive factors in the separation effect.

Neutral Loss Scan of Δ m/z 44 by UPLC-QqQ-MS

The main parameters of QqQ-MS and QqQ-MS/MS were set by convention except for the fragmentor voltage (Frag) and collision energy (CE). The fragmentor voltage for QqQ-MS was optimized to maximum quasi-molecular ion peak, and the CE value for QqQ-MS/MS was optimized to maximum fragment information for characteristic extraction. Finally, the comparative tests of reference compounds –150 eV and –20 eV were chosen as the optimal Frag and CE values, respectively.

The detection for 'unknown compounds' in *A. fruticosa* extract was performed by UPLC-QqQ-MS using neutral loss scan mode. It is worth noting that the neutral loss of CO_2 was a critical characteristic for amorfrutins deduced above. In this procedure, series experiments of neutral loss scan were carried out to detect △*m/z* 44 for the potential

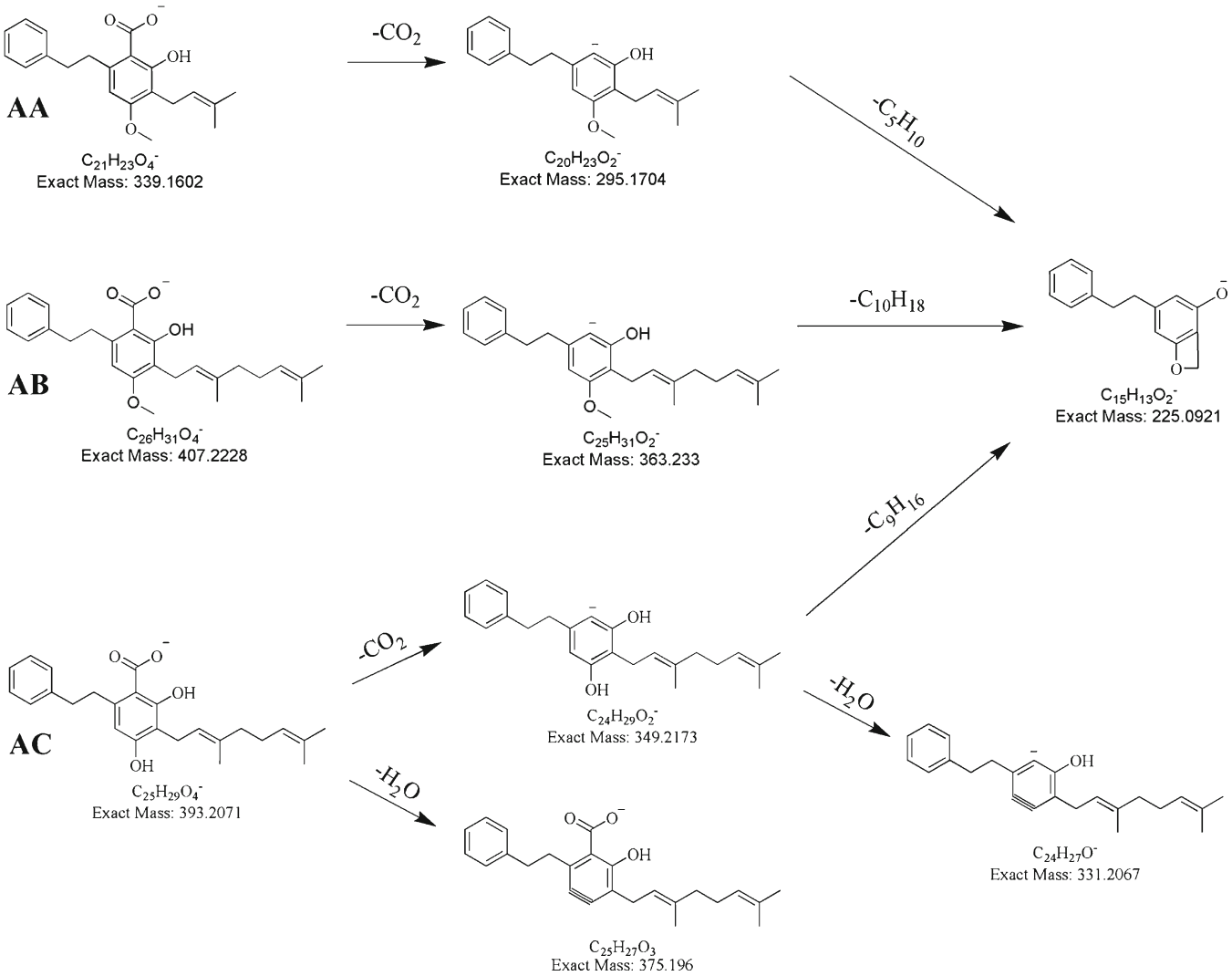


Figure 3. Proposed fragmentation pathways of reference compounds AA, AB, and AC

'unknown compounds'. The total ion current (TIC) chromatogram presented in Figure 4 shows nine suspicious peaks for amorfrutins. The chromatographic peaks with retention times of 7.05, 7.84, and 11.53 min were unambiguously identified as AC, AA, and AB by comparing the retention time, UV absorption, and MS spectra of the peaks with those of the respective reference compound. The other peaks with retention times of 5.66, 6.16, 6.87, 11.18, 4.37, and 10.89 min were marked as A1, A2, A3, A4, B1, and B2 for further structural analysis below.

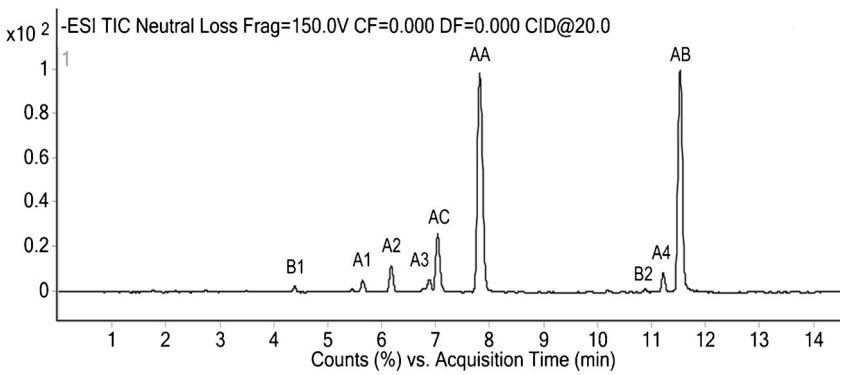


Figure 4. The TIC chromatogram of *A. fruticosa* extract analyzed by UPLC-QqQ-MS using neutral loss mode

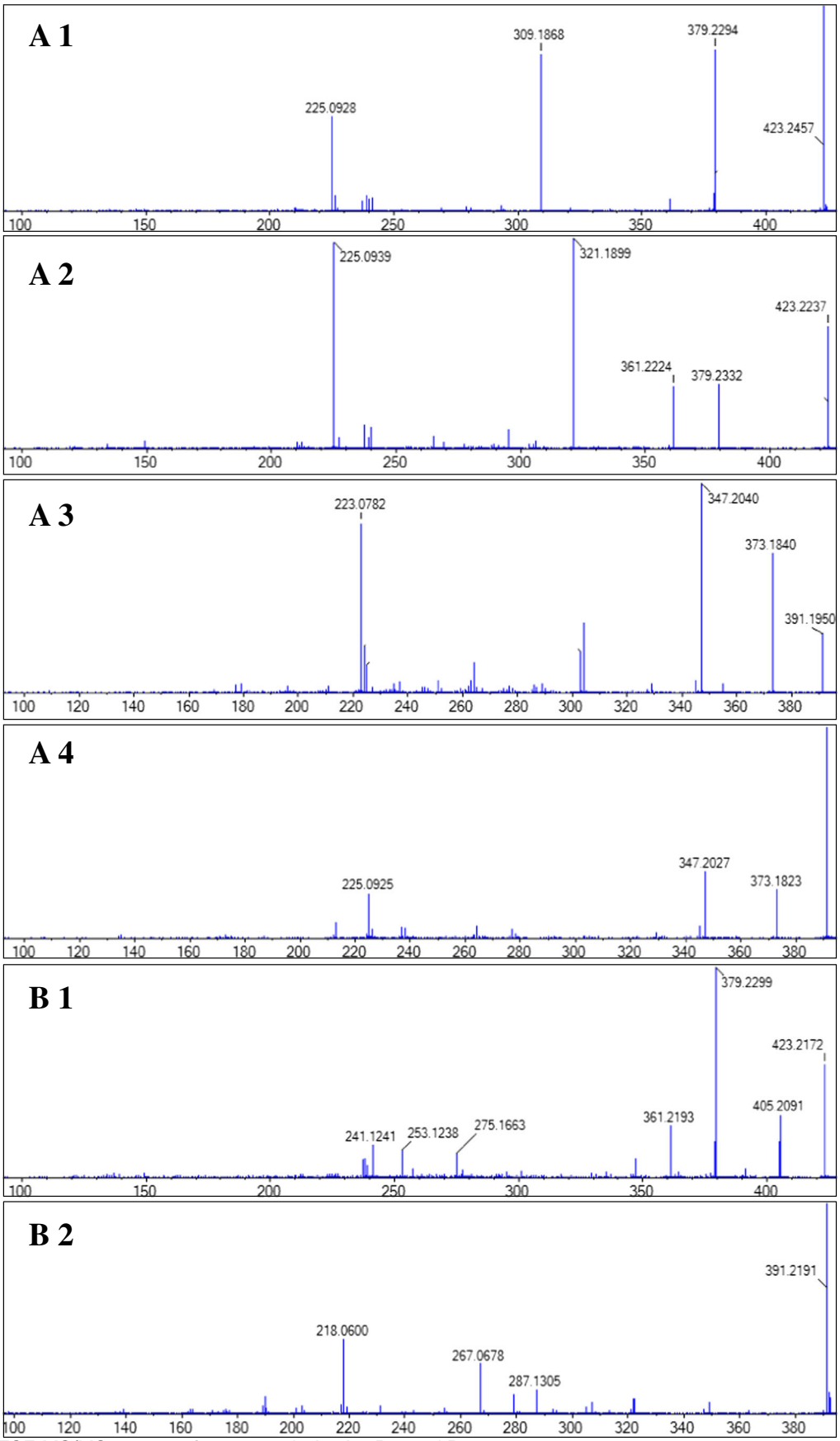


Figure 5. The TOF-MS/MS spectra of compounds A1-A4, B1, and B2

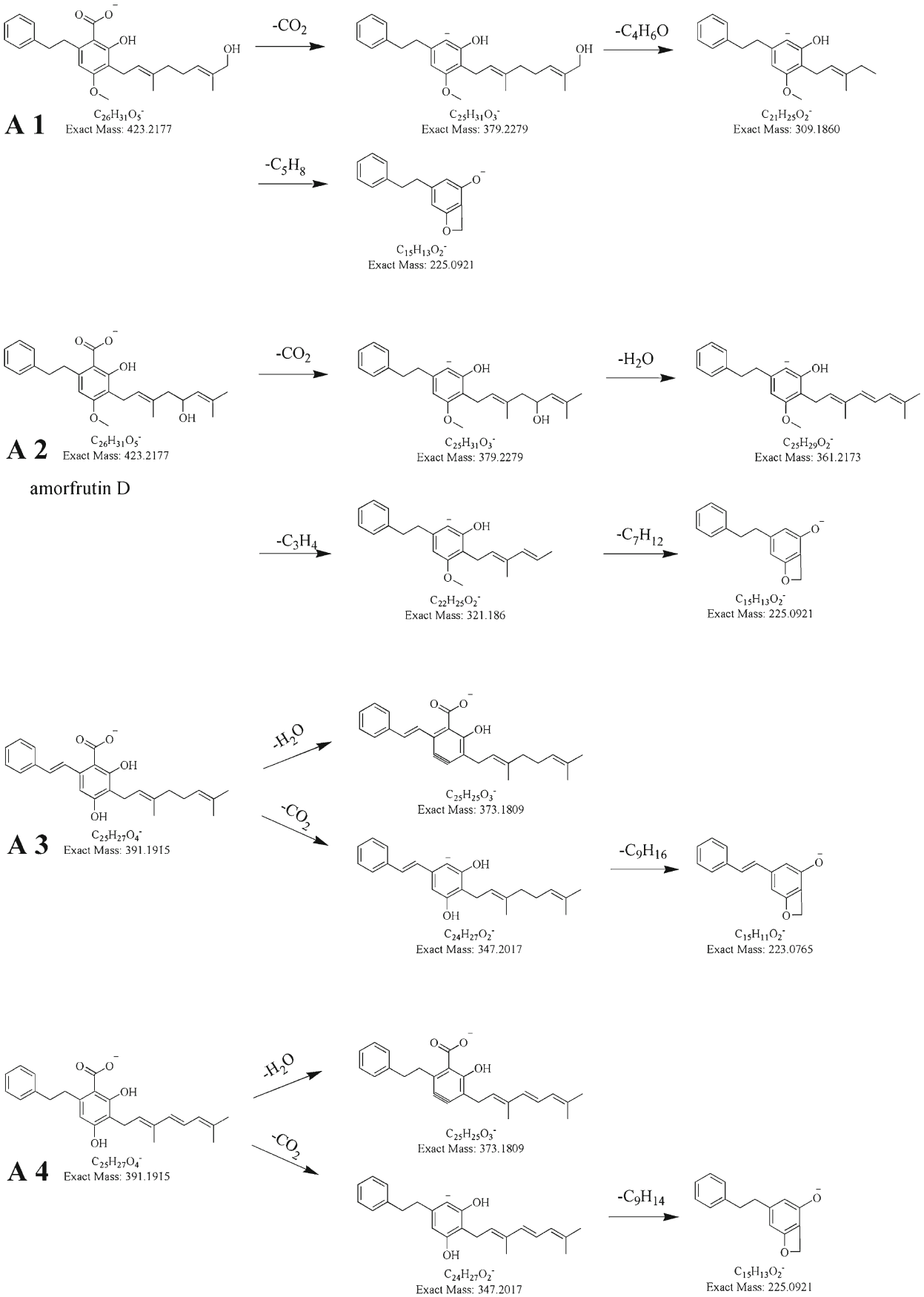


Figure 6. Proposed fragmentation pathways of compounds A1-A4

Structure Identification of Target 'Unknown Compounds' Detected Above-Mentioned by UPLC-Q-TOF-MS

The use of high-resolution (HR) MS, and particularly coupled with a hybrid quadrupole (Q) tandem HRMS is now considered to be a requirement for structural identification research [21]. The Q-HRMS instruments with low mass error could be applied for accurate assignment of chemical formula. The typical standard of ±5 ppm error leads to few formula options, especially for mass <*m/z* 500 [22]. In the present study, Q-time-of-flight (TOF) HRMS was used to achieve sufficiently high mass accuracy for chemical formula calculation. The typical fragmentation characteristics of compounds A1-A4, B1, and B2 are shown in Figure 5.

Structure Identification of A1 and A2

A1 and A2 were isomers with the same chemical formula of $C_{26}H_{32}O_5$. As the presence of *m/z* 379 $C_{25}H_{31}O_3^-$ indicating the neutral loss fragment of CO_2 and the presence of the characteristic fragment *m/z* 225 $C_{15}H_{13}O_2^-$ in TOF-MS/MS spectra, both A1 and A2 were identified as amorfrutins. The differences between A1 and A2 were the product ions of *m/z* 309.1868 $C_{21}H_{25}O_2^-$ in A1, and *m/z* 361.2224 $C_{25}H_{29}O_2^-$ and *m/z* 321.1899 $C_{22}H_{25}O_2^-$ in A2. For A2, the product ion of $C_{22}H_{25}O_2^-$ was formed from the successive elimination of H_2O and $-C_3H_4$ unit from the precursor ion $C_{25}H_{31}O_3^-$*m/z* 379.2322 $[M–H–CO_2]^-$. This feature was fully in line with amorfrutin D reported recently [8]. Amorfrutin D dehydrated easily by hydroxyl and then formed a great conjugated system of 1,3,5 hexatriene (*m/z* 361.2224 $C_{25}H_{29}O_2^-$ $[M–H–CO_2–H_2O]^-$). For A1, the product ion of *m/z* 309.1868 $C_{21}H_{25}O_2^-$ was formed from the elimination of $-C_4H_6O$ unit from the precursor ion *m/z* 379.2294 $C_{25}H_{31}O_3^-$ $[M–H–CO_2]^-$. The fact that there was no obvious elimination peak of H_2O suggested that –OH was probably at the end of geranyl. Moreover, the retention time of A1 shorter than that of A2 might support such structure inference because –OH at the end of a molecule normally results in higher polarity and weaker retention in C18 column. Thus, it was referred to A1 as a new compound and to A2 as amorfrutin D reported recently. The fragmentation pathways of A1 and A2 are summarized in Figure 6.

Structure Identification of A3 and A4

A3 and A4 were also a pair of amorfrutin isomers with a chemical formula of $C_{25}H_{28}O_4$. The substitute unit of C-5 should be –OH like AC type because of the fragment of $C_{25}H_{25}O_3^-$*m/z* 373 $[M–H–H_2O]^-$ in TOF-MS/MS spectra of A3 and A4. Compared with AC, the increased degree of unsaturation suggested that there was one more double bond existing in A3 and A4.

A fragment ion *m/z* 223.0782 $C_{15}H_{11}O_2^-$ was found in TOF-MS/MS spectrum of A3 instead of the common fragment ion *m/z* 225 $C_{15}H_{13}O_2^-$ of amorfrutins, indicating that the substitute unit of C-1 should be styryl, while the fragment ion *m/z* 225.0925 $C_{15}H_{13}O_2^-$ was observed in A4. Based on the above analysis, the structures of A3 and A4 were determined as shown in Figure 6 as well as their fragmentation pathways.

Structure Identification of B1 and B2

The molecular formula of B1 was the same as those of A1 and A2, and B2 was the same as those of A3 and A4. Furthermore, B1 and B2 fit the amorfrutins characteristics the neutral loss of CO_2 unit. However, no amorfrutins characteristic fragment peak of *m/z* 223 $C_{15}H_{11}O_2^-$ or *m/z* 225 $C_{15}H_{13}O_2^-$ was observed in TOF-MS/MS spectra. Thus, we believed that B1 and B2 were not amorfrutin derivatives. It was also suggested that the target compounds detected by UPLC-QqQ-MS may be false positive, and it was necessary to further confirm this by UPLC-Q-TOF-MS analysis.

Conclusion

In the present study, a combined application of UPLC-Q-TOF-MS and UPLC-QqQ-MS was developed to discover unknown amorfrutins from fruits of *Amorpha fruticosa*. UPLC-Q-TOF-MS analysis of amorfrutins A, B, and C reveal the mass spectrum fragmentation pathways of amorfrutins, especially the neutral loss of △*m/z* 44. UPLC-QqQ-MS analysis operated in neutral loss mode was used and six suspect compounds with the specified neutral fragment were located. Finally, four unknown amorfrutins were identified in the extract of *A. fruticose* fruits. The results indicated that the combination of QqQ-MS neutral loss scan and Q-TOF-MS molecular formula calculation was rapid and efficient for unknown compound identification, and it could be further used in trace natural product and metabolite researches discovery.

Acknowledgments

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