Separation and Characterization of Active Flavonolignans of *Silybum marianum* by Liquid Chromatography Connected with Hybrid Ion-Trap and Time-of-Flight Mass Spectrometry (LC–MS/IT-TOF)

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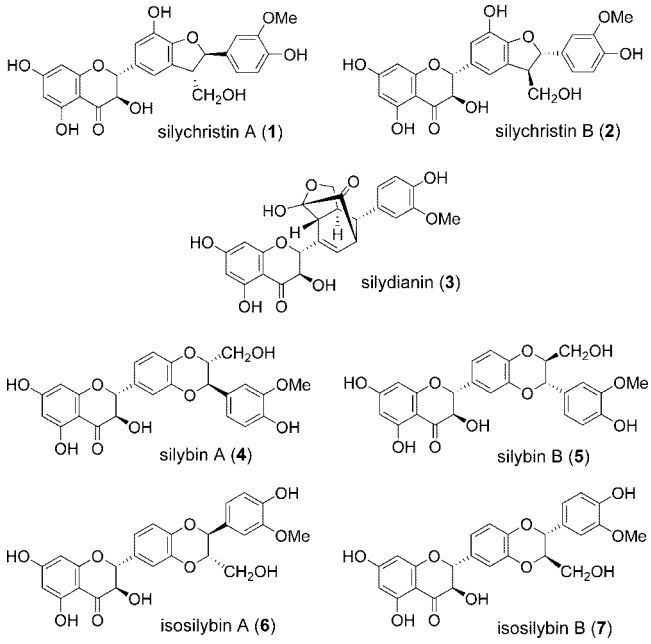
Silychristins A (**1**) and B (**2**), silydianin (**3**), silybins A (**4**) and B (**5**), and isosilybins A (**6**) and B (**7**) are major bioactive flavonolignans in silymarin, a herbal remedy derived from the milk thistle *Silybum marianum*. In this study, the seven major active flavonolignans including the diastereomers **1/2**, **4/5**, and **6/7** were completely separated using semi-micro-high performance liquid chromatography (HPLC) with a Nucleosil 100-3 C_{18} HD column and a MeOH/water/formic acid mobile phase system. The collision-induced dissociation (CID) MS/MS and MS^3 spectra of these flavonolignans were studied systematically using hybrid ion-trap and time-of-flight (IT-TOF) mass spectrometry. Efficient differentiation between the seven flavonolignans (**1**–**7**) was possible based on comparison of the resultant CID-MS/MS or MS^3 spectra. Each characteristic MS/MS or MS^3 fragmentation pattern was elucidated with high-resolution mass spectra by IT-TOF. The results with the present methodology show that liquid chromatography–mass spectrometry IT-TOF (LC–MS/IT-TOF) can be useful for general screening of active natural products from plant extracts and for the specific quality control of silymarin.

Silymarin, which is extracted from the milk thistle *Silybum marianum* (L.) Gaertn. (Asteraceae),[1] has been used for centuries as a natural remedy for treating hepatitis and cirrhosis and to protect the liver from toxic substances.[2,3] This traditional medicinal use has been supported by clinical trials; thus, silymarin could become an important hepatoprotective agent.[4,5] Silymarin is a mixture of polyphenolic flavonoids, which include silychristins A (**1**) and B (**2**), silydianin (**3**), silybins A (**4**) and B (**5**), and isosilybins A (**6**) and B (**7**) as major active flavonolignans.[6–8]



Liquid chromatography–mass spectrometry (LC–MC) is becoming an important step for the online identification of natural products in plant extract analysis. This technique has been used in many phytochemical laboratories for screening crude plant extracts.[9–11] Currently, it is possible to measure MS^n with collision-induced dissociation (CID) and high resolution mass spectra of fragment ions in CID. Khan et al. previously reported using electrospray ionization mass spectrometry (ESI-MS)/MS to study the fragmentation patterns of silymarin.[12] Barrett et al. also recently reported the separation and detailed fragmentation analysis of silychristin, silydianin, and silybin in milk thistle extract by CID-MS/MS.[13,14] However, the separation of silychristin isomers (silychristins A and B) by high pressure liquid chromatography (HPLC) has not been reported.

In this study, we report the complete separation of these seven active flavonolignans by semi-micro-HPLC with photodiode array detection (DAD). Moreover, the product ions from CID-MS/MS and CID-MS^3 spectra of these flavonolignans were systematically analyzed through the high mass accuracy capability of hybrid ion-trap and time-of-flight (IT-TOF) mass spectrometry. The internal temperature of this TOF mass spectrometry, flight tube, high voltage switch, high voltage power supply (PS), and radiofrequency (RF) oscillator, is controlled (40 ± 0.3 °C). Therefore, it is possible to obtain the exact mass for product ions without using lock mass techniques. Our primary objectives were to attain a more detailed analysis of fragmentation in CID using a high-resolution mass spectrometric technique and to credibly distinguish between the silychristins (**1**, **2**), silydianin (**3**), the silybins (**4**, **5**), and the isosilybins (**6**, **7**) for online identification.

Results and Discussion

Typical HPLC profiles of silymarin are shown in Figure 1, and the peaks numbered from **1**–**7** were completely separated. These peaks were identified as silychristins A (**1**) and B (**2**), silydianin (**3**), silybins A (**4**) and B (**5**), and isosilybins A (**6**) and B (**7**), respectively, by comparison with standard samples. The wavelength for detecting these flavonolignans was set at 288 nm, and UV spectra from 190 to 400 nm were also recorded for peak characterization with DAD. In addition, in the mass chromatogram, ($[M–H]^-$) at *m*/*z* 481 was selected to monitor for all seven flavonolignans (molecular formula $C_{25}H_{22}O_{10}$).

In the LC–MS analysis of silymarin reported by Barrett et al.,[13,14] formic acid was used as a mobile phase modifier. However, in the silychristin target peak, silychristins A (**1**) and B (**2**) overlapped. Therefore, to obtain a good resolution of adjacent peaks in a

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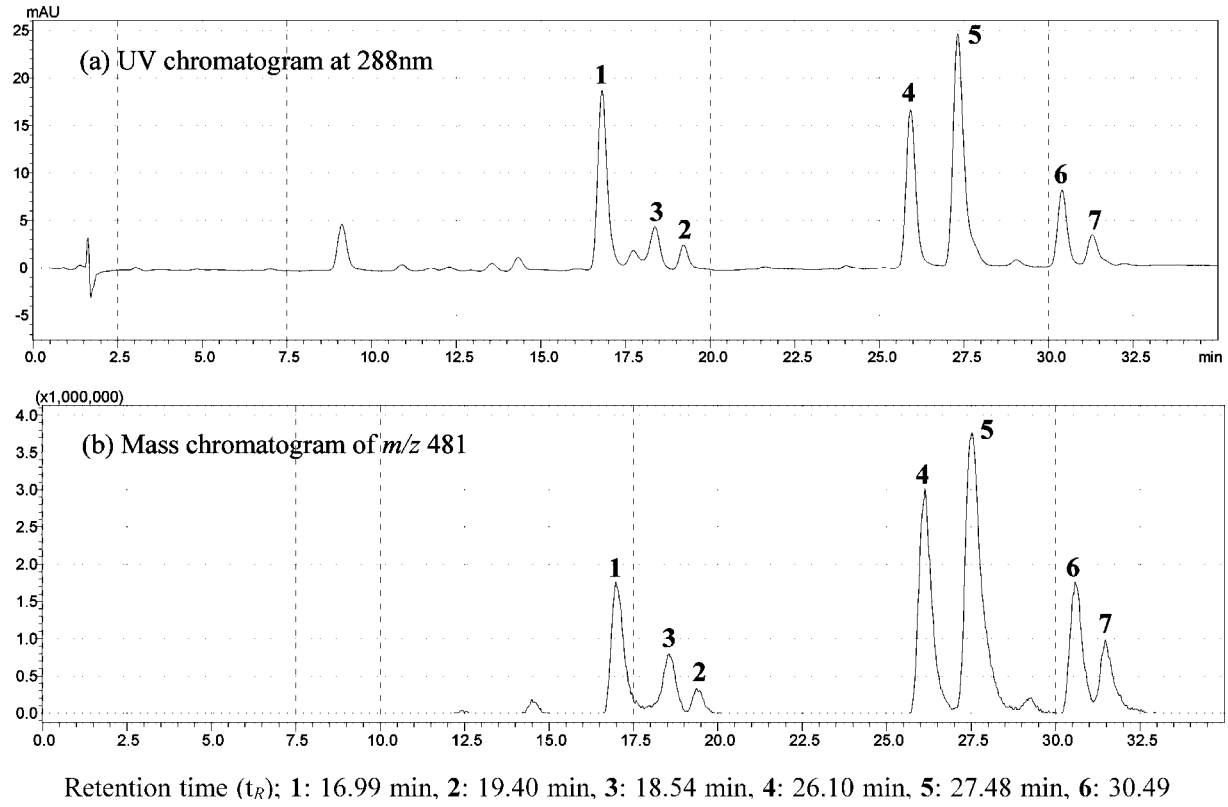

Figure 1. (a, b) LC–MS chromatograms of silymarin.

reasonably short analysis time, different mobile phases and semi-micro column compositions were screened. We found that the most suitable eluting solvent system was MeOH and 0.1% aqueous formic acid, and the best column was Nucleosil 100-3 C_{18} HD (2.0 i.d. × 125 mm) (Macherey-Nagel, Germany). The HPLC gradient profile is described in Experimental Section. The optimized LC conditions permitted a good separation of seven target flavonolignans in silymarin within 35 min.

For MS analysis, the negative-ion mode of electrospray ionization (ESI) was selected, as it easily provided extensive information via CID fragmentations. The product ion spectra of the pseudomolecular ions $[M–H]^-$ were obtained by conducting CID-MS/MS experiments. In CID-MS^3 experiments, product ions at *m/z* 463, 453, and 355, which are key ions to discriminate between the seven target flavonolignans, were chosen as precursor ions, respectively. The high mass accuracy capability of the IT-TOF instrument was used for the precise fragment ion assignments in the product ion spectra recorded.

Silychristins A and B (**1** and **2**) were detected at retention times (t_R) 16.99 and 19.40 min as peaks 1 and 2, respectively. In a CID-MS/MS experiment at *m/z* 481 $[M–H]^-$, major product ions *m/z* 463 and 355 (base peak) were observed. CID-MS^3 at *m/z* 463 and 355 showed five diagnostic fragment ions: *m/z* 283, 337, 353, 377 (base peak), and 445 resulting from *m/z* 463, and *m/z* 325 and 337 (base peak) resulting from *m/z* 355. We have proposed the structures of these product ions as shown in Figure 2, and they were supported by exact mass. However, it was not possible to define a difference between fragment patterns of the two diastereomers (**1** and **2**).

Silybins A and B (**4** and **5**) were detected at t_R 26.10 and 27.48 min as peaks 4 and 5, respectively. The CID-MS/MS obtained from the precursor ion at *m/z* 481 showed major product ions at *m/z* 257, 283, 301 (base peak), 355, 453 and 463. The CID-MS^3 from *m/z* 355, 453, and 463 showed many diagnostic fragment ions: *m/z* 175 and 327 resulted from *m/z* 355, *m/z* 229, 273 and 435 resulted from *m/z* 453, and *m/z* 239, 283 and 433 resulted from *m/z* 463. The fragment patterns of **4** and **5** were very similar except for the presence of product ion *m/z* 229 from **4**. The proposed fragmentation pathways of **4** and **5** are shown in Figure 3, and they were supported by exact mass.

Isosilybins A and B (**4** and **5**) were detected at t_R 30.49 and 31.36 min as peaks 6 and 7, respectively. In the CID-MS^3 experiment, *m/z* 273 and 435 were produced from *m/z* 453, and *m/z* 283 and 435 resulted from *m/z* 463, with *m/z* 283 observed as the base peak. Fewer diagnostic fragment ions were produced from **6** and **7** than from **4** and **5**, and moreover, the characteristic MS^3 product ions of **4** and **5** (*m/z* 433 and 239) were not observed from **6** and **7** (Figure 4). The fragment patterns of **6** and **7** were very similar except for the observation of product ion *m/z* 229 from **7**. Thus, the product ion *m/z* 229 from *m/z* 453 was detected only from **4** and **7**. The predicted fragment pathway is shown in Figure 5. This product ion (*m/z* 229) produced only from **4** and **7** could be a key ion to separate the two sets of diastereomers, **4/5** and **6/7**.

Silydianin (**3**) was detected at t_R 18.54 min as peak 3. In the CID-MS/MS experiment with *m/z* 481 $[M–H]^-$, a major product ion was observed at *m/z* 453 (base peak). CID-MS^3 with *m/z* 453 showed three diagnostic fragment ions, *m/z* 313, 327 (base peak), and 381. We have proposed the structures of these product ions as shown in Figure 6, and they were supported by exact mass.

In this study, seven active flavonolignans of silymarin, including three sets of diastereomers, were completely separated by semi-micro-HPLC. Moreover, we established the detailed structures of fragment ions from CID-MS/MS and CID-MS^3 of these flavonolignans by using the high mass accuracy capability of hybrid IT-TOF mass spectrometry. Notably, silychristins A (**1**)/B (**2**), silydianin (**3**), silybins A (**4**)/B (**5**), and isosilybins A (**6**)/B (**7**) were accurately distinguished with CID fragmentation with *m/z* 463, 453, and 355 as precursor ions. Thus, these three precursor ions (*m/z* 463, 453, and 355) are useful in identifying these seven active flavonolignans online. This new method represents a more sensitive and accurate analysis than previously reported and could be useful for the quality control of silymarin. In addition, the IT-TOF utilized in LC–MS/MS is a useful tool for identifying and elucidating the

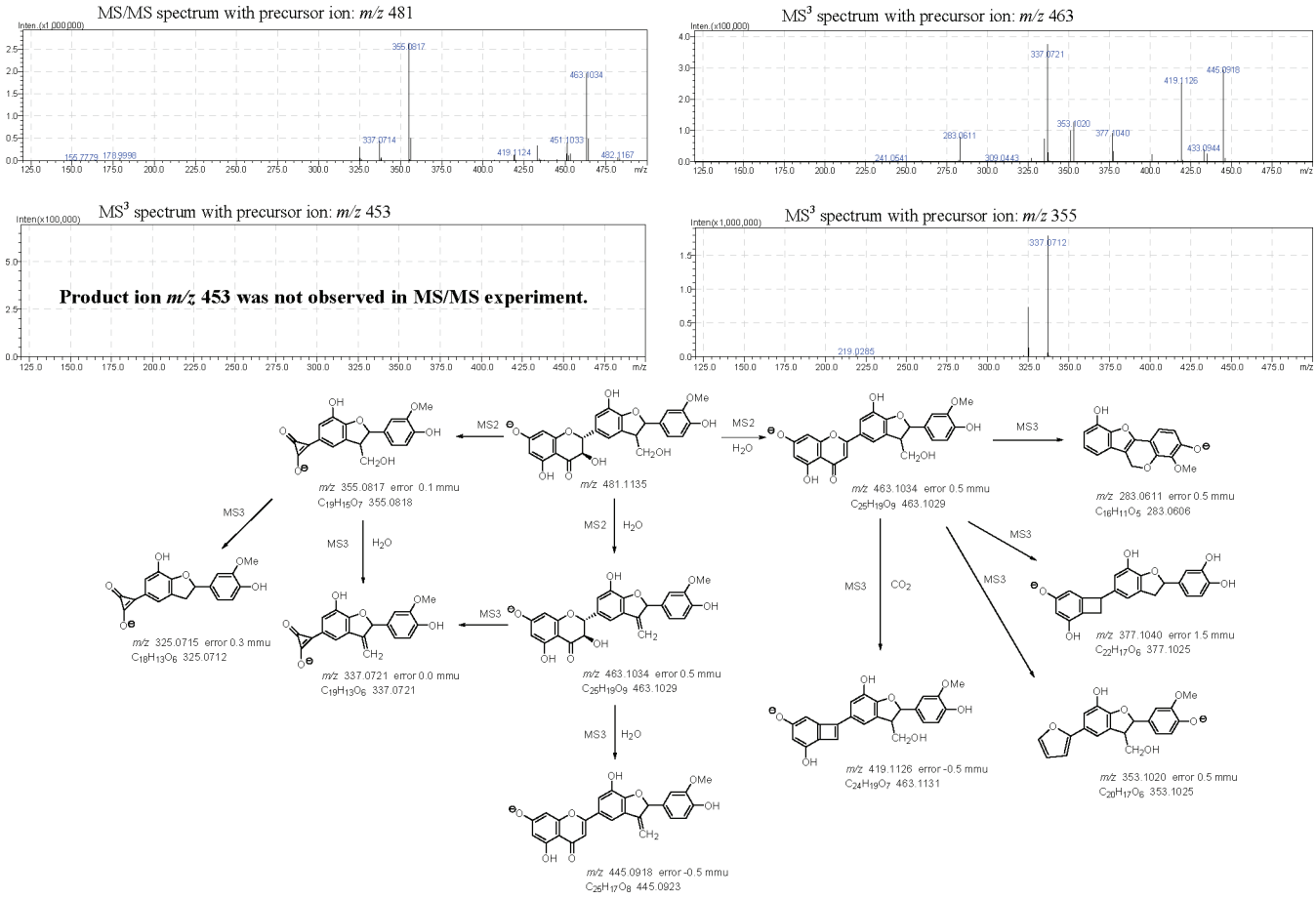

Figure 2. CID-MS/MS or MS^3 spectra and fragmentation of silychristins A (**1**) and B (**2**), obtained from the IT-TOF.

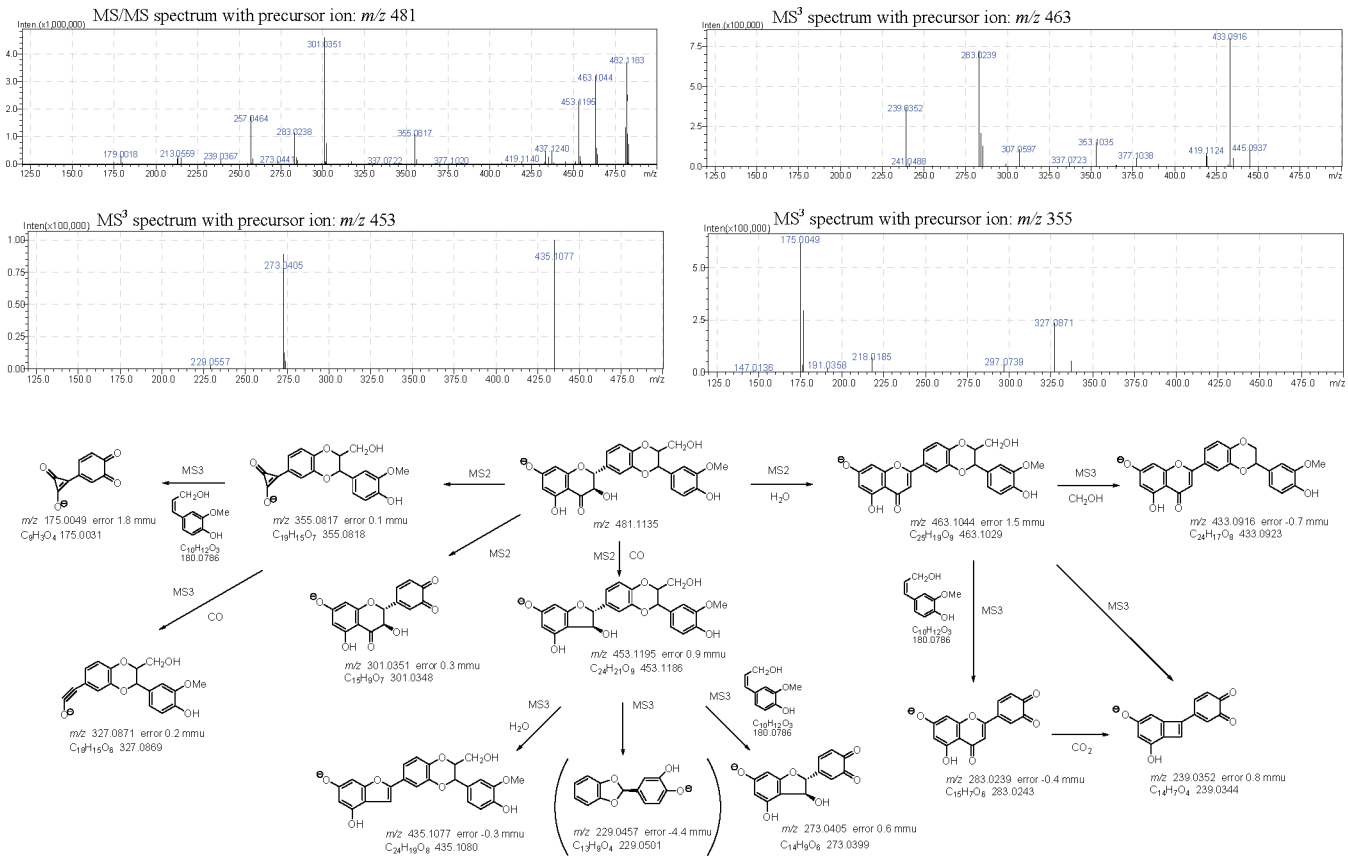

Figure 3. CID-MS/MS or MS^3 spectra and fragmentation of silybins A (**4**) and B (**5**), obtained from the IT-TOF.

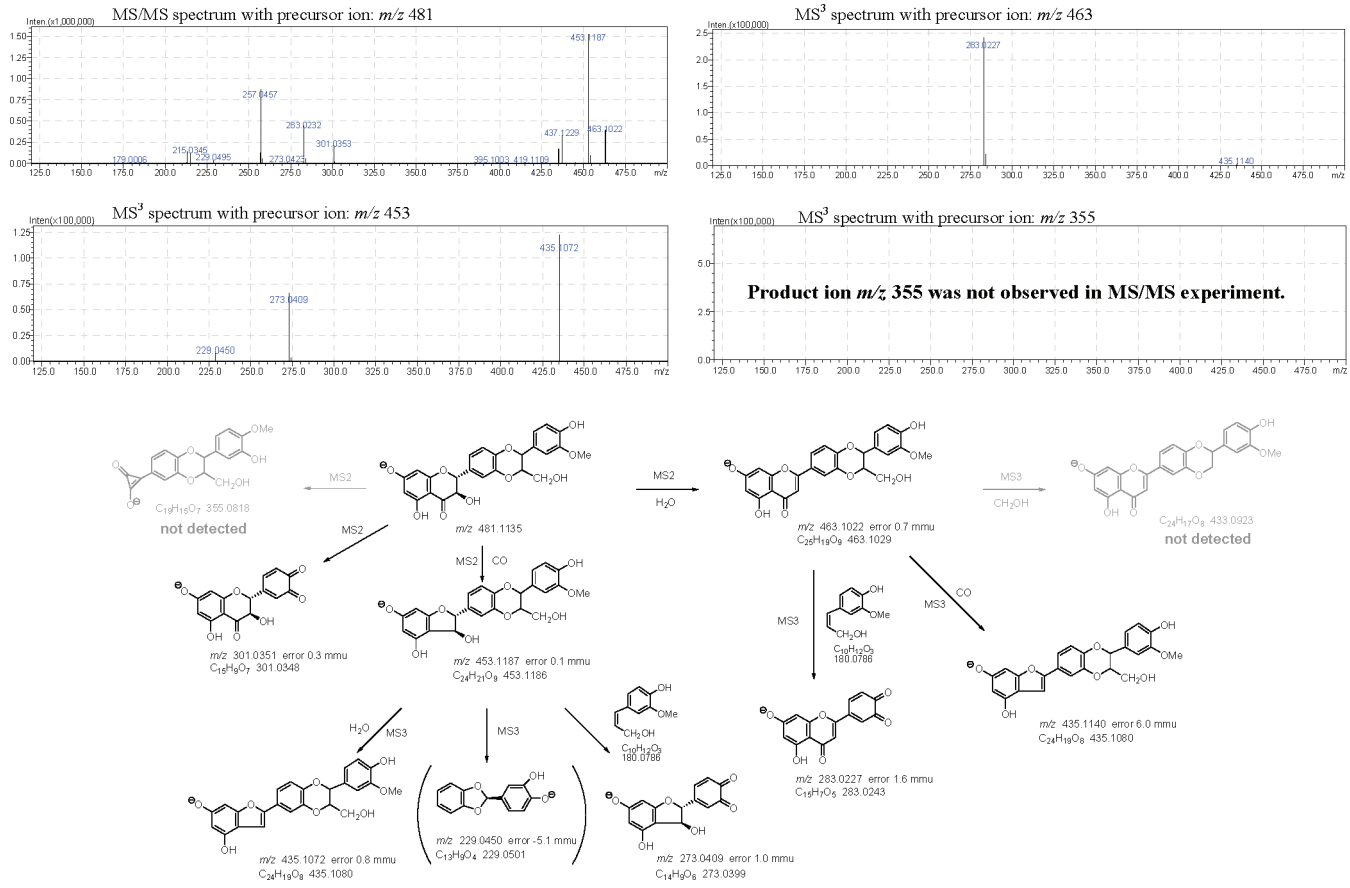

Figure 4. CID-MS/MS or MS^3 spectra and fragmentation of isosilybins A (**6**) and B (**7**), obtained from the IT-TOF.

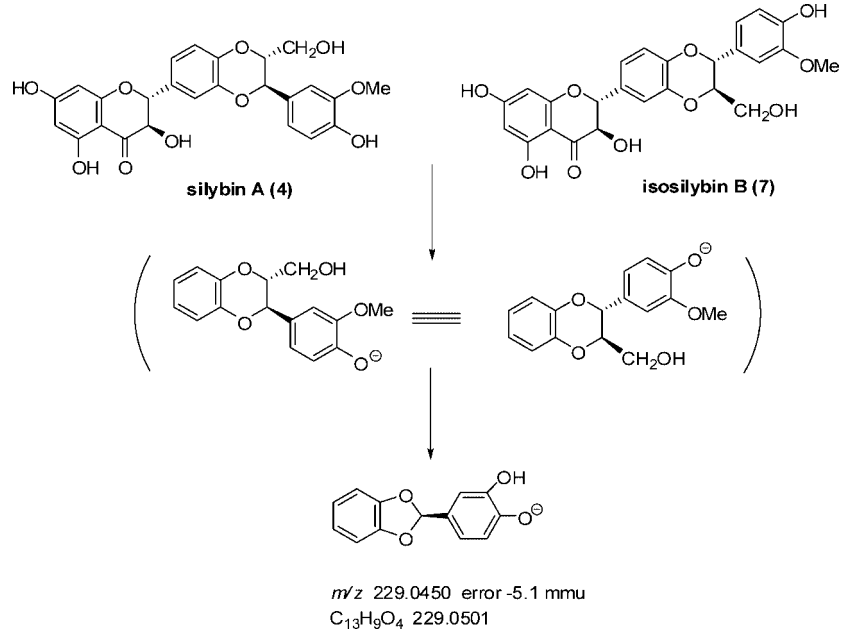

Figure 5. Predicted fragment pathway of *m/z* 229 from silybin A (**4**) and isosilybin B (**7**).

fragmentation pathway of natural products. Moreover, we anticipate that LC–MS/IT-TOF will become very useful for screening active natural products from plant extracts.

Experimental Section

General Experimental Procedures. The instruments used in this study were Shimadzu LC-20AT, SPD-M10A, and SCL-10A LC instruments (for preparative HPLC), Varian Mercury 300 and Unity Inova-500 NMR spectrometer (for NMR spectra measured in $CDCl_3$ using tetramethylsilane as an internal standard), a JASCO digital polarimeter DIP-1000 (for optical rotation, measured at 25 °C), Shimadzu DGU-20A3, LC-20AP, CBM-20A, SPD-M20A and SIL-20A LC instruments (for semi-micro-HPLC) and Shimadzu hybrid IT-TOF mass spectrometer.

Reagents and Materials. LC–MS-grade MeOH (Chromasolv, for LC–MS) and HPLC-grade MeOH (Sigma-Aldrich, for preparative HPLC) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Reagent-grade formic acid (98%) was purchased from Fluka (Buchs,

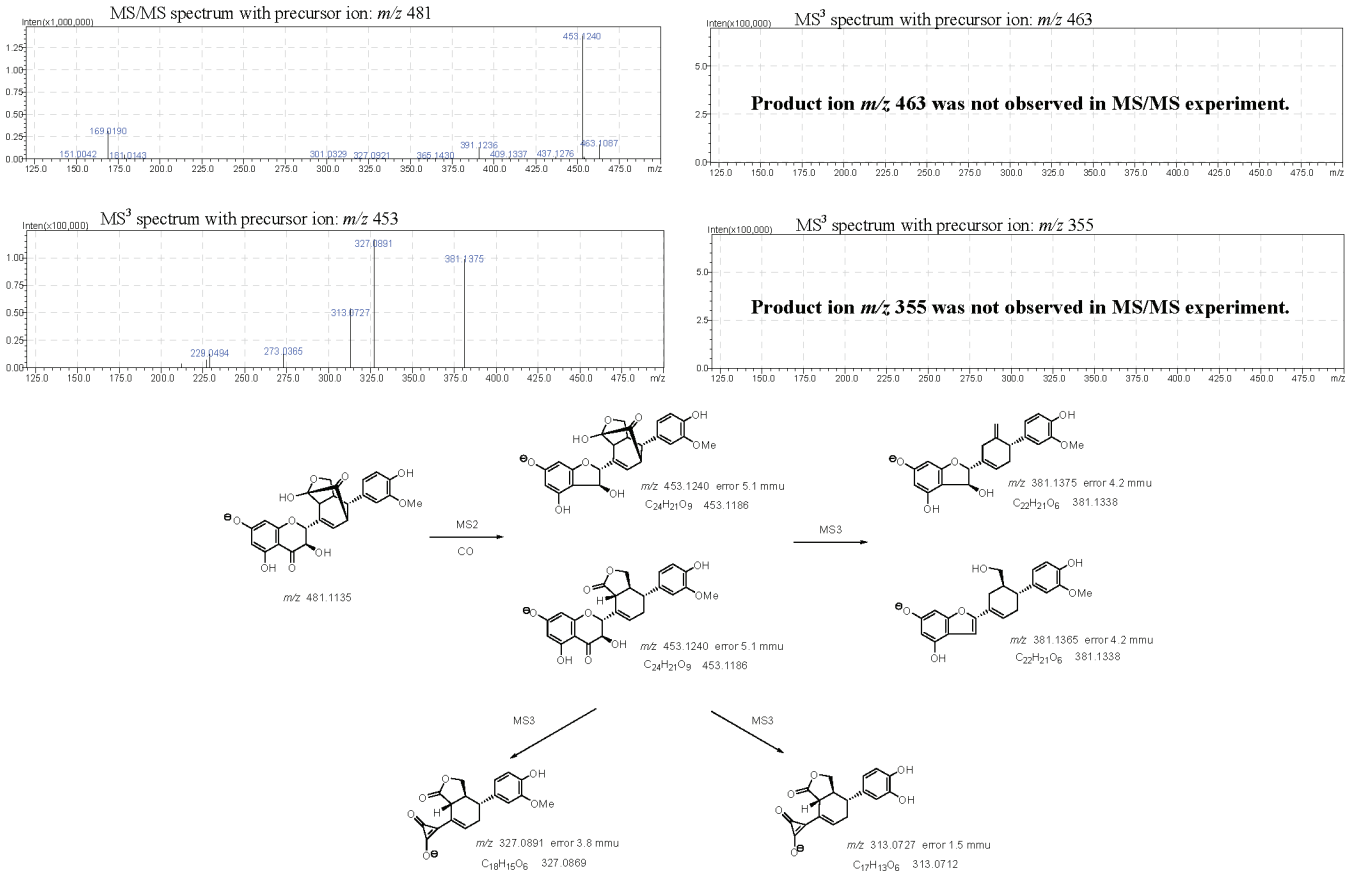

Figure 6. CID-MS/MS or MS^3 spectra and fragmentation of silydianin (**3**), obtained from the IT-TOF.

Switzerland). Ultra-pure water was prepared using a Millipore Milli-Q purification system (Bedford, MA). Milk thistle (*S. marianum*) extract powder (ID No. 78461103) was supplied by Tokiwa Phytochemical Co., Ltd. (Tokyo, Japan).

Isolation of Standard Sample. The extracts of *S. marianum* (50 g) were chromatographed by preparative HPLC (HPLC conditions: column, Alltima C_{18}-5 22 i.d. × 250 mm; mobile phase, isocratic MeOH–water (52:48); flow rate, 4.5 mL/min) to give silychristin A (**1**) (60 mg), silychristin B (**2**), (36 mg), silydianin (**3**) (35 mg), silybin A (**4**) (61 mg), silybin B (**5**) (102 mg), isosilybin A (**6**) (45 mg), and isosilybin B (**7**) (33 mg). These compounds were identified by comparison with reported $^1H/^{13}C$ NMR data and $[\alpha]_D$.[5–7]

Sample Preparation for LC–MS. MeOH extract (1 mg) was dissolved in MeOH (10 mL). The solution was chromatographed with Bond Elut C_{18}, and then diluted with MeOH to 30 ppm. One microliter was injected using an autosampler.

HPLC Analysis. HPLC was performed using a Shimadzu HPLC system. Chromatographic separation was performed on a Nucleosil 100-3 C18 HD (3 μm, 2.0 i.d. × 125 mm) (Macherey-Nagel, Duren, Germany). Mobile phase A was water containing 0.1% formic acid. Mobile phase B was MeOH. The column temperature was ambient. The HPLC flow rate was 0.2 mL/min. A sample solution of 1 μL was injected into the HPLC system. A mobile phase gradient was used with the percentage of B in A varying as follows: initial concentration, 30% B; 30 min, 55% B; 35 min, 55% B, 40 min, 30% B.

Ion-Trap and Time-of-Flight (IT-TOF) MS Analysis. CID-MS^n experiments were performed on a hybrid IT-TOF mass spectrometer with ESI interface (Shimadzu, Kyoto, Japan). The negative ESI conditions were as follows: high voltage probe, −3.5 kV; nebulizing gas flow, 1.5 L/min; CDL temperature, 200 °C; heat block temperature, 200 °C; drying gas pressure, 200 KPa. CID parameters were chosen 50% for CID energy and 50% for collision gas parameter. Ar gas was used for CID. Detector voltage of TOF was 1.6 kV. A solution of trifluoroacetic acid (TFA) and sodium hydrate was used as the standard sample to adjust sensitivity and resolution and to perform mass number calibration (ion trap and TOF analyzer).

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