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Analysis of Epipolythiodioxopiperazines in Fungus *Chaetomium cochliodes* Using HPLC-ESI-MS/MS/MS

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A series of epipolythiodioxopiperazines in the fungus *Chaetomium cochliodes* was investigated using reversed-phase liquid chromatography with diode array detection and electrospray quadrupole time-of-flight-type tandem mass spectrometry in the positive ion mode. The fragmentation of protonated molecular ions including low-abundance parent ions, $[M + H]^+$ for five known epipolythiodioxopiperazines, dethiotetra(methylthio)chetomin, chaetocochins A−C, and chetomin, was carried out using low-energy collision-induced electrospray ionization tandem spectrometry. It was found that McLafferty rearrangements occurred in the CID processes and produced a complementary pair of characteristic fragment ions containing piperazine rings (fused and unfused), especially to determine the number of S atoms on each ring. The fragmentation differential between $[M + H]^+$ and $[M + Na]^+$ was uncovered. Complementary fragmentation information obtained from $[M + H]^+$ and $[M + Na]^+$ precursor ions is especially valuable for rapid identification of epipolythiodioxopiperazines. A likely known compound, possibly related to chetoseminudin A, and three new species of epipolythiodioxopiperazines from the fungus *C. cochliodes* were identified or tentatively characterized based on tandem mass spectra of known ones.

Epipolythiodioxopiperazines (see the structure in Figure 1) are widely found as secondary metabolites of mold, with bioactivities including antitumor, antimicrobial, antinematodal, and cytotoxicity effects.[1−6] Some compounds produced from the genus *Chaetomium*, such as dethiotetra(methylthio)chetomin, chaetocochin A, and chaetocochin C, exhibit significant cytotoxicity in vitro against cancer cell lines such as Bre-04, Lu-04, and N-04.[7−9]

Structural characterization of the epipolythiodioxopiperazines is the key for the study of their bioactivities. However, this has always been a challenge, especially for the characterization of those compounds and their metabolites that are of trace amounts. UV and IR spectroscopy only provide limited structural information. Nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography offer more structural information and have become important methods for structural characterization of epipolythiodioxopiperazines.[1,8−10] However, NMR and X-ray crystallographic analyses require laborious purification and the production of monocrystals, respectively. Notably, some epipolythiodioxopiperazines are difficult to be identified even using NMR, since the numbers of S atoms in each piperazine ring are difficult to confirm.

Mass spectrometry, especially tandem mass spectrometry, has been one of the most sensitive and rapid methods for identification of organic compounds. The fragmentation mechanisms of Na^+ adduct molecular ions, $[M + Na]^+$ for epipolythiodioxopiperazines were studied using low-energy collision-induced electrospray ionization quadrupole time-of-flight tandem spectrometry (ESI-QTOF-MS/MS) and ESI-IT-MS^n.[11] Structures of biradical fragments in gas phase have attracted much scientific interest.[11−13] Two piperazine rings can be discriminated by the characteristic fragmentation pattern of McLafferty rearrangement when S-methyl groups are present. However, the number of sulfurs in each piperazine ring cannot be confirmed when only a S−S bond is present due to the loss of sulfide first. In addition, the abundances of low-mass fragment ions produced from $[M + Na]^+$ are very low, which make them unsuitable for serving as characteristic ions.

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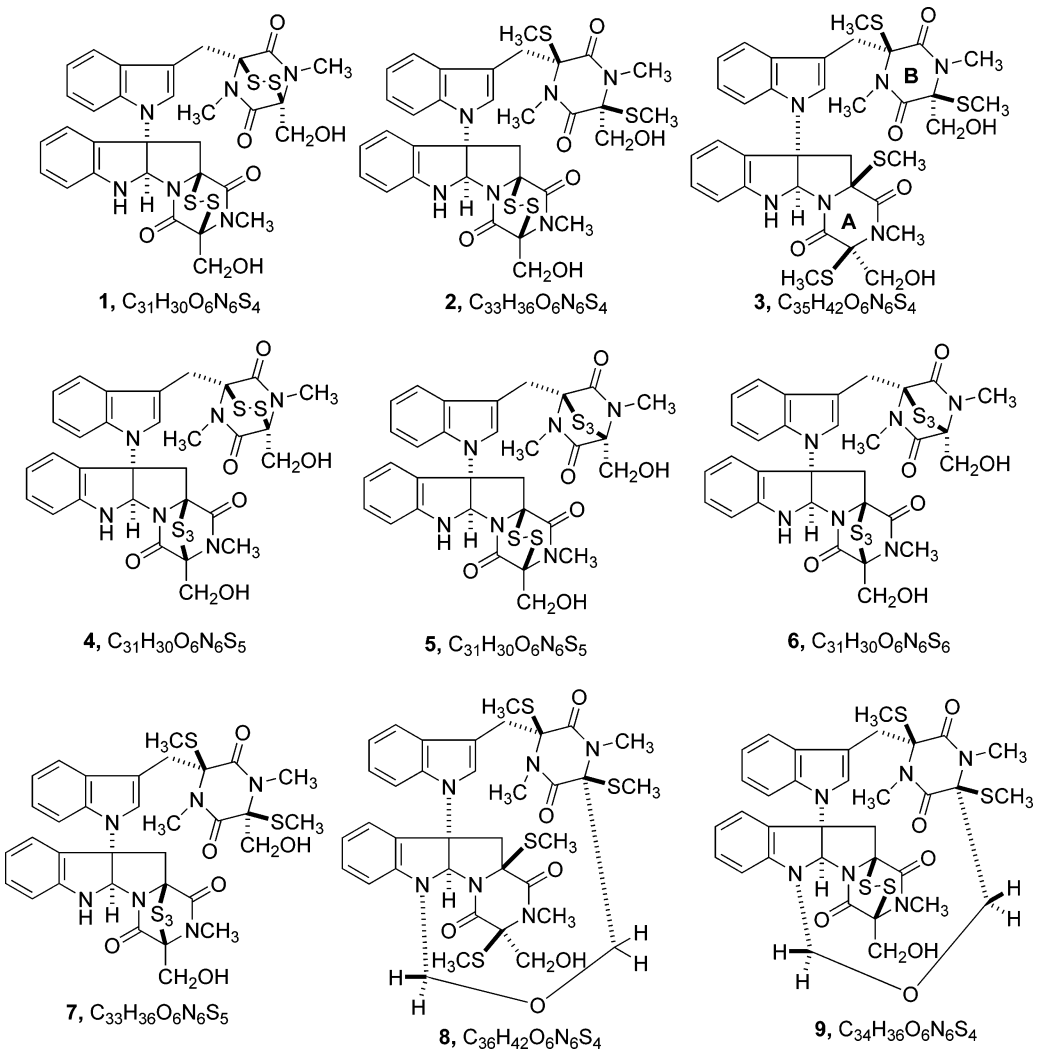

Figure 1. Epipolythiodioxopiperazines: chetomin (**1**, M_r 710.1110); chaetocochin C (**2**, M_r 740.1579); dethio-tetra (methylthio) chetomin (**3**, M_r 770.2049); chetoseminudin A (**4**, M_r 742.0825); chaetocochin D (**5**, M_r 742.0825); chaetocochin E (**6**, M_r 774.0546); chaetocochin F (**7**, M_r 772.1294); chaetocochin A (**8**, M_r 782.2049); chaetocochin B (**9**, M_r 752.1579). Compounds **5**, **6**, and **7** are new compounds.

HPLC-MS has been applied comprehensively in various fields such as natural product research and drug analysis.[14,15] Complex extracts are separated by HPLC effectively and rapidly, which minimizes the suppressing effect for analytes of low-concentration compounds during the ESI process. MS, especially ESI-IT-MS^n, provides abundant structural information for compounds identification. However, ion trap mass spectrometry cannot provide accurate masses of precursor and fragment ions. By performing automatic "on the fly" MS/MS experiments, HPLC-ESI-QTOF-MS/MS was utilized to identify compounds especially for toxicological analysis, which gave information of molecular formulas.[16,17] Unfortunately, it remains a challenge to obtain high-quality MS/MS spectra when the abundance of compounds detected is low. ESI-QTOF-MS/MS/MS has been a strong tool in the fields of natural products, synthetic chemistry, and so on,[18,19] since it can provide rapidly the abundant structural information and exact mass. Obviously, it is difficult to detect the trace compounds in the mixture due to the suppressing effect. So combination of HPLC and ESI-QTOF-MS/MS/MS, with expectation to overcome the disadvantage mentioned above, would be highly desirable for compound structural identification especially natural product and drug research.

In view of the potential application, the difficulty of structural identification and great scientific interest in fragmentation mechanisms of epipolythiodioxopiperazines, HPLC-ESI-MS/MS/MS was applied for further correlative studies on the fragmentation of protonated molecular ions in this article. According to the fragmentation information, some new epipolythiodioxopiperazines were identified. To the best of our knowledge, the method of HPLC-ESI-QTOF-MS/MS/MS was used for the first time for compound identification.

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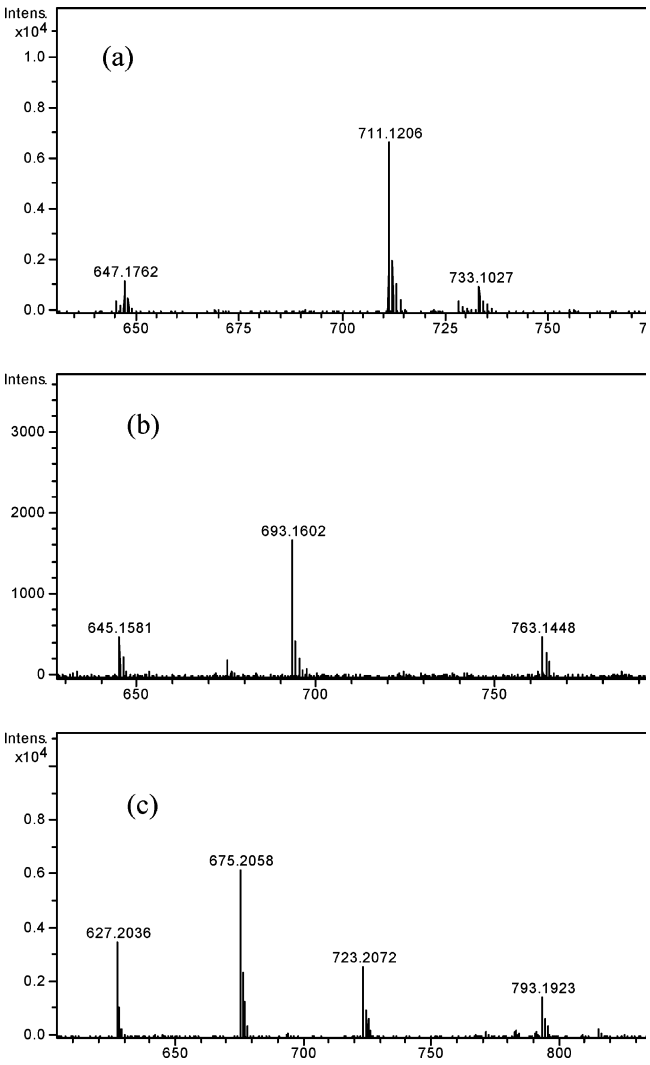


Figure 2. MS spectra of known compounds: (a) chetomin (**1**, $[M + H]^+$ at *m*/*z* 711; $[M + Na]^+$ at *m*/*z* 733), (b) chaetocochin C (**2**, $[M + H]^+$ at *m*/*z* 741; $[M + Na]^+$ at *m*/*z* 763); and (c) dethiotetra-(methylthio)chetomin (**3**, $[M + H]^+$ at *m*/*z* 771; $[M + Na]^+$ at *m*/*z* 793).

EXPERIMENTAL SECTION

Chemicals. HPLC grade methanol, acetic acid, thrice deionized water, and mass calibration standards purchased from Fisher Scientific (Pittsburgh, PA), Fluka, Haihong (Chengdu, China), and Agilent Technologies (Palo Alto, CA), respectively, were used for all analyses. AR grade methanol and ethyl acetate from Bodi Corp. (Tianjin, China) were used for preparation of sample.

Sample Preparation. Fifteen bottles of fermented solid medium (200 g/bottle) were prepared according to our previous work.[9] Starting from the fifth day, a bottle of fermented solid medium was taken out every 2 days and treated as follows: the fermented solid medium was soaked with methanol for 3 days at room temperature. After the solvent was evaporated under reduced pressure, the extracts were redissolved in water. The solution was then extracted using petroleum ether, and the residual water phase was extracted twice using ethyl acetate. The solvent of the combined organic-phase extracts was evaporated, and the dry extract was dissolved in methanol and filtered through a 0.45-μm membrane filter unit before being analyzed by HPLC−MS.

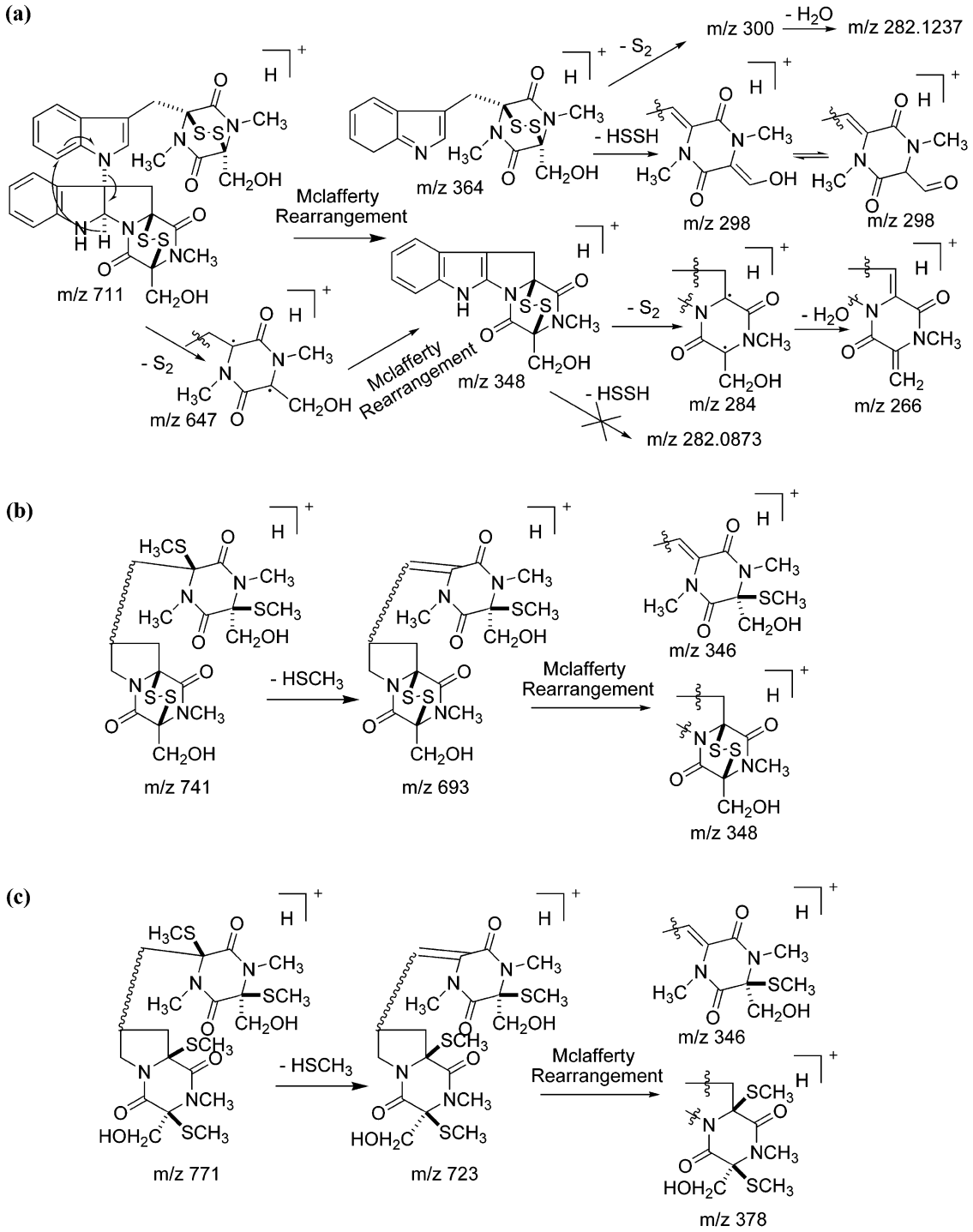
The sample taken out on the 11th day was analyzed and the known compounds were detected. Most unknown compounds with high relative abundance were observed from the sample taken out on the 23rd day. Therefore, this sample was selected for further analyses (concentration of sample, 0.1 mg/mL).

Chromatography. The Agilent 1100 series HPLC instrument was equipped with a quaternary pump, a diode-array detector (DAD), an autosampler, and a thermostated column compartment. The samples were separated using a Zorbax SB-C18 column (5 μm, 4.6 × 250 mm) from Agilent. The mobile phase consisted of methanol and water containing 0.2% (v/v) acetic acid. The flow rate was 0.7 mL/min, and column temperature was set at 30 °C. The effluent from the HPLC column was spilt using a "T" before being introduced into the MS, and the flow rate to the MS was ~0.07 mL/min. The wavelengths of the DAD detector were set at 220, 254, and 287 nm according the UV absorption of known

Table 1. Elemental Constituents of Major Ions Observed in MS Spectra of Epipolythiodioxopiperazines

compounds	formula	calculated	observed	error(ppm)
chetomin (**1**)	$C_{31}H_{30}O_6N_6S_4Na$	733.1002	733.1027	−3.43
	$C_{31}H_{31}O_6N_6S_4$	711.1182	711.1206	−3.32
	$C_{31}H_{31}O_6N_6S_2$	647.1741	647.1762	−3.24
chaetocochin C (**2**)	$C_{33}H_{36}O_6N_6S_4Na$	763.1471	763.1448	+3.06
	$C_{32}H_{33}O_6N_6S_3$	693.1618	693.1602	+2.29
	$C_{31}H_{29}O_6N_6S_2$	645.1585	645.1581	+0.51
dethiotetra(methyl-thio)chetomin (**3**)	$C_{35}H_{42}O_6N_6S_4Na$	793.1941	793.1923	+2.25
	$C_{34}H_{39}O_6N_6S_3$	723.2088	723.2072	+2.12
	$C_{33}H_{35}O_6N_6S_2$	675.2054	675.2058	−0.61
	$C_{32}H_{31}O_6N_6S$	627.2020	627.2036	−2.45
chetoseminudin A (**4**)	$C_{31}H_{30}O_6N_6S_5Na$	765.0723	765.0749	−3.45
	$C_{31}H_{31}O_6N_6S_5$	743.0903	743.0939	−4.86
	$C_{31}H_{31}O_6N_6S_3$	679.1462	679.1490	−4.23
chaetocochin D (**5**)	$C_{31}H_{30}O_6N_6S_5Na$	765.0723	765.0760	−4.89
	$C_{31}H_{31}O_6N_6S_5$	743.0903	743.0923	−2.67
chaetocochin E (**6**)	$C_{31}H_{30}O_6N_6S_6Na$	797.0442	797.0471	−3.48
	$C_{31}H_{31}O_6N_6S_6$	775.0624	775.0588	+4.61
	$C_{31}H_{31}O_6N_6S_2$	647.1741	647.1734	+1.09
chaetocochin F (**7**)	$C_{33}H_{36}O_6N_6S_5Na$	795.1192	795.1205	−1.63
	$C_{33}H_{37}O_6N_6S_5$	773.1373	773.1374	−0.15
	$C_{32}H_{33}O_6N_6S_4$	725.1339	725.1357	−2.53
	$C_{31}H_{29}O_6N_6S_3$	677.1035	677.1341	−5.28

Scheme 1. Partial Fragmentation Pathways of [M + H]$^+$ for (a) Chetomin (1), (b) Chaetocochin C (2), and (c) Dethiotetra(methylthio)chetomin (3)



epipolythiodioxopiperazines. The on-line UV/vis spectra were recorded in the range of 190−900 nm.

Mass Spectrometry. MS experiments were performed on a Bruker Daltonics BioTOF-Q mass spectrometer (Billerica, MA) in the positive mode. Accurate masses were determined by external mass calibration using the mass calibrants of MW 622.0290 and 922.0098. High-purity nitrogen gas was used as collision, nebulizer and auxiliary heated gas at a pressure of 50 psi. Sample introduction rate was 0.07 mL/min. The ESI source conditions were as follows: capillary voltage, -4500 V; end plate voltage, -4000 V; capillary exit voltage, 120-160 V; the dry gas temperature, 300 °C. The collision energy was optimized according to the signal.

MS/MS and MS/MS/MS Experiment. Two independent software programs, Bruker Daltonics HyStar and Bio TOF III Q Control, were used to control HPLC and MS separately in all MS/MS and MS/MS/MS experiments. Although it is possible to perform the automatic MS/MS (data-dependent analysis) using the combination of the two software programs mentioned above, we chose to control the HPLC and the BioTOF-Q mass spectrom-

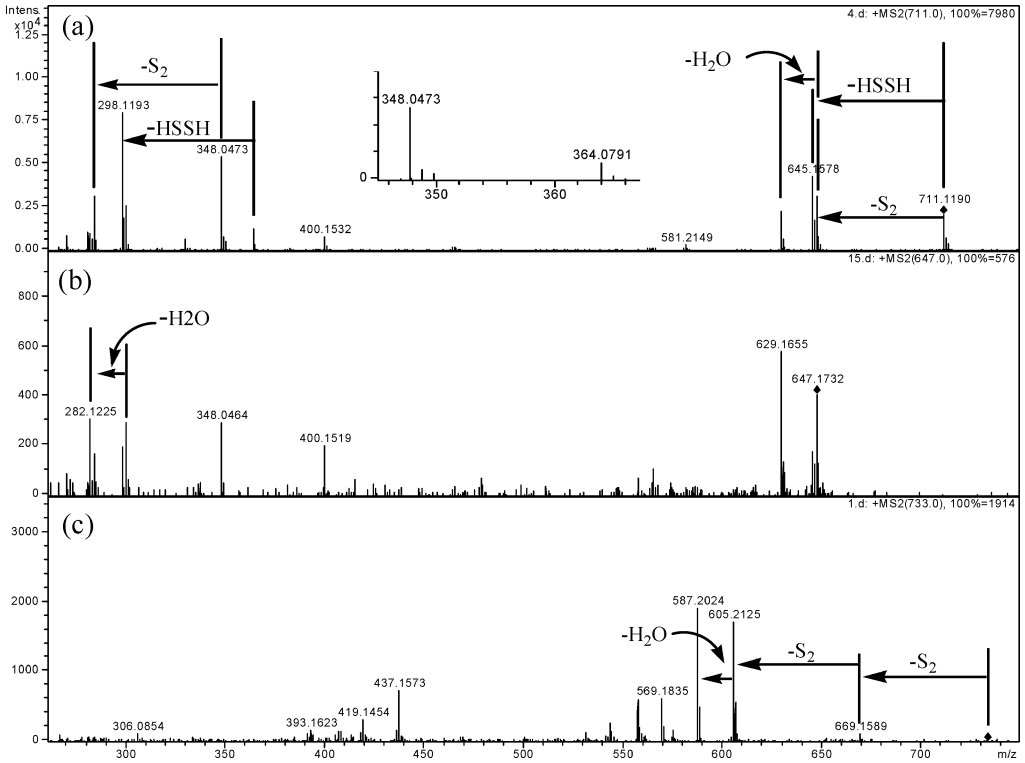


Figure 3. MS/MS and MS/MS/MS spectra of chetomin (**1**): (a) selected $[M + H]^+$ at *m*/*z* 711(collision energy at 10 eV), (b) selected ion at *m*/*z* 647 from *m*/*z* 711 (collision energy at 10 eV), and (c) selected $[M + Na]^+$ at *m*/*z* 733 (collision energy at 40 eV).

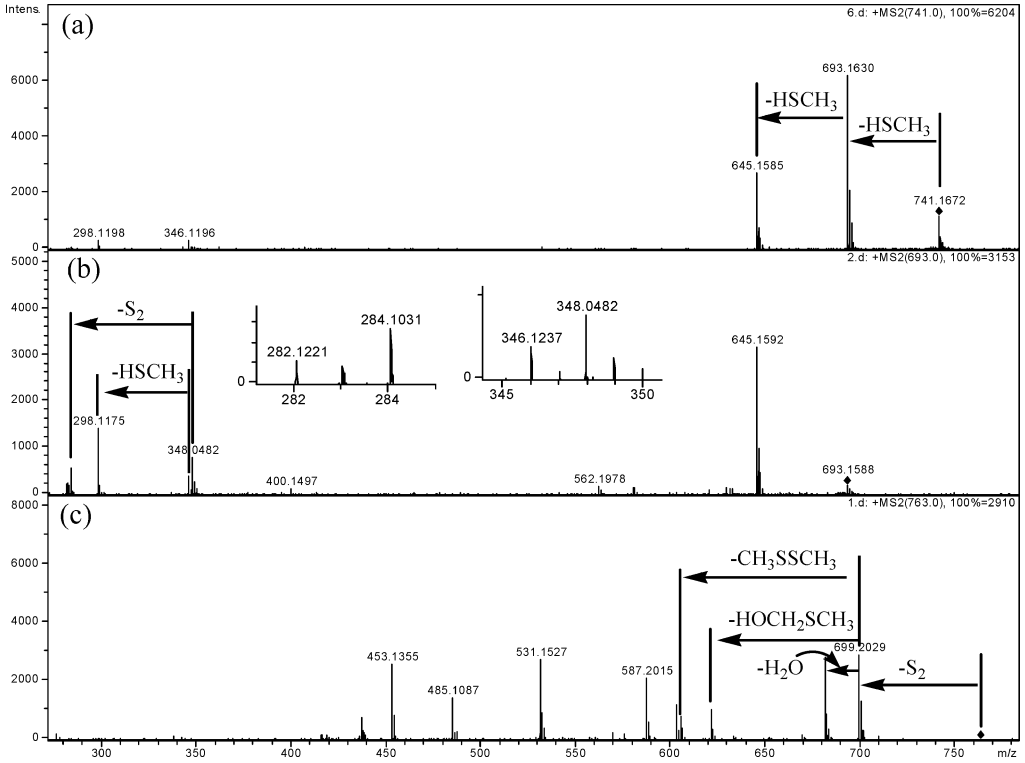


Figure 4. MS/MS and MS/MS/MS spectra of chaetocochin C (**2**): (a) selected $[M + H]^+$ at m/z 741 (collision energy at 0 eV), (b) selected ion at *m*/*z* 693 from *m*/*z* 741 (collision energy at 10 eV), and (c) selected $[M + Na]^+$ at *m*/*z* 763 (collision energy at 40 eV).

eter separately. The advantage is that parameters of MS such as collision energy, capillary exit voltage, and sampling times can be manually adjusted on the fly, which greatly improves the quality of spectra. In addition, accurate mass calibration becomes more convenient. Using the method, it is possible to obtain high-quality spectra even if the abundance of precursor ion is low. Information of product ions produced from precursor ion was obtained from MS/MS spectra. For MS/MS/MS experiments, capillary exit voltage was raised to induce in-source dissociation. Desired product ions generated through in-source dissociation were mass-

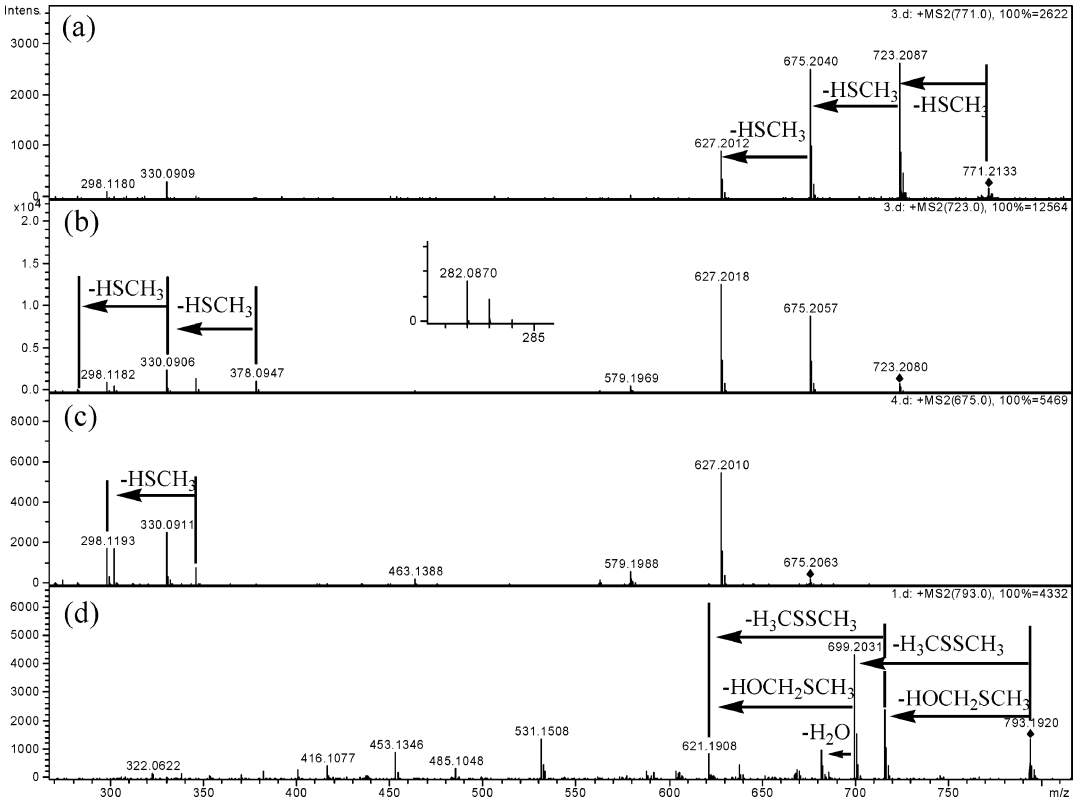


Figure 5. MS/MS and MS/MS/MS spectra of dethiotetra(methylthio)chetomin (**3**): (a) selected $[M + H]^+$ at *m/z* 771 (collision energy at 0 eV), (b) selected ion at *m/z* 723 from *m/z* 771 (collision energy at 5 eV), (c) selected ion at *m/z* 675 from *m/z* 771 (collision energy at 10 eV), and (d) selected $[M + Na]^+$ at *m/z* 793 (collision energy at 40 eV).

selected in Q1 and fragmented in Q2 to obtain corresponding MS/MS/MS spectra. Fortunately, F=fragmentation of protonated molecular ions, $[M + H]^+$, occurs readily in-source.

Molecular Formula Analysis. A make-before-break (MBB) valve was installed between the HPLC and MS to introduce the solution of mass calibration standards. The MBB valve was switched manually when the desired peak was observed. In some cases, the HPLC gradient was adjusted to make unknown compounds coelute with one or more known compounds, which serve as mass standards for checking of mass accuracy. If the mass error of a known compound is less than 5 ppm, the calibration is considered as acceptable. The isotope intensity ratios of $^{13}C/^{12}C$ and $^{34}S/^{32}S$ for $[M + H]^+$ or $[M + Na]^+$ provide the important information of the number of C and S atoms in the corresponding molecular formula. Combining the isotope intensity ratio and accurate mass, the desirable molecular formula could be obtained. Once the molecular formula of an unknown compound was determined, it might be used as a mass standard for other unknown compounds.

The above method was used for the analysis of MS/MS and MS/MS/MS spectra of unknown compounds. Some product ions that were known or easily identified (e.g., with common neutral loss) were used as mass standards for checking of mass accuracy. In addition, the molecular formula of neutral losses obtained by subtracting the observed *m/z* value of product ion from the value of calculated *m/z* value of precursor ion was used to validate the analysis above.

RESULTS AND DISCCUSSION

Mass Spectrometry Analysis of Known Compounds. The known compounds are confirmed by retention time, MS, and corresponding MS/MS spectra. The ESI-MS experiments were performed in the positive ion mode, and acetic acid was added to mobile phase to facilitate ionization. The abundance of protonated molecular ion, $[M + H]^+$ for chetomin (**1**, Figure 1) at *m/z* 711, observed is much higher than sodium adduct ion, $[M + Na]^+$ at *m/z* 733 (Figure 2a). However, for chaetocochin C (**2**, Figure 1) and dethiotetra(methylthio)chetomin (**3**, Figure 1), the abundances of protonated molecular ions at *m/z* 741 and 771, respectively, are very low, whereas that of the sodium adduct ions at *m/z* 763 and 793 are high (Figure 2b, c). The possible reason is that the sequential loss of $HSCH_3$ occurs readily for $[M + H]^+$ parent ions when the SMe group are present. Some ions with high abundance observed in the MS spectrum were fragments of the $[M + H]^+$ parent ions produced though this fragmentation pathway. The detail is delineated in the following paragraph, MS/MS and MS/MS/MS spectrometry. Incidentally, some similar phenomenon was observed in the MS analyses of chaetocochins A and B (**8** and **9**, Figure 1). The accurate masses of main ions observed in MS spectra are shown in Table 1.

MS/MS and MS/MS/MS Spectra of Known Compounds. *Chetomin (**1**).* For chetomin (Figure 1), the Mclafferty rearrangement and the neutral losses of the HSSH, S_2, and H_2O molecules are main fragmentation patterns for the selected $[M + H]^+$ parent ion at *m/z* 711 (Scheme 1a). A complementary pair of fragment ions at *m/z* 348 and 364 (Figure 3a) were formed by the Mclafferty rearrangement and further dissociated into the fragment ions at *m/z* 284 and 300, respectively, by loss of a S_2 molecule. The fragment ion at *m/z* 298 was yielded by loss of a HSSH molecule on ring B from *m/z* 364. However, the fragment ion at *m/z* 282.0873 that should be formed by the loss of a HSSH molecule

on ring A from *m/z* 348 seemed not to be observed, which indicates that the biradical structure on ring A formed by the loss of S_2 is stable. The observation is in accordance with the molecular orbital calculation by B3LYP/6-31++G**.[11] The fragment ion at *m/z* 282.1237 resulted from *m/z* 300 by loss of a H_2O molecule. A fragment ion at *m/z* 348 was observed in the MS/MS/MS spectrum of the fragment ion at *m/z* 647 (Figure 3b). This, along with the fact of much higher abundance of *m/z* 348 than that of *m/z* 364 in the MS/MS spectrum, indicates that the loss of sulfide (HSSH or S_2 molecule) occurs more readily on ring B even though the structures of rings A and B (fused and unfused piperazine rings, Figure 1) are very similar. The abundance of fragment ions in the low-mass range in the MS/MS spectrum of the selected $[M + Na]^+$ parent ion at *m/z* 733 was very low even using high collision energy (Figure 3c). The loss of the HSSH molecule from $[M + Na]^+$ ion was not observed.

*Chaetocochin C (**2**)*. Partial fragmentation pathways of chaetocochin C (Figure 1) are illustrated in Scheme 1b. In the MS/MS spectrum of the $[M + H]^+$ parent ion at *m/z* 741, the most abundant fragment peak (*m/z* 693) was due to the loss of a $HSCH_3$ molecule. This fragment further lost another $HSCH_3$ molecule and became the fragment ion at *m/z* 645 (Figure 4). The fragment ion at *m/z* 346 observed in the MS/MS/MS spectrum of the fragment ion at *m/z* 693 indicates the SMe is located at ring B. In addition, the loss of the HSSH molecule at ring A was not observed. In the MS/MS spectrum of the selected $[M + Na]^+$ parent ion at *m/z* 763, the main losses of *m/z* 94, 78, 64, and 18 correspond to $H_3CSCSCH_3$, HOH_2CSCH_3, S_2, and H_2O molecules, respectively. The losses of the $HSCH_3$ and HSSH molecules in the MS/MS spectrum of $[M + Na]^+$ were not observed.

*Dethiotetra(methylthio)chetomin (**3**)*. The fragmentation patterns of dethiotetra(methylthio)chetomin (Figure 1) are similar to those of compounds **1** and **2**. Sequential losses of the $HSCH_3$ molecules were observed in the MS/MS spectrum of the $[M + H]^+$ at *m/z* 771 (Figure 5). The fragment ion at *m/z* 378 observed in the MS/MS/MS spectrum of the fragment ion at *m/z* 723 indicates that the loss of the $HSCH_3$ molecule first occurs on ring B (Scheme 1c). For the $[M + Na]^+$ parent ion at *m/z* 793, the losses of 94 ($H_3CSCSCH_3$) and 78 Da (HOH_2CSCH_3) are the primary fragmentation pathway, whereas the loss of the $HSCH_3$ molecule is negligible.

In the MS/MS spectra of chaetocochin A (**8**) and B (**9**) (Figure 1), similar fragmentation patterns described in the previous section were observed (see Supporting Information). The fragment ions at *m/z* 310 and 358 appear to be the characteristic ions of $[M + H]^+$ for chaetocochin A at *m/z* 783 and B at *m/z* 753, indicating the two piperaziners are linked by an acetal group. Notably, the proton may be located at either the N or O atom, which is the drive of fragmentation of the acetal group. Additionally, the fragment ions at *m/z* 342 and 390 observed in the MS/MS spectra of the $[M + H]^+$ for chaetocochin A indicate the presence of the acetyl group and SMe at ring A. The fragment ions at *m/z* 360 and 296 (loss of S−S from *m/z* 360) in the MS/MS and MS/MS/MS spectra of the $[M + H]^+$ for chaetocochin B indicates the presence of the acetyl group and S−S at ring A.

Structural Analysis of a Likely Known Compound and Three Unknown Compounds. *Chetoseminudin A (**4**)*.[13] Chetoseminiudin A (Figure 1) was first reported by Ishibashi and co-workers. As observed in the MS spectrum shown in Figure 6a, the accurate masses of $[M + H]^+$ and $[M + Na]^+$ at *m/z* 743.0929 and 765.0749, respectively, correspond to the molecular formula of chetoseminudin A: $C_{31}H_{30}O_6N_6S_5$. The MS/MS spectra of **4** are very similar to those of chetomin (**1**) (Figures 3 and 7), only one S atom difference between their molecular formulas. This indicates the skeleton structure of **4** should be the same as **1**. A

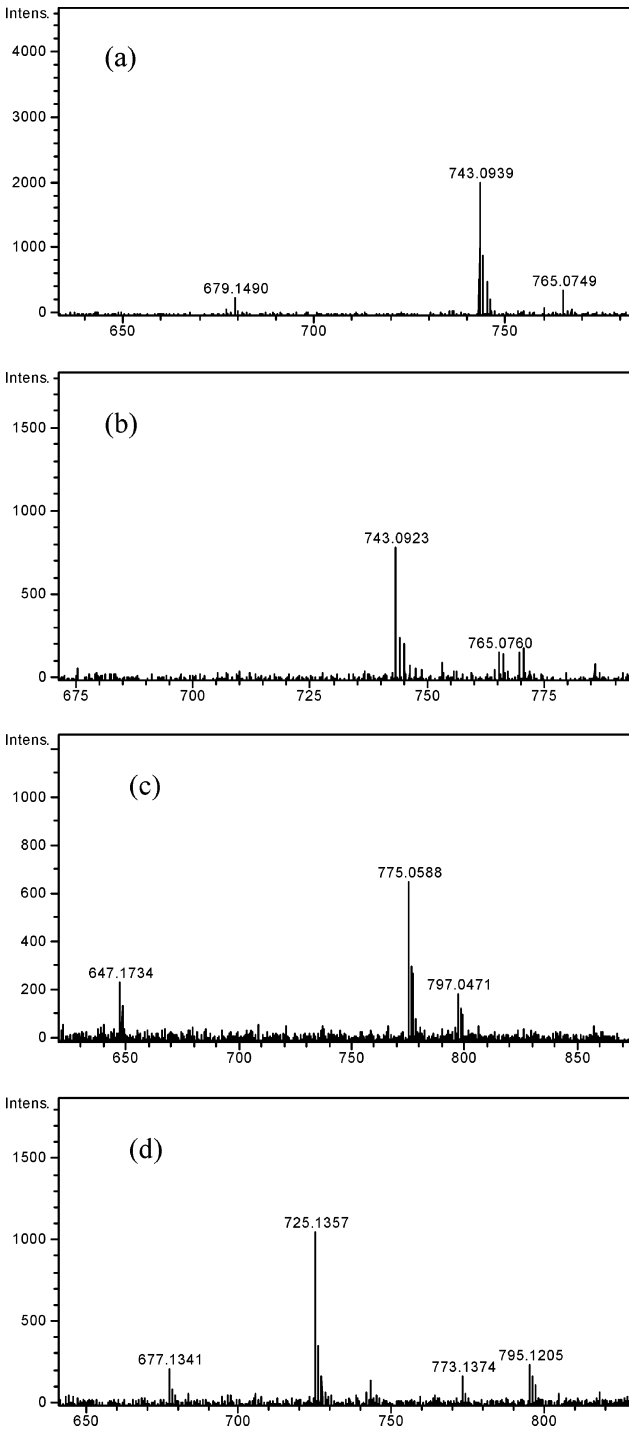


Figure 6. MS spectra of likely known and known and new compounds: (a) chetoseminudin A (**4**, $[M + H]^+$ at *m/z* 743, $[M + Na]^+$ at *m/z* 765), (b) chaetocochin D (**5**, $[M + H]^+$ at *m/z* 743, $[M + Na]^+$ at *m/z* 765), (c) chaetocochin E (**6**, $[M + H]^+$ at *m/z* 775, $[M + Na]^+$ at *m/z* 797), and (d) chaetocochin F (**7**, $[M + H]^+$ at *m/z* 773, $[M + Na]^+$ at *m/z* 795).

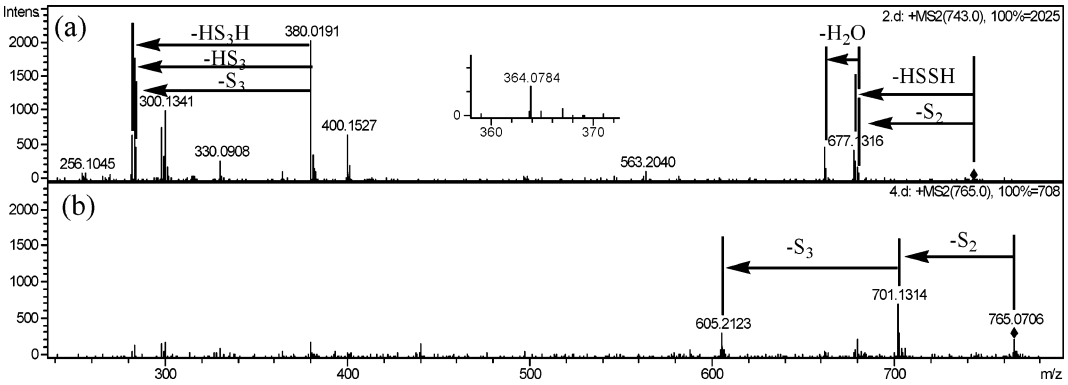


Figure 7. MS/MS spectra of chetoseminudin A (**4**): (a) selected $[M + H]^+$ at *m/z* 743(collision energy at 20 eV) and (b) selected $[M + Na]^+$ at *m/z* 765 (collision energy at 20 eV).

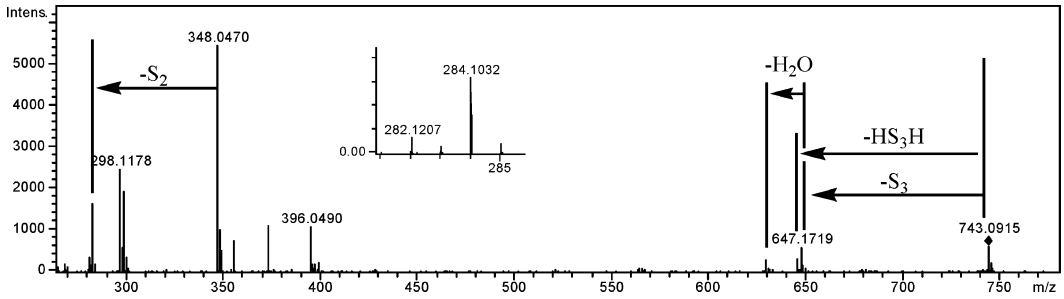


Figure 8. MS/MS spectra of selected $[M + H]^+$ at *m/z* 743 for chaetocochin D (**5**) (collision energy at 10 eV).

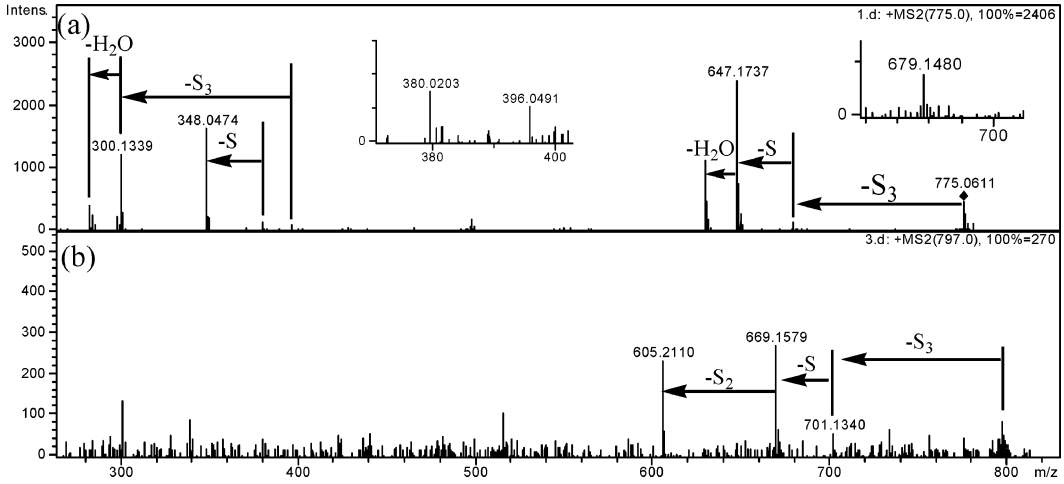
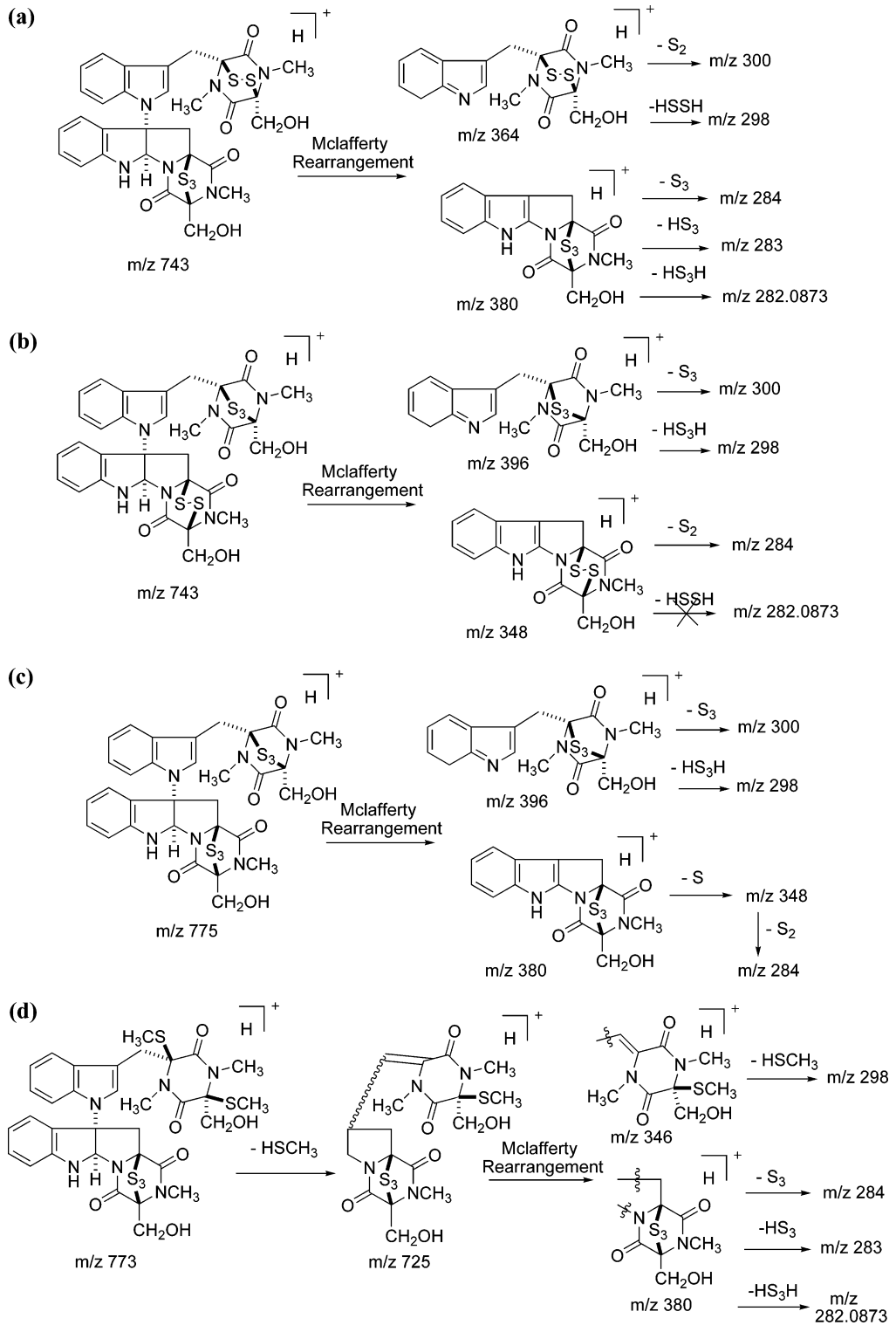


Figure 9. MS/MS spectra of chaetocochin E (**6**): (a) selected $[M + H]^+$ at *m/z* 775 (collision energy at 10 eV) and (b) selected $[M + Na]^+$ at *m/z* 797 (collision energy at 20 eV).

complementary pair of fragment ions at *m/z* 380 and 364 in the MS/MS spectrum of $[M + H]^+$ at *m/z* 743 (Figure 7a), possibly formed by the Mclafferty rearrangement, indicates that two S atoms are on ring B and three on ring A (Scheme 2a). Notably, the numbers of S atoms on each piperazine ring are difficult to confirm even using NMR. The formation of a high-abundance fragment ion at *m/z* 677 by loss of a HSSH molecule on ring B from *m/z* 743 and the fact of much higher abundance of *m/z* 380 than that of *m/z* 364 in the MS/MS spectrum indicate that the loss of sulfide at ring B occurs more readily. This is in agreement with the analysis of **1**. In addition, the product ions at *m/z* 283 and 282.0873 are formed by loss of a HS_3 (97 Da) and a HS_3H (98 Da) molecule on ring A, respectively, from the fragment ion at *m/z* 380. The low-abundance fragment ion at *m/z* 581 is formed possibly by loss of a S_3 molecule from *m/z* 677. In the MS/MS spectrum of $[M + Na]^+$ at *m/z* 765, the product ion at *m/z* 701 is formed by loss of a S_2 molecule from *m/z* 765 and further produces *m/z* 605 through loss of a S_3 molecule. This further supports the above structural analysis.

*Chaetocochin D (**5**).* The accurate masses of $[M + H]^+$ and $[M + Na]^+$ at *m/z* 743.0923 and 765.0760 (Figure 6b), respectively, also correspond to the molecular formula $C_{31}H_{30}O_6N_6S_5$. Therefore, it is an isomer of **4** and could be **5** (Figure 1). A complementary pair of fragment ions at *m/z* 348 and 396 in the MS/MS spectrum of $[M + H]^+$ parent ion at *m/z* 743 (Figure 8), possibly formed by the Mclafferty rearrangement, indicates that the S_2 group is on ring A and S_3 group on ring B (Scheme 2b). High-abundance fragment ions at *m/z* 647 and 645 formed by loss of a S_3 and a HS_3H molecule from *m/z* 743, respectively, and much higher abundance of *m/z* 348 than that of *m/z* 396 in the MS/MS

Scheme 2. Partial Fragmentation Pathways of $[M + H]^+$ for (a) Chetoseminudin A (4), (b) Chaetocochin D (5), (c) Chaetocochin E (6), and (d) Chaetocochin F (7)



spectrum indicate that the loss of the sulfide part occurring at ring B is easier. The loss of the HSSH molecule at ring A in the spectrum was not observed.

*Chaetocochin E (**6**)*. The accurate masses of $[M + H]^+$ and $[M + Na]^+$ parent ions at *m/z* 775.0588 and 797.0471 (Figure 6c), respectively, provide the molecular formula $C_{31}H_{30}O_6N_6S_6$, which corresponds to **6** (Figure 1). Fragment ions observed for **6** were similar to **1**, **4**, and **5**. This means that **6** has the same skeleton structure. A complementary pair of fragment ions at *m/z* 380 and 396 in the MS/MS spectrum of the $[M + H]^+$ parent ion at *m/z* 775 (Figure 9a) were possibly formed by the Mclafferty rearrangement, indicating that the S_3 groups are on ring A and B (Scheme 2c). Highly abundant fragment ions at *m/z* 348 and 647 were formed from *m/z* 380 and 679, respectively, possibly by loss of one S atom on ring A. In the MS/MS spectrum of the $[M + Na]^+$ parent ion

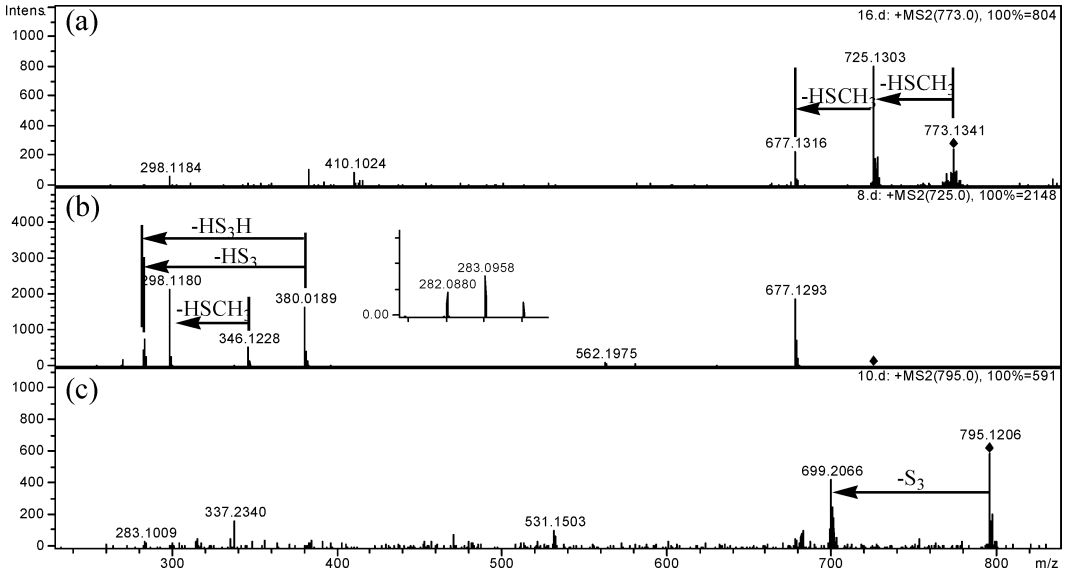


Figure 10. MS/MS and MS/MS/MS spectra of chaetocochin F (**7**): (a) selected $[M + H]^+$ at *m/z* 773 (collision energy at 0 eV), (b) selected ion at *m/z* 725 from *m/z* 773 (collision energy at 15 eV), and (c) selected $[M + Na]^+$ at *m/z* 795 (collision energy at 20 eV).

at *m/z* 797, the fragment ions at *m/z* 701, 669, and 605 are produced by sequential loss of a S_3, a S, and a S_2 molecule from *m/z* 797.

*Chaetocochin F (**7**)*. The accurate masses of $[M + H]^+$ and $[M + Na]^+$ at *m/z* 773.1374 and 795.1205 (Figure 6d), respectively, give the molecular formula $C_{33}H_{36}O_6N_6S_5$ for **7** (Figure 1). Since the MS, MS/MS, and MS/MS/MS spectra (Figure 10) of **7** are very similar to those of chaetocochin C (**2**), the skeleton structure of **7** should be the same as **2**. A complementary pair of fragment ions at *m/z* 380 and 346 in the MS/MS/MS spectrum of the precursor ion at *m/z* 725, possibly formed by the Mclafferty rearrangement, indicates that the S_3 group is on ring A and SMe on ring B (Scheme 2d). The sequential losses of the $HSCH_3$ molecules observed in the MS/MS spectrum of the protonated precursor ion $[M + H]^+$ and the primary loss of the S_3 molecule in the MS/MS spectrum of the sodiated precursor ion $[M + Na]^+$ further supports the structural analysis of **7**.

CONCLUSION

Fragmentation mechanisms of protonated molecular ions, $[M + H]^+$, for five representative epipolythiodioxopiperazines, were elucidated using HPLC-ESI- MS/MS/MS in low-energy collision-induced dissociation in the positive ion mode. The McLafferty rearrangement yielded two characteristic ions containing intact piperazine rings, which provided information about the number of S atoms on each ring. Different fragmentation pathways between $[M + H]^+$ and $[M + Na]^+$ precursor ions were observed: the loss of the $HSCH_3$ molecule was dominant for $[M + H]^+$ when S-methyl was present, whereas the loss of the S_2 molecule appears to be first for $[M + Na]^+$ when the S−S bond was present; the fragmentation patterns of losses of the HSSH and HS_3H molecules were observed for $[M + H]^+$, but not for $[M + Na]^+$. In addition, when rings A and B are very similar, the fragmentation differential between them could be distinguished by MS/MS and MS/MS/MS experiments: the loss of sulfide at ring B occurred more readily and the biradical structure appeared to be stable on ring A. Notably, chetoseminudin A and three new epipolythiodioxopiperazines were found in fungus *Chaetomium cochliodes* and were identified based on tandem mass spectra of known compounds.

In summary, HPLC-ESI-QTOF-MS/MS/MS has proved to be a powerful tool for compound identification, especially for natural product research that is of trace amounts. Manual adjustment of mass spectrometry parameters on the fly make it possible to obtain high-quality MS/MS spectra of low-abundance precusor ions. Complementary information obtained from the fragmentation experiments of $[M + H]^+$ and $[M + Na]^+$ precursor ions is valuable for rapid identification of epipolythiodioxopiperazines. This is especially important for discremination of the number of S atoms on each piperazine ring, which is very challenging even for NMR. The fragmentation differential between $[M + H]^+$ and $[M + Na]^+$ precursor ions, as well as between very similar rings A and B, are of interest.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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