Exploring Chemical Structures From Cortex Lycii, Based on Manual and Automatic Analysis of the HPLC-Q-TOF-MS Data

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Abstract
Cortex Lycii, the root barks of Lycium barbarum and L. chinense, known as “di gu pi” in traditional Chinese herbal drugs, is an important ingredient of formulations used for treating a variety of diseases. During the last 3 decades, more than 70 chemical entities have been separated and purified from either the aqueous or aqueous ethyl alcohol extracts of Cortex Lycii. In this study, high-performance liquid chromatography together with quadrupole-time-of-flight mass spectrometry (MS) was employed to explore new analog structures from aqueous ethyl alcohol extracts (50%, v/v), which led us to discover 4 new phenolic amides and a new cyclic peptide. The structure-based manual screening method, on the basis of the analysis of the fragmentation pathway of the previously known compounds, was used to make a preliminary analysis of the negative total ion chromatography and negative extract ion spectra. Three ions at m/z 472.1, 314.1, and 445.2 were assigned to phenolic amides, and by further analysis of their MS/MS data, the structure of 1, corresponding to one of them (m/z 314.1), was illustrated as an analog of the known compound KN1. A parent ion at m/z 856.1 was assigned to a cyclic peptide analog (2) in the manual analysis procedure. Furthermore, the MS/MS data were profiled on the Global Natural Product Social Molecular Networking (GNPS, https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) workflow to weave a visualization molecular network. Three more new analog ions (m/z 604.3 [3], 597.3 [4], and 611.3 [5]) were found in the aggregation of KN5 and KN7, and their structures were all determined by comparisons with known compounds. This manual and networking automatic screening method may provide a sensitive and efficient procedure to facilitate the mining of novel trace components.

Keywords
cortex lycii, trace components, HPLC-Q-TOF-MS, networking automatic screening

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The dried root barks of Lycium barbarum L. and L. chinense Mill. (Solanaceae), called Cortex Lycii, and known in China as “di gu pi”, are important herbal drugs in traditional Chinese medicine (TCM). They are used as ingredients of formulations to treat various diseases, such as diabetes mellitus, night sweats, coughs, hematemesis, hypertension, and ulcers in China, Japan, and Korea. Previous phytochemical studies on this drug have reported more than 70 compounds assigned to various classes including alkaloids, phenolic amides, cyclic peptides, dipeptide, cyclic diterpenes, and pentacyclic triterpenoids from either the water or (and) ethanol extracts (Figure 1). The crude extracts and chemical monomeric entities from Cortex Lycii showed various pharmacological properties including antioxidant, free radical scavenging, antitumor, antimicrobial, antifungal, antiviral, antidiabetic, and antihypotensive activities.

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Figure 1. The chemical structures of known compounds.
For the acceleration of modernization of the TCM process and better understanding the mechanism of pharmacological action at the molecular level, studies on the complex chemical constituents of TCMs have become one of the hottest subjects. Previous works indicated that the cyclic peptides, lyciumins A and B from Cortex Lycii, showed activities against angiotensin converting enzyme and angiotensin and were expected to be applied in treating clinical hypertension. Lyciumins, bearing a unique cyclic chemical skeleton with a rare linkage between the indole N1 of tryptophan (Trp) and the Cα of glycine, has continuously attracted the attention of chemists and pharmacologists for a long time.

Natural products have continually been the most important source for the development of new medicines for their attractive complex structures and noticeable pharmacological activities. Discovering novel chemical structures from natural resources could play critical roles in enriching the chemical molecular library of natural products and also could provide fundamental skeletons for the designing of new structural drug molecules by chemical synthesis. In the past decades, with the development of mass spectrometry (MS), especially tandem mass spectrometry (MS/MS), numerous natural product structures have been discovered. In our previous work, 20 new structures were discovered on the basis of a manual screening and automatic networking workflow through a reanalysis of the liquid chromatography (LC)-MS/MS data for the fermentation broth of Streptomyces sp. SS.20,21

Early studies on Cortex Lycii provided fundamental chemical structures for discovering novel components in trace amounts by high-performance liquid chromatography (HPLC)-MS. Especially based on the MS/MS fragment patterns of known compounds, the analogs’ structures could be established by analysis of their similar secondary fragment ions or neutral losses, and by comparisons of their MS/MS fragmentation behaviors with those of known compounds (Figure 1). In this study, an HPLC-quadrupole-time-of-flight (Q-TOF)-MS method was employed to explore novel structures from the 50% ethanol (EtOH) aqueous extract of Cortex Lycii; 4 new phenolic amides (1, 3-5) and a new cyclic peptide (2) were established using manual and automatic screening analysis of MS/MS data. Herein, we reported the sample pretreatment and mass data analysis methods.

Experimental Materials and Methods

Chemicals and Materials

Methanol of HPLC grade was supplied by Titan Scientific Co., Ltd. Shanghai, China. Water repurified with a Milli-Q academic water purification system (Millipore, USA) was used for HPLC analysis. Acetic acid, ammonia, and ammonium acetate were of analytical reagent grade from Beijing Chemical Reagent Plant (Beijing, China).

A Sep-Pack C18 cartridge, the solid phase extraction column, was purchased from Waters Co., Ltd. (Milford, MA, USA). A CAPCELL PAK C18 AQ (4.6 × 250 mm, 5.0 µm, Shiseido, Kyoto, Japan) chromatography column was used for HPLC–MS/MS analysis. HPLC–MS/MS data were acquired using an Agilent 1100 (LC) in tandem with an Agilent 6510 Q-TOF MS (Agilent Technologies, Santa Clara, CA, USA).

Plant Material

Cortex Lycii, the root barks of Lycium barbarum and L. chinense (50 Kg), was purchased from Tong-Ren-Tang Pharmacy, Xihongmen shop, Xihongmen Town, Daxing District, Beijing City of People’s Republic of China, in December 2018. The material was identified by Professor Yufeng Ren of the school of biological sciences and engineering of North Minzu University, and the voucher specimen was numbered as NMUDGP-20181218 in plant specimen room.

Sample Preparation

Air dried, powdered Cortex Lycii (100 g) was extracted on an ultrasonic water bath with 100 mL of 50% aqueous EtOH at 20°C for 3 × 2 hours. The extract was evaporated to dryness under reduced pressure, and the residue, as a 2.0 mL water solution, was passed through a Sep-Pack C18 cartridge; the adsorbed components were eluted with purified water, 50% methanol aqueous solution, and HPLC grade methanol to yield samples 1-3. All samples were centrifuged for 10 minutes at 12 000 rpm at 4°C, and the supernatant used as the test samples.

HPLC-MS/MS Analysis

A 2-phase gradient elution system including phase 1 and phase 2 was used for the HPLC-Q-TOF-MS procedure. HPLC grade methanol was used in phase 1, while in phase 2, a solution of 0.1% ammonium acetate solution (pH 8.0) was employed. Linear gradient started with 10% mobile 1 and then reached 90% over 70 minutes. The flow rate was set to 0.8 mL/min, and the effluent was equally separated by a 3-way joint into the diode array detector (DAD) detector and the Q-TOF detector, respectively. The DAD was monitored at 254 nm, and the column temperature was maintained at 25°C. Twenty microliters of the test sample was injected.

The MS data were acquired with an electrospray ionization source; the capillary voltage was 4 kV, the capillary outlet voltage 135 V, the taper hole voltage 65 V, the desolvation temperature 350°C, the dry airflow speed 10 L/min, the atomizer pressure 40 psi, the collision energy 8-45 V, the scanning range m/z 100-2000, and the detection mode was negative ion (ESI−).

The molecular network was created following standard GNPS’s workflows provided in the website of https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp. In brief, raw data were transformed into mzXML format using the MS Convert program of ProteoWizard 3.0 and then analyzed using GNPS. The parameters are presented in supplemental Table S1.
Results and Discussion

Extraction of the Molecular Ions of Known Compounds

In order to investigate the new structural analogs, the accurate molecular ions of known phenolic amides (KN1–KN9, series 1, Figure 1) and cyclic peptides (KN10–KN13, series 2, Figure 1) were extracted from the negative total ion chromatograms (−TIC) of samples 1–3. The negative extracted ion overlaid spectra (Figure 2) showed that almost all the known compounds could be extracted (abundances > 10^5), except compounds KN3 and KN4. The abundances of compounds KN1 and KN9 were higher than other known compounds, which verified that phenolic amides are the main chemical components of Cortex Lycii (Figure 2(a)).

Figure 2(b) shows the extracted ions belonging to 4 cyclic peptides (KN10–KN13). The different abundance of peaks corresponding to KN10–KN13 indicated that lyciumin A (KN10) is the main cyclic peptide in Cortex Lycii. The ordinate difference between Figure 2(a) (<3.0 × 10^5) and 2(b) (<1.0 × 10^6) indicated that the contents of cyclic peptides were lower than those of phenolic amides. The peak for the dipeptide of lyciumamide (KN14) was lower than that of the cyclic peptides, indicating that the dipeptide is in trace amount in the extract of Cortex Lycii.

Fragmentation Patterns of Phenolic Amides and Cyclic Peptides in Negative Mode

Fragmentation behaviors in positive MS mode of phenolic amides and cyclic peptides have been discussed in the literature. The fragment sites of phenolic amides mainly occurred at or around the amido bond in positive mode. However, for cyclic peptides, the losses of side chains were the main MS/MS features. Herein, we established the fragmentation behaviors of those compounds in negative mode by detailed analysis of the MS/MS of known compounds, especially on the basis of the MS/MS analysis of KN1, KN2, KN7, KN9, and KN10.

The fragmentation behaviors of KN1 and KN2. The chemical structure of KN1 is very similar to that of KN2 except for the difference of a methoxyl group and a double bond, which is conjugated to the carbonyl group (Figure 1). Herein, the MS/MS of the ions at m/z 300.1 and 312.1 for KN1 and KN2 were extracted, respectively (Figure 3(a) and (b)), and the fragmentation patterns for KN1 and KN2 were established based on their reasonable neutral losses (Figure 3(a-1) and (b-1)). For KN1 (Figure 3(b)), the higher abundant fragment ions at m/z 178.1 and 121.1 indicated that the losses in the
negative mode were mainly induced by the negative center of the 3,4-dihydroxyphenyl group, while for KN2, a 3-methoxyl-4-hydroxyl-substituted mode reduced the acidity that was caused by the intramolecular hydrogen bond of 4-OH with 3-OMe. A special fragment ion at $m/z$ 197.1, which was produced by losing a CH$_3^+$, gave support to the negative charge center around this phenyl also being an important active site. Also, the ions at $m/z$ 190.1 and 135.1 could be determined by the neutral losses of a 3-methoxyl-4-hydroxylphenyl and 3-methoxyl-4-hydroxylphenyl acryloyl group, respectively (Figure 3(b-1)). The fragment ions at $m/z$ 178.1 and 148.1 should be induced by the negative center around the 4-hydroxylphenyl group.

The fragmentation behaviors of KN5 and KN7. The MS/MS extracted chromatograms of the molecular ions of KN5 and KN7 are presented in Figure 4(a) and (b), respectively, and the fragment patterns for KN5 and KN7 in Figure 4(a-1) and (b-1). Comparison of the chemical structures of KN5 and KN7 indicated that the acetylbutylammonia group in KN5 could provide another negative charge center that reduces the cleavages concentrated on the terminal sites and to produce ions at $m/z$ 427, 442, 455, and 471 in a relatively lower abundance. Fragment ions at $m/z$ 478, 463, and 299 were the common ions of KN5 and KN7, which indicated that these could be used as the characteristic ions for determining the chemical structures.
analogs of phenolic amides bearing the identical substituted stilbene group.

The fragmentation behaviors of cyclic peptides. The negative mode fragmentation behaviors of cyclic peptides in Cortex Lycii have not been studied before. Herein, the MS/MS of the molecular ions at $m/z$ 872.4, 895.4, and 962.4 for KN10, KN11, and KN13 were extracted as shown in Figure 5(a), (b), and (c), respectively. Due to the lower abundance of the ion for KN12 ($<10^{-4}$), the MS/MS data for KN12 were not collected. Based on the reasonable losses for peptide compounds, the fragmentation patterns for KN10, KN11, and KN13 were provided as Figure 5 (a-1) (for KN10), (b-1) (for KN10), and (c-1) (for KN13). The loss of HCHO from the threonine (Thr) residue in the cyclotetrapeptide skeleton was the dominant neutral loss in the fragmentation patterns of cyclic peptides. The neutral loss of 208 Da in KN10 (M-664), KN11 (M-687), and KN13 (M-754) could be used as the evidence for determining the presence of a proline dipeptide moiety in the branched peptide chain. The 90 Da difference between the ions at $m/z$ 591 and 574 in KN13 with those at $m/z$ 501 and 484 in KN10 and KN11 could be caused by the different amino acids in the cyclotetrapeptide skeleton, a phenylalanine (Phe) in KN13 replacing the glycine (Gly) in KN10 and KN11. For the same reason, the difference of 23 Da corresponding to the chain peptide residue of 371 Da (M-501) in KN10 with that of 394 Da (M-501) in KN11 was identical to the difference in the molecular weight (MW) of tryptophan (Try, 204 Da) and tyrosine (Tyr, 181 Da).

Manual Exploration of the New Structural Chemical Constituents

The characteristic ions at $m/z$ 478.1, 463.2, and 299.1 were extracted in the total ion chromatography (TIC) of samples 1–3 for exploring new structural analogs of phenolic amides (Figure 6(a)). The peaks with retention times of 23.5 and 29.9 minutes corresponded to compounds KN1 and KN7, respectively. In addition, 3 new peaks at 22.5, 26.0, and 30.0 minutes with molecular ions of $m/z$ 472.1, 314.1, and 445.2 displayed similar MS/MS fragment ions to those of known phenolic amides. Detailed analysis of their MS/MS spectra, especially of comparisons of their neutral losses and characteristic ions, a structure corresponding to one of them ($m/z$ 314.1) was determined, as shown in Figure 7(a). However, the chemical structures corresponding to the ions at $m/z$ 472.1 and 445.2 could
not be illustrated on the basis of their MS data. The MS/MS for the ions at \( m/z \) 472.1 and 445.2 are provided in the supplemental Figures S1 and S2, respectively.

The characteristic ions, including those at \( m/z \) 501.2, 471.2, 387.1, and 410.1, were used as common ions for exploring cyclic peptides. Figure 6(b) shows the extracted peaks for the known KN10, KN11, and KN13 at retention times of 22.5, 24.1, and 27.3 minutes, respectively. In addition, a new ion at \( m/z \) 856.2, corresponding to the peak at 25.2 minutes in sample 2 (P4 marked *), displayed similar fragment ions to those of the known compounds in Figure 5, especially the presence of the ions at \( m/z \) 501.2, 484.2, 471.2, and 454.2 in relative clear abundance, which indicated that this compound also has a cyclotetrapeptide skeleton identical to that of KN10 and KN11 (Figure 7(b)). However, a fragmentation ion at \( m/z \) 371.1 corresponding to the side chain was 16 Da lower than that in KN10 (\( m/z \) 387.1) suggesting the lack of 1 oxygen atom in this new compound. A similar neutral loss of 208 Da (M-648) corresponding to the diproline residue indicated that the new compound also bore this structure at the terminal of the side chain. Therefore, the difference between compound P4 with KN10 should occur at the moiety of the Tyr, most likely replaced by a phenylalanine (Phe). This speculation was supported by the fragmentation ion of a Phe-cyclotetrapeptide at \( m/z \) 648.2 in this compound rather than a Tyr-cyclotetrapeptide (\( m/z \) 664.2) in KN10.

**Automatic Exploration of New Structural Chemical Constituents Through Networking Workflow**

The HPLC-MS/MS data were reanalyzed on the GNPS platform, and the results disposed in visualization networking (Figure 8). A total of 527 nodes were profiled in the molecular net and 33 sets were composed of at least 3 molecular nodes. In this networking map, the molecular ions corresponding to KN1–KN5, KN7, KN10, KN11, KN13, and KN14 appeared in different sets. Moreover, the complex ions of KN10-Cl\(^-\) (\( m/z \) 908.4), KN11-Cl\(^-\) (\( m/z \) 931.4), and KN13-Cl\(^-\) (\( m/z \) 998.4) were also detected. By detailed analysis of this network, new compounds 1 and 2 that were illustrated by manual
analysis of the TIC above were also profiled in this map, and compound 1 showed a relation to the known KN1, while compound 2 showed a connection to those cyclic peptides. Besides, there are 3 nodes with ions at $m/z$ 604.3 (3), 597.3 (4), and 611.3 (5) that were not reported in previous work, which showed a connection with compounds KN5 and KN7 (Figure 8(b)).

The molecular ion for 3 at $m/z$ 604.3 was 30 Da lower than that of KN5, which implied that compound 3 had 1 methoxyl group less than that of KN5, and this was supported by detailed comparisons of their MS/MS (Figure 9). A weak fragmentation ion at $m/z$ 562.3 was 42 Da lower than that of the molecular ion, which indicated that the acetyl group continued to be located in the structure of 3. A fragment ion at $m/z$ 448.2, 30 Da lower than the ion at $m/z$ 478.2 in KN5 (Figure 4-A2), indicated that the methoxyl group was missing from those substituents around two aromatic rings. The structure of KN5 bearing a 3,5-dimethoxyl-4-hydroxyl phenyl indicated that the absence of this methoxyl group should occur in this place. Therefore, compound 3 should have a structure with a 3-methoxyl-4-hydroxyl phenyl replacing that of a 3,5-dimethoxyl-4-hydroxyl phenyl in KN5.

Figure 7. The tandem mass spectra for compounds 1 (a) and 2 (b) and the fragmentation for (a-1) 1 and (b-1) 2.

Figure 8. The network (a) and the selectively amplifying sets signed in red cycle in (a), for discovering new structures (b).
The molecular ion of $4 \ (m/z \ 597.3)$ showed 44 Da lower than that of KN7, indicating that $4$ should be one of the analogs of KN7. The MS/MS of $4$ (Figure 10) showed similar ion distribution to that of KN7 (Figure 4-B2), especially the base peak at $m/z \ 434.2$, which was 44 Da less than that at $m/z \ 478.2$, as well as ions at $m/z \ 419.1$ (in $4$) and $m/z \ 463.2$ in KN7, $m/z \ 405.2$ (in $4$) and $m/z \ 449.2$ in KN7, and $m/z \ 255.1$ (in $4$) and $m/z \ 299.1$ in KN7, which indicated that the chemical difference between $4$ and KN7 was mainly concentrated on the substituted divinyl ethylene moiety. The key ion at $m/z \ 460.2$, with a neutral loss of 137 Da, was identical to a 3-methoxyl-4-hydroxyl benzyl group, which indicated that the 3,5-dimethoxyl-4-hydroxyl styrene group in KN7 is replaced by a 3-methoxyl-4-hydroxyl phenylethyl in $4$. Due to the 44 Da molecular difference between $4$ and KN7, another lacking 1 oxygen atom was identified in the other 3,4-dihydroxyphenyl group of KN7. Therefore, the structure for $4$ was illustrated as that in Figure 10(b).

The 30 Da lower molecular weight of $5 \ (m/z \ 611.2)$ than KN7 implied that $5$ also lacked 1 methoxyl in comparison with KN7, which was supported by the fragmentation ions at $m/z \ 448.1, 433.1, 419.1$, and 269.1 (Figure 11). Like compound $3$, compound $5$ also bears a 3-methoxy-4-hydroxyl phenyl moiety rather than the 3,5-dimethoxy-4-hydroxyl phenyl in KN7.

The above work described two methods of profiling MS/MS data for exploring new structures. To date, numerous new technologies and methods have been developed for a detailed investigation of natural products with new structures. Systematic isolation and separation of natural products from natural sources (especially plant materials) have been intensively investigated during the last century, and with the help of modern structural analysis technologies, the absolute chemical structures of numerous natural products have been determined, and quite a few of them are in clinical use, either as they are or after structural modification. However, it is becoming more and more difficult to discover new series of chemical structures from known resources, most likely due to repeated discoveries and due to their very trace contents. Therefore, using more sensitive and efficient equipment and technology has become the main choice to explore new natural products. HPLC–NMR, HPLC–MS–NMR, and HPLC–MS/MS were designed to analyze systematically TCM, and microbial secondary metabolites, as well as drug metabolic pathways of important steps in drug discovery.

Automatic profiling of huge MS/MS data in the network is an auxiliary tool for the rapid screening of new compounds by clustering compounds with specific MS/MS ions and neutral losses. The molecular networking method is a visualization tool and was developed as a public networking platform (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp). This aims to be an open-access knowledge base for community-wide organizations and the sharing of raw, processed, or identified MS/MS spectrometric data. In this work, by manual analysis, together with the aid of the GNPS platform, the HPLC-Q-TOF-MS data of Cortex Lycii was
investigated that has led us to discover 4 new phenolic amides and 1 new cyclic peptide analog. Although in the past work, more than 70 compounds have been found from extracts of Cortex Lycii, there are still a large number of mass spectra in the visualization map (Figure 8) that have not been assigned and may correspond to new types of structures.

Conclusions
A structure-based manual screening and automatic networking of HPLC-Q-TOF-MS data developed in the analysis of a TCM for systematically exploring new structures led to the discovery of 4 new phenolic amides (1, 3-5) and 1 new cyclic peptide (2). The main contribution of this article is to provide a new method for exploring new structural compounds in TCMs using tandem mass spectrometric data and molecular networking.

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