Identification and Analysis of Secondary Metabolites from CH₃OH/H₂O Extract of *Metacordyceps Neogunnii*

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Abstract: Secondary metabolites are considered to be the major compounds in *Cordyceps* with anti-tumor, anti-aging and immunity-enhancing effects. The molecular structures of secondary metabolites form the basis for the development and utilization of *Cordyceps*. *Metacordyceps neogunnii* is an important *Cordyceps* resource, but less study has been made on the molecular structure of its secondary metabolites. In this study, gas chromatography-mass spectrometry (GC/MS) and ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) were used to analyze and identify the secondary metabolites from CH₃OH/H₂O extract of *Metacordyceps neogunnii*. The results show that a total of 22 compounds were identified by GC-MS, including 2 n-alkanes, 1 isoparaffin, 1 cycloalkane, 6 olefins and 13 esters. The predominant compounds were (*E*)-ocimene, (*E*)- β -ocimene, methyl oleate, dioctyl adipate, methyl palmitate and methyl linoleate, obtained by means of GC/MS. Five distinct classes of secondary metabolites were speculated: 3 polypeptides, 2 esters, 1 isoflavone, 1 isoindrone and 3 amides, from which 10 compounds were detected using UPLC-Q-TOF/MS. (*R*)-N-((1-(((9H-fluorene-9-yl) methoxy) carbonyl) pyrrolidine-2-yl) methyl)-N-(2-(6-benzoylamino-9H-purin-9-yl) acetyl) glycine, benzyl (5-(2-((3-(2,3-dihydrobenzo[b] [1,4] dioxin-6-yl)-4-carbonyl-4H-chromene-7-yl) oxo) acetyl amino) amyl) carbamate, 5, 5'-((propane-2,2-diyl (4,1-phenylene)) bis (oxo)) bis (2-(naphthalene-1-yl) isoindolone-1,3-dione), 1-dodecylazepine-2-one and other compounds were the first detected in *Metacordyceps neogunnii*.

1 Introduction

Metacordyceps neogunnii (*M. neogunnii*) T.C. Wen & K.D. Hyde, namely, *Cordyceps gunnii* (Berk.) Berk. early reported in China which is widely present in Guizhou, Hunan and Anhui provinces [1], has various effects such as analgesia, sedation, improvement of human immunity, anti-tumor, anti-aging, promoting sleep and enhancing memory [2-5]. It has chemical composition and medicinal value similar to those of *Cordyceps sinensis*. Furthermore, it is characterized with such merits as wide ecological amplitude and short cultivation period [6], thus more suitable for large-scale cultivation through modern submerged fermentation. Secondary metabolites of *Cordyceps sinensis* are considered to be major compounds with various effects, from which it is highly likely to find new bioactive substances or lead compounds for drugs [7]. However, at present, the research on *M. neogunnii* mainly focuses on strain identification, genomics analysis and preliminary pharmacological effects, with little on secondary metabolites [8].

Gas chromatography-mass spectrometry (GC/MS) can be used for the qualitative analysis of organic compounds, which is a powerful means to obtain molecular structure information, but it is only suitable for volatile organic compounds with low molecular weight (< 500 Da) [9]. This technology has already been applied in the analysis of volatile components of *Cordyceps sinensis* [10]. Ultra performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS), due to its extremely high sensitivity, can be used to distinguish compounds with very close molecular weight differences or even compounds with the same molecular weight but different element composition. It can be used to have a qualitative analysis of trace substances in samples with complex components, capable of accurately and rapidly obtaining molecular structure information of an organic compounds, which has been widely applied in environmental science, medicine, drug research and development and other fields [11,12]. The study of active metabolites is an inevitable link for the efficient development and utilization of *M. neogunnii* resources. In order to further explore the secondary metabolites of *M. neogunnii* and enrich the natural product library of

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cordyceps fungi, based on the principle of the dissolution in the similar material structure, the enrichment and separation of group components of secondary metabolites in *M. neogunnii* were carried out. The molecular structure of secondary metabolites was analyzed by GC/MS and UPLC-Q-TOF/MS, and the function characteristics of these compounds were briefly discussed, which provided data support and scientific basis for the further development and utilization of these compounds.

2 MATERIALS AND METHODS

2.1 Materials, reagents and main instruments

M. neogunnii was provided by Professor Wen Ting-chi (Guizhou Biochemical Engineering Center, Guizhou University, China). The petroleum ether used in the experiment was analytical reagent (Xilong Scientific Co., Ltd., Guangdong Province, China), which was used after rotary evaporation and distillation, methanol was chromatographically pure (CINC High Purity Solvents (Shanghai) Co., Ltd., China). The main instruments including the Agilent 7890A-5975C Gas Chromatography-Mass Spectrometer (Agilent, USA); UPLCH-CLASS/QTOF G2-XS Ultra High Performance Liquid Chromatography Tandem Quadrupole Time-of-Flight Mass Spectrometer (Waters, USA); 1810D Ultra-pure water purification system (Shanghai SENCO Technology Co., Ltd., China); TG16-WS Tabletop High Speed Centrifuge (Shanghai Lu Xiangyi centrifuge instrument Co., Ltd., China); DHG-9101-2A electric heating constant temperature blast drying oven (Sanfa Scientific Instruments, Shanghai City, China); AUY220 electronic balance (Shimadzu Technology Co., Ltd.); XL-600B multifunctional crusher (Xiaobao Electric Co., Ltd. Yongkang City, Zhejiang Province, China).

2.2 Sample preparation

M. neogunnii was pulverized to pass through a 100-mesh sieve followed by desiccation in a vacuum at 60 °C for 24 h before use. Subsequently, *Cordyceps* power (2 g) and 60 % methanol (30 mL) were added to a 100 mL triangular flask and exhaustively extracted under ultrasonic radiation. Extract of 60 % methanol was concentrated by distillation to obtain Extract I. Extract I was extracted with equal amount of petroleum ether using a separatory funnel for 3 times. And 3 extractions of petroleum ether were merged, concentrated and purified through a 0.45µm microporous membrane to obtain the extract II (MNE_{PE}). In addition, the raffinate of petroleum ether (MNR_{PE}) was purified by centrifugation before analysis. MNE_{PE} was analyzed with GC/MS and MNR_{PE} analyzed by UPLC-Q-TOF/MS.

2.3 Sample analysis

The processed MNE_{PE} was analyzed by GC/MS. The injection volume was 0.5µL, the injector temperature was set at 290 °C, the split ratio was 5:1, and the chromatographic column was HP-5MS elasticity quartz capillary column (30 m×250µm×0.25µm). The carrier gas was high purity nitrogen (99.999 %) at a flow rate of 1.0 mL/min, the oven was programmed to start at 60 °C, increased to 160 °C with 5 °C /min, hold for 1 min, then increased from 160 °C to 190 °C with 5 °C /min, hold for 1 min, increased from 190 °C to 280 °C with 3 °C /min, hold for 9 min. The solvent delay was 3.00min, the ion source temperature was set at 230 °C with the electron energy at 70 eV and the mass range at *m/z* 50-500, Electron Impact mode. The MSD ChemStation software was used to process the data, and the mass spectra obtained were identified by comparing with the NIST08 standard spectra, the compounds were quantitatively analyzed by normalization method.

The processed MNE_{PE} was analyzed by UPLC-Q-TOF/MS. (ACQUITY HSS T3 column (2.1 mm×100 mm, 1.8µm) was used with mobile phase consisted of 0.1 % formic acid water-0.1 % acetonitrile at the flow rate of 0.3mL/min, the column temperature was set at 30 °C and the injection volume was 1µL. Gradient elution conditions: 0~5 min, 95 % A; 5~50 min, 95 %A; 50~55 min, 5 %A; 55~57 min, 5 %A; 57~60 min, 95 %A. Electrospray ionization source, detected in positive ion mode. The ion source temperature was set at 120 °C; the desolvation gas temperature at 80 °C with a desolvation gas flow of 900L/h; the collision energy was 3 eV; the capillary voltage was set at 3.1 kV, the cone voltage was up to 35 V; the cone gas flow was set to 50L/h; the scan range of primary mass spectrometry parent ion was from 50 to 1200 *m/z*; the scan range of second-order mass spectrometry fragment ion was from 50 to 1000 *m/z*). Compounds were identified using Massfragment in Masslynx software and its molecular weight and molecular structure formula were summarized according to relevant literature. Massfragment can automatically identify ion fragments of compounds by using a series of unique chemical intelligence algorithms.

3. RESULTS AND DISCUSSION

3.1 Analysis of MNE_{PE}

The compounds of MNE_{PE} were analyzed and identified by GC/MS. 22 compounds were identified, including 9 hydrocarbons and 13 ester compounds, with the relative contents of 7.24 % and 92.80 %, respectively. Total ion chromatography of MNE_{PE} and the main compounds were summarized in Table 1.

3.1.1 Hydrocarbon compounds in MNE_{PE}

Hydrocarbon compounds in MNE_{PE} include 2 normal alkanes, 1 isoparaffin and 6 olefins, with relative content distribution of 0.23 %, 0.24 % and 6.77 %, respectively (As seen in Table 1). An alkane is an organic compound, existing in higher eukaryotes such as mammals, insects and birds, and has effects of anti-desiccation, nerve protection, signal transmission and

regulation [13]. *N*-alkanes belong to the biomarker compound of fungi[14]. 2,6,10,14-Tetramethylhexadecane (Peak 8), also known as phytane, is one kind of regular isoprenoid alkanes and has the characteristics of a biomarker compound as well. The detection of these compounds is of great significance for the study of the origin and genetic properties of entomogenous fungi.

Table 1. Compounds identified by GC/MS in MNE_{PE}

Peak	RT (time)	Compound	Structure	MF	SI (%)	RC (%)
1	4.488	1-methyl-4-(prop-1-en-2-yl) cyclohex-1-ene		C ₁₀ H ₁₆	94	0.22
2	5.091	(<i>E</i>)-ocimene		C ₁₀ H ₁₆	96	3.85
3	5.806	(<i>E</i>)-β-ocimene		C ₁₀ H ₁₆	97	2.32
4	11.315	Allo-ocimene		C ₁₀ H ₁₆	97	0.23
5	27.036	(1 <i>S</i> ,2 <i>R</i> ,5 <i>S</i>)-2,6,6,8-Tetramethyltricyclo[5.3.1.0 ^{1,5}] undec-8-ene		C ₁₅ H ₂₄	93	0.05
6	32.339	(1 <i>S</i> ,8 <i>aR</i>)-1-isopropyl-4,7-dimethyl-1,2,3,5,6,8 <i>a</i> -hexahydronaphthalene		C ₁₅ H ₂₄	94	0.10
7	36.557	hexadecane		C ₁₆ H ₃₄	90	0.09
8	40.989	2,6,10,14-tetramethylhexadecane		C ₂₀ H ₄₂	93	0.24
9	47.659	diisobutyl phthalate		C ₂₀ H ₃₀ O ₄	90	0.90
10	50.266	methyl palmitate		C ₁₇ H ₃₄ O ₂	99	5.52
11	51.532	dibutyl phthalate		C ₁₆ H ₂₂ O ₄	94	54.47
12	56.466	(9 <i>Z</i> ,12 <i>Z</i>)-methyl octadeca-9,12-dienoate		C ₁₉ H ₃₄ O ₂	99	4.28
13	56.763	(<i>E</i>)-methyl octadec-9-enoate		C ₁₉ H ₃₆ O ₂	99	15.33
14	57.77	methyl stearate		C ₁₉ H ₃₈ O ₂	99	0.98
15	58.489	(10 <i>E</i> ,12 <i>Z</i>)-methyl octadeca-10,12-dienoate		C ₁₉ H ₃₄ O ₂	91	0.17
16	60.103	(9 <i>Z</i> ,11 <i>E</i>)-methyl octadeca-9,11-dienoate		C ₁₉ H ₃₄ O ₂	98	0.18
17	60.234	docosane		C ₂₂ H ₄₆	82	0.14
18	63.919	(<i>E</i>)-2-ethylhexyl 3-(4-methoxyphenyl)acrylate		C ₁₈ H ₂₆ O ₃	93	0.42

19	64.402	(<i>E</i>)-octyl 3-(4-methoxyphenyl)acrylate		C ₁₈ H ₂₆ O ₃	91	0.09
20	66.598	bis(2-ethylhexyl) adipate		C ₂₂ H ₄₂ O ₄	97	8.52
21	69.156	hexadecyl 2-ethylhexanoate		C ₂₄ H ₄₈ O ₂	92	0.17
22	70.911	dioctyl phthalate		C ₂₄ H ₃₈ O ₄	95	1.74

RT retention time, *MF* molecular formula, *SI* similarity with NIST08 standard spectra, *RC* relative content by peak area

The major olefins in MNE_{PE} were terpenes with a relative content of 6.62 %, mainly including 4 compounds (Table 1), namely, D-limonene (Peak 1), Ocimene (Peak 2 & 3) and Allo-Ocimene (Peak 4). D-limonene is a natural monoterpene, widely existing in citrus, vegetables and spices, and has anti-inflammatory, antibacterial, anti-tumor and other effects [15]. Its anti-cancer mechanism mainly lies in chemical prevention, inhibition of cell cycle, induction of cell apoptosis, and inhibition of tumor metastasis and invasion [16]. Its role in cancer prevention has attracted extensive attention; so far it has been widely used in many industries and fields such as food, pesticide, chemical industry and medicine [17]. Ocimene is an acyclic monoterpene with an aromatic odor, which can be used in the production of flavors and fragrances. It has three types, containing α , cis- β and trans- β , and the isomer is allo-ocimene. Ocimene is also an important communication signal molecule, but the current research on ocimene and β -ocimene is not sufficient enough, and the mechanisms of its medicinal functions remain to be further explored [18].

3.1.2 Esters in MNE_{PE}

MNE_{PE} contained 13 esters with a relative content of 92.80 %, consisting of phthalates, the fatty acid methyl ester and other esters. Phthalates, detected as ioctyl hexanedioate (Peak 9, 0.90 %), diisobutyl phthalate (Peak 11, 54.47 %) and dibutyl phthalate (DBP, Peak 22, 1.74 %), are mainly plasticizers. DBP is widely used in plastic products, cosmetics, lubricants and other fields. Strain fermentation culture was carried out with DBP by some researchers by using cordyceps *militaris* and *paecilomyces verticillatus* as raw materials. The results showed that 6 % DBP was capable of increasing the biomass of bacterial strains. The total production of cordycepin was significantly higher than that of the control group without organic phase [19]. It is important to increase the content of cordycepin for using mycelium as medicine and functional food. Therefore, DBP should be further developed as the microbial production of secondary metabolites.

(*E*)-2-ethylhexyl 3-(4-methoxyphenyl)acrylate (Peak 18) and (*E*)-octyl 3-(4-methoxyphenyl) acrylate (Peak 19) accounting for 0.51 % (Table 1), are mainly used in skin care cosmetics. Among them, octyl methoxycinnamate is also a therapeutic drug for photosensitive dermatitis,

which can effectively absorb the ultraviolet rays in sunlight and prevent the human skin from being red, black and sunburn.

There are 6 kinds of the fatty acid methyl ester (Table 1) in MNE_{PE}, namely, methyl oleate (Peak 10, 4.15 %), (*9Z,12Z*)-methyl octadeca-9,12-dienoate (Peak 12, 4.28 %), (*E*)-methyl octadec-9-enoate (Peak 13, 15.33 %), methyl stearate (Peak 14, 0.98 %), (*10E,12Z*)-methyl octadeca-10,12-dienoate (Peak 15, 0.17 %), (*9Z,11E*)-methyl octadeca-9,11-dienoate (Peak 16, 0.18 %), with a total relative content of 26.46 %. In addition to more methyl esters contained in *Cordyceps*, methyl esterification of methanol extract under the action of ultrasonic radiation during the extraction process may also lead to higher content of MNE_{PE}. In general, there are more fatty acids in MNE_{PE} of *M. neogunnii*, mainly unsaturated fatty acids (linoleic acid and oleic acid), and a small quantity of saturated fatty acids, such as palmitic acid, stearic acid and arachidic acid, which are consistent with the research results of our previous work [20]. Linoleic acid is ω -6 series polyunsaturated fatty acid, which has the effects of reducing cholesterol and inhibiting tumor cells, and can be converted into arachidonic acid to generate prostaglandins through an unsaturated action [21]. Oleic acid is ω -9 series monounsaturated fatty acid, which has the effects of regulating the concentration ratio of high and low density lipoprotein cholesterol in serum, preventing cardiovascular diseases, and reducing cholecystitis and cholelithiasis [22]. Although being a saturated fatty acid, stearic acid is rapidly converted into oleic acid in vivo without affecting the concentration of cholesterol.

3.2 Analysis of MNR_{PE}

The compounds of MNR_{PE} were analyzed and identified with UPLC-Q-TOF/MS. MassLynx 4.1 was used to analyze the total ion current in the positive ion mode. 10 compounds and their fragmentation pathway were analyzed and deduced combined with ion fragmentation, retention time, accurate mass-to-charge ratio, online database Chemspider and references.

3.2.1 UPLC-Q-TOF/MS analysis and possible fragmentation pathway

Compound 1 (*R*)-N-((1-((9H-fluorene-9-yl) methoxy) carbonyl) pyrrolidine-2-yl) methyl)-N-(2-(6-benzoyl-

amino-9H-purine-9-yl) acetyl) glycine

Compound 1 had a retention time of 1.507 min. The mass spectrometry signal at m/z 660.2551 $[M+H]^+$ was obtained by using the Massfragment function in Masslynx, and the mass spectrum peaks at 201.1246, 341.1833, 571.2112 and 642.2471 appeared in the secondary mass spectrum. According to the database matching, the molecular formula was $C_{36}H_{33}N_7O_6$ and the molecular weight was 659.2492. It was speculated that the compound may be (*R*)-*N*-((1-(((9H-fluorene-9-yl) methoxy) carbonyl) pyrrolidine-2-yl) methyl)-*N*-(2-(6-benzoylamino-9H-purine-9-yl) acetyl) glycine, and the secondary fragment ions were $[M+H-H_2O]^+$, $[M+H-C_3H_7NO_2]^+$, $[M+H-C_{18}H_{11}N_2O_4]^+$ and $[M+H-C_{23}H_{19}N_6O_5]^+$. Its possible fragmentation pathway in Figure 2.

Figure 2. Possible bond-breaking pathways of compound 1

Compound 2 Ethyl 1-(2-(2-(2,4-dicarbonyl-1,3-diazaspiro [4.4] nonane-3-yl) acetoxy) acetyl) piperidine-4-carboxylate

Compound 2 had a retention time of 1.941 min. The mass spectrum signal at m/z 410.1931 $[M+H]^+$ was obtained, and the mass spectrum peaks at 392.1821, 366.1844, 272.1593 and 201.1250 appeared in the secondary mass spectrum. According to the database matching, the molecular formula was $C_{19}H_{27}N_3O_7$ and the molecular weight was 409.1849. It was speculated that the compound may be ethyl 1-(2-(2-(2,4-dicarbonyl-1,3-diazaspiro [4.4] nonane-3-yl) acetoxy) acetyl) piperidine-4-carboxylate, and the secondary fragment ions were $[M+H-CH_2NO_4]^+$, $[M+H-C_7H_6O_3]^+$ and $[M+H-C_{10}H_{11}O_4]^+$. Its possible fragmentation pathway in Figure 3.

Figure 3. Possible bond-breaking pathways of compound 2

Compound 3 L-tyrosyl-L-valyl-L-alanyl-L-glutamic acid

Figure 4. Possible bond-breaking pathways of compound 3

Compound 3 had a retention time of 2.174 min. The quasi-ion molecular peak at m/z 481.2297 $[M+H]^+$, and

its characteristic fragments $[M+H-H_2O]^+$, $[M+H-C_5H_9O_5]^+$ and $[M+H-C_{10}H_9NO_4]^+$ were obtained. According to its fragmentation rule, it is preliminarily speculated that the compound may be L-tyrosyl-L-valyl-L-alanyl-L-glutamic acid, the molecular formula was $C_{22}H_{32}N_4O_8$ and the molecular weight was 480.2220. Its possible fragmentation pathway in Figure 4.

Compound 4 L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid

Compound 4 had a retention time of 2.697 min. The quasi-ion molecular peak at m/z 467.2466 $[M+H]^+$, and its characteristic fragments $[M+H-CO_2]^+$, $[M+H-C_7H_{12}N_2O_5]^+$, $[M+H-C_6H_9NO_4]^+$ and $[M+H-C_2H_9O_5]^+$, were obtained. According to its fragmentation rule, it was preliminarily speculated that the compound may be L-tyrosyl-L-valyl-L-alanyl-L-aspartic acid, with molecular formula being $C_{21}H_{30}N_4O_8$ and molecular weight being 466.2064. Its possible fragmentation mode in Figure 5.

Figure 5. Possible bond-breaking pathways of compound 4

Compound 5 Benzyl (5-(2-((3-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)-4-oxo-4H-chromene-7-yl) oxy) acetamido) pentyl) carbamate

Compound 5 had a retention time of 2.697 min. The quasi-ion molecular peak at m/z 573.2822 $[M+H]^+$, and its characteristic fragments $[M+H-C_3H_6O_3]^+$, $[M+H-C_8H_{11}O_2]^+$ and $[M+H-C_9H_4O_4]^+$, were obtained. According to its fragmentation rule, it was preliminarily speculated that the compound may be Benzyl (5-(2-((3-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)-4-oxo-4H-chromene-7-yl) oxy) acetamido) pentyl) carbamate, with molecular formula being $C_{32}H_{32}N_2O_8$ and molecular weight being 572.2159. Its possible fragmentation pathway in Figure 6.

Figure 6. Possible bond-breaking pathways of compound 5

Compound 6 (*E*)-2-ethoxy-4-((2-(2-(naphthalene-1-yloxy) acetyl) hydrazono) methyl) phenyl 3,4,5-trimethoxybenzoate

Compound 6 had a retention time of 3.975 min. The quasi-molecular ion peak at m/z 559.2077 $[M+H]^+$ was obtained. The mass spectrum peaks at 187.1078, 231.0333, 327.1116 and 541.1935 appeared in the secondary mass spectrum. According to database matching, the molecular formula was $C_{31}H_{30}N_2O_8$ and the molecular weight was 558.2002. It is presumed that the compound may be (*E*)-2-ethoxy-4-((2-(2-

(naphthalene-1-oxo) acetyl) hydrazono) methyl) phenyl 3,4,5-trimethoxybenzoate, and the secondary fragment ions were $[M+H-H_2O]^+$, $[M+H-C_{13}H_{12}NO_2]^+$, $[M+H-C_{20}H_{24}O_4]^+$ and $[M+H-C_{19}H_{16}NO_6]^+$. Its possible fragmentation pathway is shown in Figure 7.

Figure 7. Possible bond-breaking pathways of compound 6

Compound 7 5,5'-((propane-2,2-diylbis (4,1-phenylene)) bis (oxo)) bis (2-(naphthalene-1-yl) isoindoline-1,3-dione)

Figure 8. Possible bond-breaking pathways of compound 7

Table 2. Possible bond-breaking pathways of compound 8

Theoretical value	256.2636	172.1705	158.1553	144.1388
Measured value	256.2640 (-0.4.mDa)	172.1701 (+0.4.mDa))	158.1545 (+0.8.mDa)	144.1388 (-0.0.mDa)
Fragment				
Molecular formula	C ₁₆ H ₃₄ NO (-none)	C ₁₀ H ₂₂ NO (-C ₆ H ₁₂)	C ₉ H ₂₀ NO (-C ₇ H ₁₄)	C ₈ H ₁₈ NO (-C ₈ H ₁₆)

Compound 9 1-dodecylazepan-2-one

Compound 9 had quasi-ion molecular peak at m/z 282.2792 $[M+H]^+$, with a retention time of 30.630 min. which characteristic fragments of $[M+H-CH_3]^+$, $[M+H-H_2O]^+$, $[M+H-C_2H_2O]^+$, $[M+H-C_3H_6O]^+$ and $[M+H-C_{10}H_{20}]^+$, were obtained. It was preliminarily speculated that the compound may be 1-dodecylazepan-2-one with molecular formula of C₁₈H₃₅NO and molecular weight of 281.2719. Its possible fragmentation pathway in Figure 9.

Figure 9. Possible bond-breaking pathways of compound 9

Compound 10 2-methyl heptadecan amide

Compound 10 had a retention time of 32.207 min. The mass spectrum signal at m/z 284.2958 $[M+H]^+$ was obtained, and the mass spectrum peaks at 228.2290, 214.2174 and 200.2009 appeared in the secondary mass spectrum. According to the database matching, the molecular formula was C₁₈H₃₇NO and the molecular

Compound 7 had a retention time of 5.068 min. The quasi-molecular ion peak at m/z 771.2470 $[M+H]^+$ was obtained, and the mass spectrum peaks at 599.1622, 725.2394, 743.2527 and 753.2352 appeared in the secondary mass spectrum. According to the database matching, the molecular formula was C₅₁H₃₄N₂O₆ and the molecular weight was 770.2417. It was speculated that the compound may be 5,5'-((propane-2,2-diylbis (4,1-phenylene)) bis (oxo)) bis (2-(naphthalene-1-yl) isoindoline-1,3-dione), and the secondary fragment ions were $[M+H-H_2O]^+$, $[M+H-CH_2O_2]^+$ and $[M+H-CO]^+$. Its possible fragmentation pathway in Figure 8.

Compound 8 2-methyl pentadecanamide

Compound 8 had a retention time of 29.900 min. The quasi-ion molecular peak at m/z 256.2636, the mass spectrum peaks at 172.1705, 158.1553 and 144.1388 (Table 3) appeared in the secondary mass spectrum, and its fragment ions were $[M+H-C_6H_{12}]^+$, $[M+H-C_7H_{14}]^+$ and $[M+H-C_8H_{16}]^+$. According to the database matching, the molecular formula was C₁₆H₃₃NO and the molecular weight was 255.2562. It was presumed that the compound may be 2-methyl pentadecanamide. Its possible fragmentation mode in table 2.

weight was 283.2875. It is presumed that the compound may be 2-methyl heptadecan amide, and the secondary fragment ions were $[M+H-C_4H_8]^+$, $[M+H-C_5H_{10}]^+$ and $[M+H-C_6H_{12}]^+$. Its possible fragmentation pathway in Figure 10.

Figure 10. Possible bond-breaking pathways of compound 10

3.2.2 Analysis of Compounds

Nitrogen is the basis of life and plays an important role in life activities. The nitrogenous compounds have high biological activity. 10 nitrogen-containing compounds were analyzed from MNR_{PE}, including 3 polypeptides (peaks 1, 3 and 4), 2 esters (peaks 3 and 6), 1 isoflavone (peak 5), 1 isoindolinone (peak 7) and 3 amides (peaks 8, 9 and 10).

The polypeptide, with a small molecular weight, can control the growth, development, immune regulation and metabolism of the human body. It is characterized with

the sensitiveness to tumors, little drug resistance and low cost [23]. Although the biotechnological technology of synthetic peptides is relatively mature, the peptides extracted from *Cordyceps* are used as raw materials to synthesize isomerized peptides, which belong to a novel anti-tumor polypeptide [24, 25]. Zhu et al [26] isolated and purified the peptides with high acidic amino acid residues from the mycelium of *Paecilomyces gunnii*, which had a strong analgesic effect but without morphine dependence. Given the strong analgesic effect and non-addiction of secondary metabolites of polypeptides in *M. neogunnii*, to develop new analgesic and detoxification drugs will be an important direction for the development and utilization of *M. neogunnii* in the future.

Isoflavone compound, with C₆-C₃-C₆ as the mother stone, is one of the effective components of many traditional Chinese medicines and has been included into *The Chinese Pharmacopoeia* [27]. Isoflavone has a variety of activities such as cardiovascular protection [28], anti-tumor [29], osteoporosis prevention [30], nerve protection, neurodegeneration resistance [31], blood sugar reduction [32], and immune system regulation [33]. Its anti-cancer properties are prominent, and it can inhibit the growth and spread of cancer cells, effective to cancer cells only. Wang et al [34] confirmed that isoflavone compounds had an inhibitory effect on the proliferation of prostate cancer cell PC-3.

Isoindrones are benzo heterocyclic compounds [35] with excellent pharmaceutical activities, like analgesic, anti-inflammatory, antibacterial, anti-tumor, anti-diabetes, anti-cardiovascular diseases and other activities [36, 37]. The parent ring structure of isoindrone is contained in Falipamil, Lenalidomide, Indoprofen and other drugs, which plays an important role in the development and utilization of new drugs.

Amide compounds are widely found in nature. Many alkaloids, such as colchicine, febrifugine, ergotone and other molecular structures, contain amide bonds (-CONH-) [37]. Amide bond (or peptide bond) is the basic structure of protein. Hexa-lactam skeleton widely exists in natural products and small-molecule active compounds, which has an important biological significance as well as research value [38,39]. For example, compound UK224671 containing the skeleton has neurohormone 2 (NK2) receptor antagonism [37], and AM-8553 has a better effect for MDM2-p53 inhibition [38].

4. Conclusions

M. neogunnii, a new and importance *Cordyceps* resource to be developed, underwent the compound enrichment and separation with organic solvents. Secondary metabolites of *M. neogunnii* were separated and analyzed combined with GC/MS and UPLC-Q-TOF/MS, and related biological activities were discussed. In this paper, 22 compounds, mainly hydrocarbons and esters, were identified in MNE_{PE}. 10 nitrogen-containing compounds, including peptides, amides, isoflavones, isoindrones, esters and other compounds, were analyzed from MNR_{PE}.

The carbon number of each compound was greater than 16, and its molecular weight ranged from 200 to 780. Such findings have not been reported in other literatures so far. The results revealed that the secondary metabolites of *M. neogunnii* were diverse, reflecting that *M. neogunnii* contained abundant secondary metabolites with important pharmacological effects. It is necessary to further explore *M. neogunnii* by means of multi-layer separation analysis, which may provide the scientific basis for its rational development and utilization, as well as the reference for other *Cordyceps* fungi.

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