Analysis of Triterpenoids in *Ganoderma lucidum* Using Liquid Chromatography Coupled with Electrospray Ionization Mass Spectrometry

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Triterpenoids extracted from *Ganoderma lucidum* (Leyss. ex Fr.) Karst were separated and characterized using optimized reversed-phase liquid chromatography with diode array detection and electrospray ion trap tandem mass spectrometry (HPLC-DAD-ESI-MS^n). They could be classified into five types depending on the fragmentation behavior. All triterpenoids gave $[M - H]^-$ and $[2M - H]^-$ ions by electrospray ionization monitored in the negative ion mode; in addition, compounds of types III and IV gave prominent $[M - H - H_2O]^-$ ions and the unsaturated bond at C-20, 22 would reduce the abundance of $[M - H - H_2O]^-$ ion. The key fragmentation information was cleavage at C- and D-rings despite the predominant losses of H_2O and CO_2. Compounds with hydroxyls at C-7 and C-15 would produce a list of **b**, **b** – 1, **b** – 2, and **b** – 16 ions attributed to cleavage of D-ring; if the second alcohol at C-15 were oxidized to ketone, the prominent cleavage would occur at C-ring and produce a group of ions of **a**; if C-7 were oxidized to ketone, transference of two hydrogen atoms would occur during the cleavage of rings and a list of ions about **a** + 2 and/or **b** + 2 would appear instead. The above fragmentations and regularities in fragmentation pathways were reported for the first time, and were implemented for the analysis of triterpenoids in *G. lucidum*. The chloroform extract was separated on a Zorbax SB-C_{18} column, eluting with an acetonitrile-0.2% acetic acid gradient. A total of 32 triterpenoids, including six new ones, were identified or tentatively characterized based on the tandem mass spectra of the HPLC peaks. (J Am Soc Mass Spectrom 2007, 18, 927–939) © 2007 American Society for Mass Spectrometry

Ganoderma lucidum (Leyss. ex Fr.) Karst, a medicinal fungus called "Lingzhi" in China, is a commonly used Chinese herb and an important ingredient in traditional Chinese medicine herbal formulations for the prevention and treatment of various types of diseases, such as cancer, hepatopathy, arthritis, hypertension, neurasthenia, debility, etc. Lingzhi has long been used as a folk remedy for promotion of health and longevity in China and other oriental countries. The most attractive character of this kind of medicinal fungus is its immunomodulatory and antitumor activities [1–7].

Clearly, the activities of *G. lucidum* are mainly due to polysaccharides and/or triterpenoids of the fungus. Over 130 triterpenoids have been isolated from the fruiting bodies, cultured mycelia, and spores during the past two decades [8, 9]. Some of the triterpenoids showed antiandrogenic [10], antihepatitis B [11], antioxidant [12, 17], antitumor [13, 15], anticomplement [14], antimicrobial [16], anti-HIV-1 [18], selectively inhibit eukaryotic DNA polymerase [19], and angiotensin converting enzyme-inhibitory activities [20]. Thus, a valuable and convincing method for characterization of triterpenoids of *G. lucidum* is necessary for quality control of the herbal medicine.

Mass spectrometry is the most selective technique for the rapid qualitative determination of known compounds as well as the identification of unknown com-

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pounds from extracts of natural products [21–24]. Some methods were established for separation and detection of triterpenoids in *G. lucidum* [25, 26]. In this study, an integrated approach consisting of LC/ESI-MS and tandem mass spectrometry (MS^n) has been used for the identification of triterpenoids in *G. lucidum* extracts. To the best of our knowledge, we report here for the first time a comprehensive analysis for the triterpenic acids occurring in *G. lucidum* based on MS^n technique. Using ESI-MS, it has been possible to obtain structurally significant fragmentation ions of triterpenoids. The present approach could be applied to studies of triterpenoids in crude extracts of *G. lucidum*. A total of 32 compounds were identified or tentatively characterized from the chloroform extract of *G. lucidum* (Figure 1, Table 1); six of them are reported for the first time.

Experimental

Chemicals

Ganoderic acid A, B, AM_1, C_2, D, G, H, J, K, ganoderenic acid B, and 3β-hydroxy-4, 4, 14-trimethyl-7, 11, 15-trioxochol-8-en-24-oic acid were isolated from the fruit bodies of *G. lucidum*. All these structures were fully characterized by direct comparison of their NMR and MS spectra data with those reported in the literatures [27–33]. Their purities were over 95% determined by HPLC/UV analysis. HPLC-grade acetonitrile (MeCN) (Burdick and Jackson-Honeywell Intl., Muskegon, MI) and ultra-pure water were used for all analyses. The chloroform and methanol for sample preparation and the acetic acid (CH_3COOH) used in the mobile phase were of AR grade, purchased from Beijing Chemical Corp. (Beijing, China).

Sample Preparation

G. lucidum was obtained from the cultivation base of Green Valley Pharmaceutical Co. Ltd. (Shanghai, China). A 2.0 g powder of dried samples was extracted with 40 mL $CHCl_3$ in an ultrasonic water bath for 20 min. This extraction was repeated twice. The extracted solution was mixed and filtrated through analytical filter paper; then the filtered solution was evaporated at 35 °C to dryness in vacuum. The dry extract was dissolved in 5 mL methanol and filtrated through a 0.45 μm membrane filter unit. Then 3 μL of each sample solution was analyzed by HPLC.

Chromatography

The analyses were performed on an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector (DAD), an autosampler, and a column compartment. The sample was separated on a Zorbax SB-C_{18} column (5 μm, 4.6 × 250 mm; Agilent). The mobile phase consisted of acetonitrile (CH_3CN) and water containing 0.2% (vol/vol) CH_3COOH, with a gradient from 30 to 32% CH_3CN over the first 40 min, then to 40% in 20 min, and held at 40% CH_3CN for another 5 min. The flow rate was 1.0 mL/min, and column temperature was set at 35 °C. The DAD detector was monitored at 252 nm, and the on-line UV spectra were recorded in the range of 190 to 400 nm.

Mass Spectrometry

A Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was connected to the Agilent 1100 HPLC instrument via an ESI interface. The LC effluent was introduced into the ESI source in a post-column splitting ratio of 2:1. Ultrahigh-purity helium (He) was used as the collision gas and high-purity nitrogen (N_2) as the nebulizing gas. The MS detector was optimized by injecting a 5 μL/min flow of triterpenoid standards (0.1 mg/mL in methanol) to obtain maximum intensities of $[M - H]^-$ ions. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.0 kV; sheath gas (N_2), 40 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 270 °C; capillary voltage, −30 V; tube lens offset voltage, −25 V. For full scan MS analysis, the spectra were recorded in the range of *m/z* 80 to 1200. A data-dependent acquisition was set so that the two most abundant ions in full scan MS would trigger tandem mass spectrometry (MS^n, n = 2 to 4). The collision energy for MS^n was adjusted to 41% in LC/MS analysis, and the isolation width of precursor ions was 3.0 *m/z*.

Results and Discussion

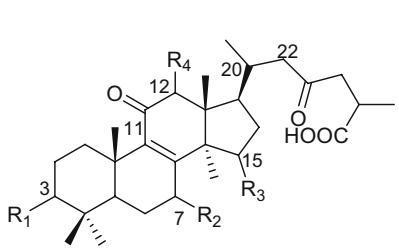
ESI-MS^n Analysis of Pure Standards

ESI-MS spectra in both negative and positive modes were examined in this study. Negative mode ESI was found to be sensitive for triterpenic acids. Pure compounds (0.1 mg/mL in MeOH) were injected into the ESI source by continuous infusion. All triterpenoids gave $[M - H]^-$ and $[2M - H]^-$ ions in their negative ion mass spectra. The $[M - H]^-$ were selected for CID fragmentation to produce MS/MS spectra. The prominent MS/MS ions were selected for further MS^n analysis (n = 3 to 4). The collision energy ranged from 30 to 50%.

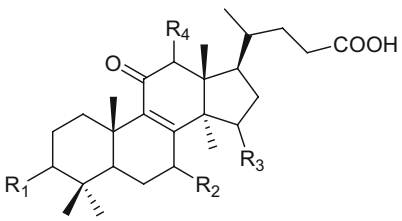
The compounds could be classified into five groups according to their chemical structures and fragmentation patterns. The dominant fragmentation pathways of the compounds studied are losses of H_2O and CO_2; however, the cleavages of C-ring and D-ring, which produce **a**, **b**, **c**, **d**, **e** ions etc. (Figure 1), are characteristic features. The major fragmentation pathways are given in Scheme 1.

Fragmentation of Ganoderic Acid A and Ganoderic Acid C_2 (Type I)

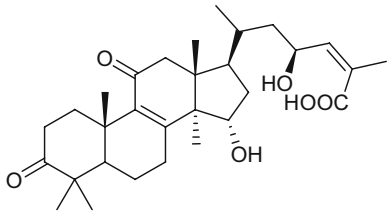
When applied to an collision energy of 40%, the $[M - H]^-$ ion at *m/z* 515 of ganoderic acid A (Figure 2a)



A



B



C

No.	Compound name	Type	R_1	R_2	R_3	R_4	Double bond	MW
1	12-hydroxyganoderic acid C_2	A	β-OH	β-OH	α-OH	OH	–	534
2	Ganoderic acid C_2	A	β-OH	β-OH	α-OH	H	–	518
3	Ganolucidic acid B	A	β-OH	H	α-OH	H	–	502
4	Lucidenic acid N	B	β-OH	β-OH	=O	H	–	460
5	Ganoderic acid C_6	A	β-OH	=O	=O	β-OH	–	530
6	3?-hydroxy-4,4,14-trimethyl-7,11,15-trioxochol-8-en-24-oic acid	B	β-OH	=O	=O	H	–	458
7	Ganoderic acid G	A	β-OH	β-OH	=O	β-OH	–	532
8	Ganoderenic acid B	A	β-OH	β-OH	=O	H	$\Delta_{20,22}$	514
9	Ganoderic acid B	A	β-OH	β-OH	=O	H	–	516
10	Lucidenic acid E	B	β-OH	=O	=O	β-OAc	–	516
11	Ganoderic acid AM_1	A	β-OH	=O	=O	H	–	514
12	Ganoderenic acid K	A	β-OH	β-OH	=O	β-OAc	$\Delta_{20,22}$	572
13	Ganoderic acid K	A	β-OH	β-OH	=O	β-OAc	–	574
14	7,15-dihydroxy-4,4,14-trimethyl-3,11-dioxochol-8-en-24-oic acid	B	=O	OH	OH	H	–	460
15	Elfvingic acid A	A	=O	=O	β-OH	α-OH	$\Delta_{20,22}$	528
16	Ganoderic acid A	A	=O	β-OH	α-OH	H	–	516
17	Ganoderic acid H	A	β-OH	=O	=O	β-OAc	–	572
18	12,15-bis(acetyloxy)-3-hydroxy-7,11,23-trioxo-lanost-8-en-26-oic acid	A	OH	=O	OAc	OAc	–	616
19	Ganolucidic acid A	A	=O	H	α-OH	H	–	500
20	12-hydroxy-3,7,11,15,23-pentaoxo-lanost-8-en-26-oic acid	A	=O	=O	=O	OH	–	528
21	Lucidenic acid A	B	=O	β-OH	=O	H	–	458
22	12-hydroxyganoderic acid D	A	=O	β-OH	=O	OH	–	530
23	Ganoderenic acid D	A	=O	β-OH	=O	H	$\Delta_{20,22}$	512
24	Lucidenic acid F	B	=O	=O	=O	H	–	456
25	Ganoderic acid D	A	=O	β-OH	=O	H	–	514
26	Lucidenic acid D	B	=O	=O	=O	β-OAc	–	514
27	Ganoderic acid F	A	=O	=O	=O	H	–	512
28	12-acetoxyganoderic acid D	A	=O	β-OH	=O	OAc	–	572
29	3-acetylganoderic acid H	A	β-OAc	=O	=O	β-OAc	–	614
30	Ganolucidic acid D	C	–	–	–	–	–	500
31	12-acetoxyganoderic acid F	A	=O	=O	=O	β-OAc	–	570
32	Ganoderic acid J	A	=O	=O	α-OH	H	–	514

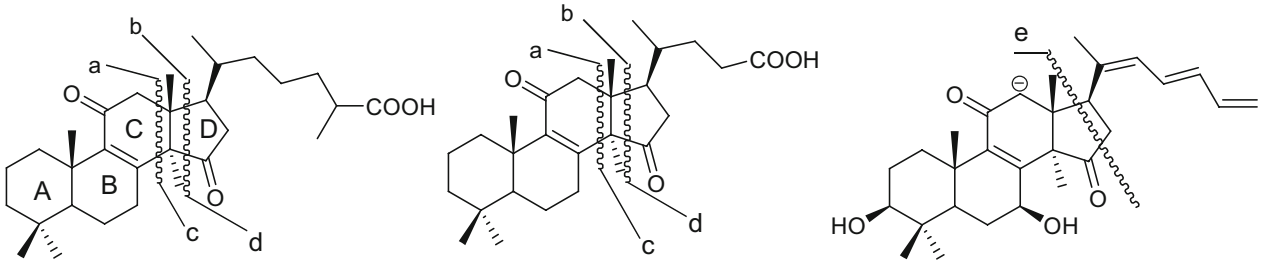


Figure 1. Chemical structures of the triterpenoids identified from *Ganoderma lucidum* and the characteristic fragmentation pathways of triterpenic acids.

Table 1. Identification of Triterpenic Acids from the Chloroform Extract of *Ganoderma glucidum*

No.	Retention time (min)	Assigned identity	UV λ_{max} (nm)	$[M-H]^-$ and $[2M-H]^-$ *m/z*	HPLC/ESI-MS^n *m/z* (% base peak)
1[b]	6.74	12-Hydroxyganoderic acid C_2	–	533	MS^2[533]: 515(100), 486(20) MS^3[533→515]: 497(7), 485(100), 467(21), 453(20), 423(10), 303(7) MS^4[533→515→485]: 467(100), 423(25), 405(11), 288(11)
2[a]	14.91	Ganoderic acid C_2	258	517,1035	MS^2[517]: 499(100), 438(14), 304(6) MS^3[517→499]: 481(30), 456(7), 437(100), 407(6), 302(15), 287(17) MS^4[517→499→437]: 437(80), 422(16), 407(100)
3	16.63	Ganolucidic acid B	–	501	MS^2[501]: 483(100), 458(6), 439(49), 421(7), 289(93), 287(31)
4	18.13	Lucidenic acid N	258	459, 919	MS^2[459]: 441(100), 423(30), 397(41), 385(52), 331(17), 303(10), 289(56), 288(11), 263(34), 260(11), 249(28), 195(16) MS^3[459→441]: 441(25), 423(100), 397(41), 263(21), 249(24) MS^4[459→441→423]: 408(100)
5	18.50	Ganoderic acid C_6	254	529,1059	MS^2[511]: 481(9), 467(100), 438(6) MS^3[511→467]: 467(33), 451(9), 449(12), 437(100), 424(22), 319(6), 303(19), 301(49) MS^4[511→467→437]: 437(100), 419(22)
6[a]	20.16	3β-hydroxy-4,4,14-trimethyl-7,11,15-trioxochol-8-en-24-oic acid	–	457, 915	MS^2[457]: 439(94), 421(82), 413(76), 397(100), 395(40), 385(52), 383(16), 382(27), 379(6), 338(10), 303(42), 287(9), 249(77), 163(46) MS^3[457→397]: 382(100)
7[a]	21.20	Ganoderic acid G	256	531,1063	MS^2[513]: 498(11), 469(100), 454(47), 452(33), 437(23), 304(10), 302(21), 266(48) MS^3[513→469]: 451(31), 290(13), 265(100)
8[a]	21.27	Ganoderenic acid B	248	513,1027	MS^2[495]: 480(19), 452(34), 451(100), 437(13), 331(12) MS^3[495→451]: 433(29), 407(6), 331(74), 315(9), 303(26), 287(31), 263(100), 249(18), 241(7) MS^4[495→451→263]: 247 (100)
9[a]	23.14	Ganoderic acid B	258	515,1031	MS^2[497]: 453(100), 439(8), 304(30), 288(6), 250(13) MS^3[497→453]: 435(9), 409(6), 287(16), 263(26), 249(100)
10	24.09	Lucidenic acid E	–	515,1031	MS^2[515]: 473(100) MS^3[515→473]: 458(10), 455(67), 443(100), 437(15), 429(6), 427(9), 413(13), 411(28), 394(12), 384(10), 370(7), 352(16), 330(23), 304(9), 289(17), 274(6) MS^4[515→473→443]: 443(19), 425(100), 400(6), 381(18), 326(13)
11[a]	24.47	Ganoderic acid AM_1	270	513,1027	MS^2[513]: 495(24), 451(100), 436(24) MS^3[513→451]: 436(100), 249 (1) MS^4[513→451→436]: 435(69), 421(100)
12[b]	26.03	Ganoderenic acid K	250	571	MS^2[553]: 538(37), 511(13), 494(8), 467(100), 450(8)
13[a]	27.38	Ganoderic acid K	256	573,1147	MS^2[555]: 513(22), 511(77), 496(29), 470(100), 452(43), 304(13), 303(18), 266(11) MS^3[555→469]: 469(8), 451(31), 290(53), 265(100) MS^4[555→469→265]: 237(100)
14[b]	28.36	7,15-dihydroxy-4,4,14-trimethyl-3,11-dioxochol-8-en–24-oic acid	–	459, 919	MS^2[459]: 441(86), 423(7), 415(26), 397(11), 300(69), 299(44), 285(100) MS^3[459→285]: 285(100), 269(47), 187(7)
15	29.70	Elfvingic acid A	256	527,1055	MS^2[509]: 479(8), 465(100), 435(6) MS^3[509→465]: 465(67), 435(100), 421(7), 317 (1), 301(13), 299(33) MS^4[509→465→435]: 435(100), 417(12)
16[a]	31.84	Ganoderic acid A	–	515,1031	MS^2[515]: 497(100), 480(9), 454(6), 436(19), 302(9), 301(6) MS^3[515→497]: 479(47), 435(100), 300(10), 299(8), 285(18) MS^4[515→497→435]: 435(100), 420(14), 405(82), 378(6), 365(10)

Table 1. Continued

No.	Retention time (min)	Assigned identity	UV λ_{max} (nm)	$[M-H]^-$ and $[2M-H]^-$ *m/z*	HPLC/ESI-MS^n *m/z* (% base peak)
17[a]	31.91	Ganoderic acid H	–	571	MS^2[553]: 515(29), 512(100), 510(22), 468(22) MS^3[553→511]: 481(9), 467(100) MS^4[553→511→467]: 467(37), 437(100), 423(8), 301(43)
18[b]	33.55	12,15-bis(acetyloxy)-3-hydroxy-7,11,23-trioxo-lanost-8-en-26-oic acid	–	615	MS^2[597]: 553(100), 511(21) MS^3[597→553]: 523(7), 511(67), 509(100), 493(66), 467(25), 449(23) MS^4[597→553→509]: 479(9), 467(95), 449(100)
19	34.61	Ganolucidic acid A	220,258	499, 999	MS^2[499]: 481(100), 455(7), 437(77), 287(57), 285(88), 233(15) MS^3[499→481]: 437(100), 285(59)
20	36.75	12-hydroxy-3,7,11,15,23-pentaoxo-lanost-8-en-26-oic acid	–	527	MS^2[509]: 465(100) MS^3[509→465]: 465(56), 435(100), 317 (1), 301 (17), 299(40) MS^4[509→465→435]: 435(100)
21	39.12	Lucidenic acid A	256	457, 915	MS^2[457]: 442(14), 439(100), 421(25), 395(43), 383(24), 329(7), 323(6), 287(56), 285(12), 261(34), 258(13), 247(7), 209(14), 193(15), 149(15) MS^3[457→439]: 424(59), 421(71), 323(36), 287(28), 261(100)
22	39.77	12-hydroxyganoderic acid D	256	529,1059	MS^2[511]: 496(24), 493(24), 467(100), 452(69), 449(70), 434(30), 317(10), 301(14), 300(6), 299(20), 263(69) MS^3[511→467]: 449(22), 288(51), 263(100)
23	42.92	Ganoderenic acid D	246	511,1023	MS^2[493]: 479(8), 478(25), 450(34), 449(100), 435(18), 329(10), 286(7) MS^3[493→449]: 431(8), 405(27), 335(7), 329(53), 301(15), 285(100), 283(9), 261(99), 247(12), 172(9), 149(11)
24	45.92	Lucidenic acid F	250	455, 911	MS^2[455]: 437(84), 425(14), 419(56), 411(53), 395(100), 393(22), 383(35), 380(39), 377(10), 339(9), 301(55), 247(52), 245(36), 209(12), 207(7), 163(32), 149(9) MS^3[455→395]: 380(100)
25[a]	47.20	Ganoderic acid D	256	513,1027	MS^2[495]: 451(100), 437(15), 301(31), 286(10), 284(11), 247(11) MS^3[495→451]: 436(6), 433(11), 407(28), 285(56), 261(10), 247(100), 149(68) MS^4[495→451→247]: 149(100)
26	49.94	Lucidenic acid D	252	513,1027	MS^2[513]: 471(100) MS^3[513→471]: 456(14), 453(57), 441(100), 438(7), 435(12), 428(8), 411(7), 409(20), 391(10), 349(9), 328(23), 301(8), 300(10), 287(16), 272(9) MS^4[513→471→441]: 441(21), 423(100), 397(10), 379(19), 340(20), 324(13)
27	51.50	Ganoderic acid F	260	511,1023	MS^2[511]: 493(39), 449(100), 435(17) MS^3[511→449]: 434(100), 431(7) MS^4[511→449→434]: 433(100), 419(97)
28[b]	52.60	12-acetoxyganoderic acid D	256	571,1143	MS^2[553]: 538(6), 511(51), 509(77), 494(46), 476(10), 467(100), 450(75), 302(16), 301(21), 263(12) MS^3[553→467]: 468(19), 449(33), 407(8), 288(56), 263(100)
29[b]	53.06	3-acetylganoderic acid H	–	613	MS^2[595]: 553(100), 552(21), 510(17) MS^3[595→553]: 523(8), 509(100) MS^4[595→553→509]: 509(64), 479(100), 465(14), 449(52), 345(13), 343(41)
30	54.47	Ganolucidic acid D	258	499, 999	MS^2[499]: 481(60), 437(100), 287(15), 285(45) MS^3[499→437]: 287(6), 285(100) MS^4[499→437→285]: 285(100), 269(66), 213(8), 187(8)
31	57.68	12-acetoxyganoderic acid F	256	569,1139	MS^2[551]: 509(100), 508(14), 466(15) MS^3[551→509]: 479(10), 465(100), 436(6) MS^4[551→509→465]: 465(47), 449(12), 447(12), 435(100), 422(9), 317 (1), 301(13), 299(38)

Table 1. Continued

No.	Retention time (min)	Assigned identity	UV λ_{max} (nm)	$[M-H]^-$ and $[2M-H]^-$ *m/z*	HPLC/ESI-MS^n *m/z* (% base peak)
32[a]	59.26	Ganoderic acid J	272	513,1027	MS^2[513]: 451(100), 437(28), 422(21), 301 (1), 285 (1) MS^3[513→451]: 451(8), 436(74), 433(7), 421(100) MS^4[513→451→421]: 421(55), 403(100), 380(13), 305(6)

–UV absorption maximum unavailable because of low intensities or overlapped
[a]Structures confirmed by comparison with reference standards.
[b]Reported for the first time.

produced a prominent ion at *m/z* 497 by eliminating a molecule of H_2O (Δm = 18). The *m/z* 497 ion was further subjected to MS^3 analysis to produce signals at *m/z* 479 and 435, formed by the sequential losses of H_2O and carbon dioxide (CO_2, Δm = 44) through a rearrangement process at the side chain, as suggested in Scheme 1. The *m/z* ion 435 then underwent successive losses of CH_3 (Δm = 15) to generate an ion at *m/z* 405. The above product ions were the dominant fragments of ganoderic acid A; moreover, some minor signals at *m/z* 285 (**b** – 16), 301 (**b**), 300 (**b** – 1), and 299 (**b – 2**) were also observed in MS^n (*n* = 2 to 3) spectra, especially in the MS^3 spectrum (Figure 3). We assumed that the fragmentation should result from cleavage of the D-ring (Figure 1), and this provided characteristic information for compounds with skeletons such as that of ganoderic acid A. The fragmentation pathway is depicted in Scheme 1.

Ganoderic acid C_2 gave similar ESI-MS^n (*n* = 1 to 4) spectra to those of ganoderic acid A, except that the corresponding ions were heavier by 2 Da, which resulted from the difference of the group at C-3. Hence, it could be concluded that different groups at C-3 do not change the main fragmentation pathway of the compounds investigated.

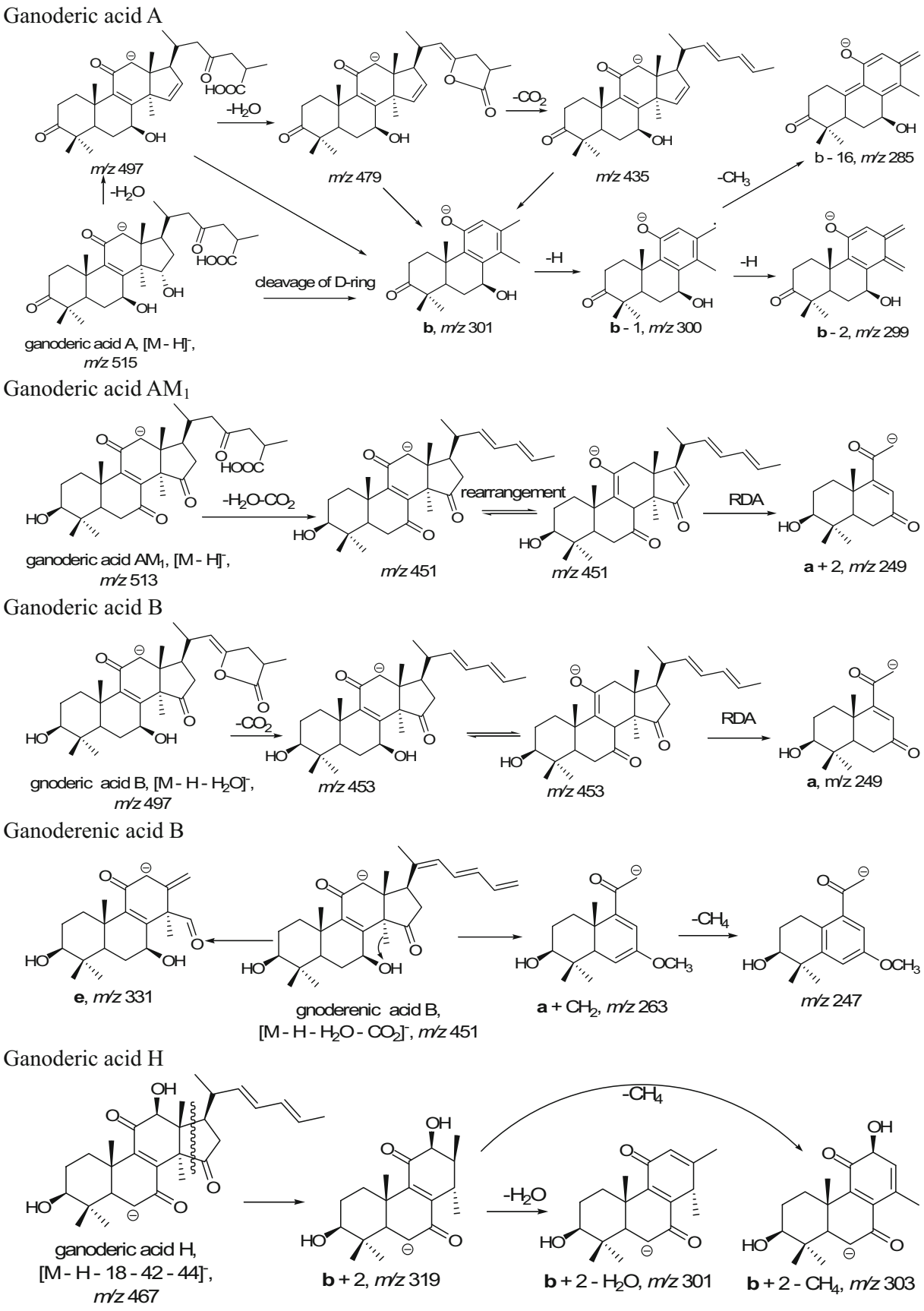
Fragmentation of Ganoderic Acid AM_1 and Ganoderic Acid J (Type II)

Ganoderic acid AM_1 gave the $[M - H]^-$ ion at *m/z* 513, which produced ions at *m/z* 495 and 451 in the MS/MS spectrum, originating from neutral loss of H_2O and CO_2 similar to the results of ganoderic acid A; but ions involving cleavage of the rings were hardly observed; the very minor **a** + 2 ion at *m/z* 249 indicated a characteristic rearrangement in the process of cleavage of the rings for the structures with 7-carbonyl (see Scheme 1). MS^3 and MS^4 spectra of ganoderic acid AM_1 yielded abundant product ions at *m/z* 436 and 421 respectively, through loss of CH_3 (Δm = 15).

The fragmentation behavior of ganoderic acid AM_1 could be applied to ganoderic acid J. The $[M - H]^-$ ion at *m/z* 513 gave a prominent product ion at *m/z* 451; the MS^3 spectrum of the *m/z* 451 ion was somewhat different from that of ganoderic acid AM_1; it gave two abundant ions at *m/z* 436 and 421 (Figure 4). We suggested that this might be due to the different substitute at C-15. In such a case, loss of a molecule of H_2O could be observed in the MS^4 spectrum.

Fragmentation of Ganoderic Acid B, D, G, K, and Ganoderenic Acid B (Type III)

The fragmentation behavior of ganoderic acid B were very different from those of compounds of types I and II. In its full scan MS spectrum, the prominent signal was not the $[M - H]^-$ ion at *m/z* 515 but the $[M - H - H_2O]^-$ ion at *m/z* 497 (Figure 2b), resulting from a



Scheme 1. Major fragmentation pathways of some triterpenoids.

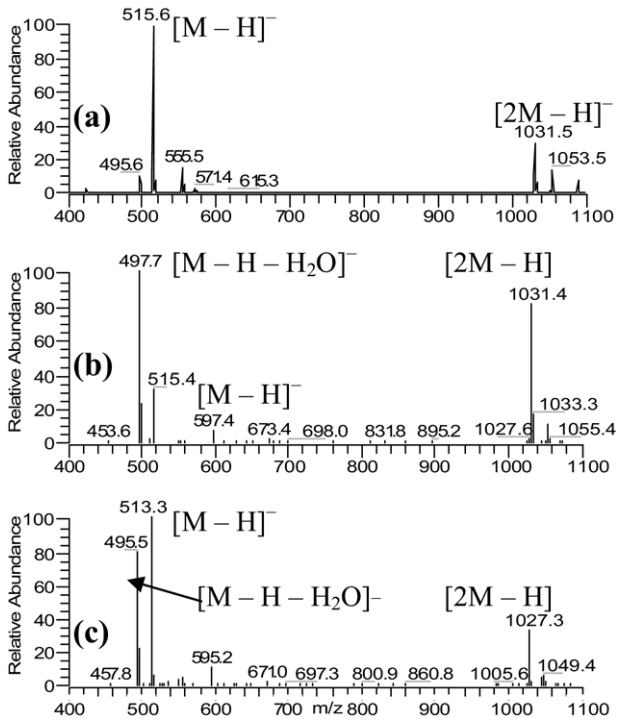


Figure 2. Full scan MS spectra for ganoderic acid A (**a**), ganoderic acid B (**b**), and ganoderenic acid B (**c**).

rearrangement process at the side chain, as suggested in Scheme 1. We assumed that this was the characteristic fragmentation feature for structures with 15-hydroxy-7-oxo groups. The *m/z* 497 ion was then subjected to MS/MS analysis and produced a prominent ion at *m/z* 453, formed by loss of a molecule of CO_2 (Δm = 44). Figure°4d°shows°the°MS^3°spectrum°of°the°*m/z* 453°ion; the most prominent product ion was at *m/z* 249 (**a**), followed by the **a** + CH_2 ion at *m/z* 263 along with **b** – H – CH_3 ion at *m/z* 287, corresponding to rearrangement and cleavage of the C- and D-rings, respectively (see Scheme 1).

All issues discussed above for ganoderic acid B could apply here for other compounds of type III. Ganoderic acid D had almost identical ESI-MS^n (*n* = 1 to 4) spectra with those of ganoderic acid B, except that the corresponding ions were lighter by two mass units, resulting from the different substitution at C-3. Ganoderic acid G was a monohydroxylated derivative at C-12 of ganoderic acid B and, thus, all its corresponding ions (**a** ion at *m/z* 265, **b** ion at *m/z* 319) were heavier by 16 Da than those of ganoderic acid B. Ganoderic acid K was a acetylated derivative at C-12 of ganoderic acid G and its $[M – H – H_2O]^-$ ion at *m/z* 555 yielded an ion in the MS/MS spectrum at *m/z* 513, originating from a neutral loss of 42 Da (CH_2 = CO). We assumed that this reaction involved the elimination of the acetyl at C-12. The obtained ion then showed the same fragmentation patterns as those of ganoderic acid G. Some characteristic°signals°were°shown°in°Table°2.

The mass spectrum of ganoderenic acid B was somewhat different from that of ganoderic acid B. Its full scan MS spectrum gave both $[M – H]^-$ ion (*m/z* 513) and $[M – H – H_2O]^-$ ion (*m/z* 495) in abundant signals (Figure 2c).°We°suggested°that°this°character°could°be applied°for°all°other°ganoderenic°acids.°The°$[M – H – H_2O]^-$ ion at *m/z* 495 showed similar fragmentation patterns°to°those°of°ganoderic°acid°B,°but°the°abundances of some ions were very different. The **a** + CH_2 ion (*m/z* 263) was a base peak in the MS^3 spectrum, followed°by°**e** ion°at°*m/z* 331°(see°Figure°1°and°Scheme 1), along with **b** – H – CH_3 ion at *m/z* 287; the **a** ion at *m/z* 249 was less abundant on the contrary to that of ganoderic acid B.

Fragmentation of Ganoderic Acid H (Type IV)

Ganoderic acid H was an acetoxylated derivative at C-12 of ganoderic acid AM_1, but its fragmentation behavior was somewhat different from those of ganoderic acid AM_1. The most prominent ion in its full scan mass spectrum was the $[M – H – H_2O]^-$ ion at *m/z* 553 like that of ganoderic acid K. Neutral losses of CH_2 = CO (Δm = 42, *m/z* 553→511), and CO_2 (Δm = 44, *m/z* 511→467) were observed in the MS^2 and MS^3 spectra, respectively. The obtained *m/z* 467 ion then gave abundant signals at *m/z* 301 and 303 in the MS^4 spectrum along with the base peak at *m/z* 437. We assumed that the two ions should be **b** + 2 – H_2O and **b** + 2 – H – CH_3 ions°(Figure°5c),°involving°the°transfer°of°two°hydrogen atoms from part **d** to part **b** in the process of cleavage of the D-ring (Scheme 1); this could be the characteristic feature for structures with 12-hydroxy-7-oxo groups.

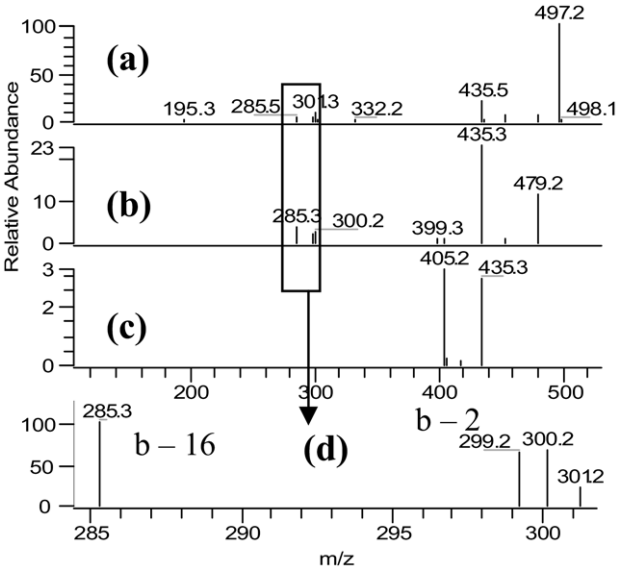


Figure 3. ESI-MS spectra for ganoderic acid A: (**a**) MS/MS spectrum of $[M – H]^-$ ion at *m/z* 515 ; (**b**) MS^3 spectrum of *m/z* 497 (515→497); (**c**) MS^4 spectrum of *m/z* 435 (515→497→435); (**d**) the box zoomed in for **b**, **b** – 1, **b** – 2 and, **b** – 16 ions.

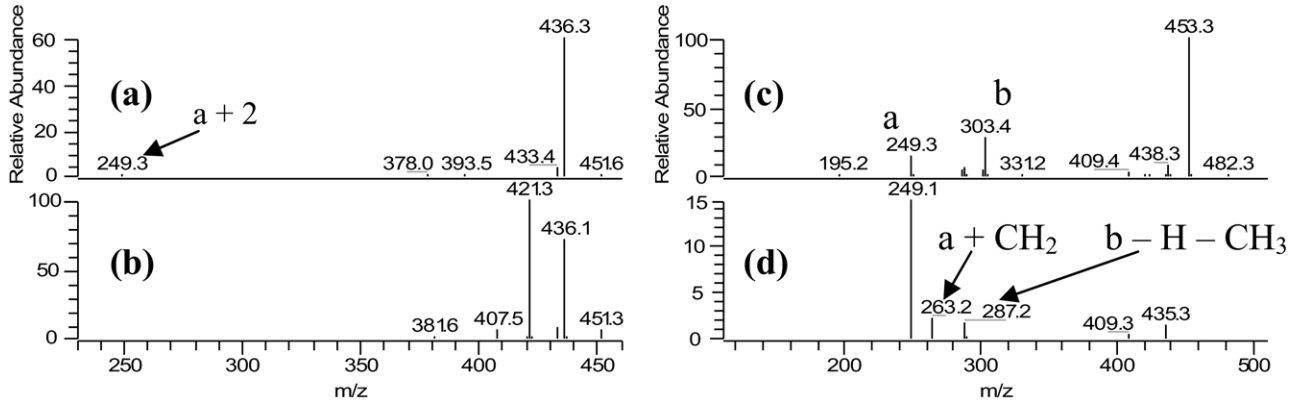


Figure 4. ESI-MS spectra for ganoderic acids: MS^3 spectra for ion at *m/z* 451 of ganoderic acid AM_1 (**a**) and ganoderic acid J (**b**); MS/MS spectrum at *m/z* 497 (**c**) and MS^3 spectrum at *m/z* 453 (**d**) of ganoderic acid B.

Fragmentation of 3β-Hydroxy-4, 4, 14-Trimethyl-7, 11, 15-Trioxochol-8-en-24-oic Acid (Type V)

The structure of this type of compound has a skeleton of lanostane containing 27 carbon atoms and is called lucidenic acid. Part of the structure is similar to that of ganoderic acid except the side chain at C-17. Thus, some characteristic fragmentation features of ganoderic acid could be applied for lucidenic acid.

Figure°5d°shows°the°MS/MS°spectrum°of°the°[M°–H]$^-$ ion at *m/z* 457 for 3β-hydroxy-4, 4, 14-trimethyl-7,

Table 2. Characteristic ESI-MS spectral information for Type III triterpenoids

	Full scan (*m/z*, %)						
No.	$[M - H]^-$	$[M - H - H_2O]^-$	a	a + 14	b	b – 16	b – 29
7	531 (7)	513 (100)	265		319	303	290
8	513 (100)	495 (80)	249	263	303	287	
9	515 (25)	497 (100)	249	263	303	287	
12	571 (86)	553 (100)	265			303	290
13	573 (15)	555 (100)	265			303	290
22	529 (7)	511 (100)	263		317	301	288
23	511 (80)	493 (100)	247	261	301	285	
25	513 (20)	495 (100)	247	261	301	285	
28	571 (1)	553 (100)	263			301	288

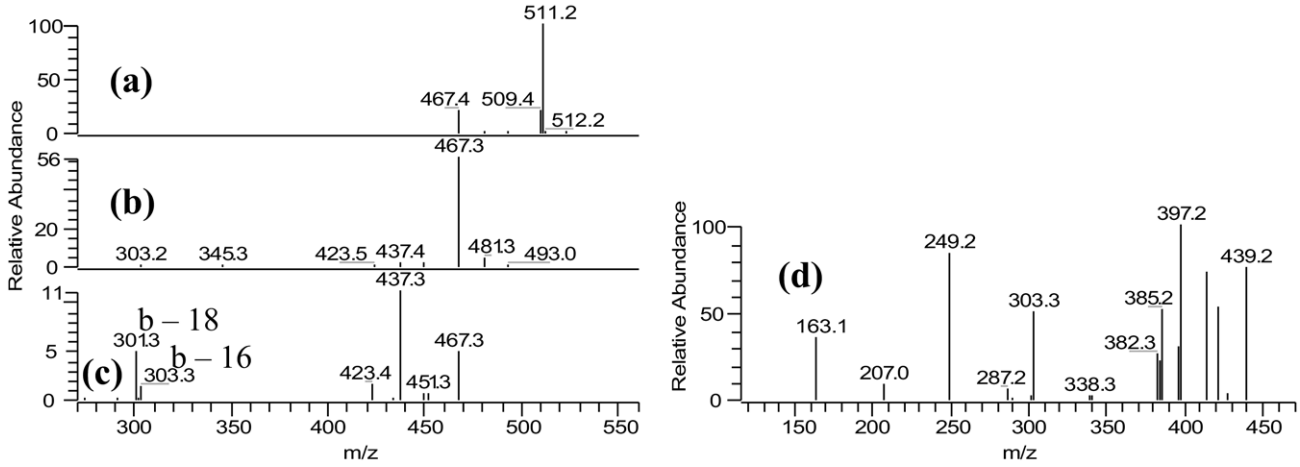


Figure 5. ESI-MS spectra for ganoderic acid H: (**a**) MS/MS spectrum of $[M - H - H_2O]^-$ ion at *m/z* 553; (**b**) MS^3 spectrum of *m/z* 511 (553→511); (**c**) MS^4 spectrum of *m/z* 467 (553→511→467); (**d**) MS/MS spectrum of $[M - H]^-$ ion at *m/z* 457 for 3β-hydroxy-4, 4, 14-trimethyl-7, 11, 15-trioxochol-8-en-24-oic acid.

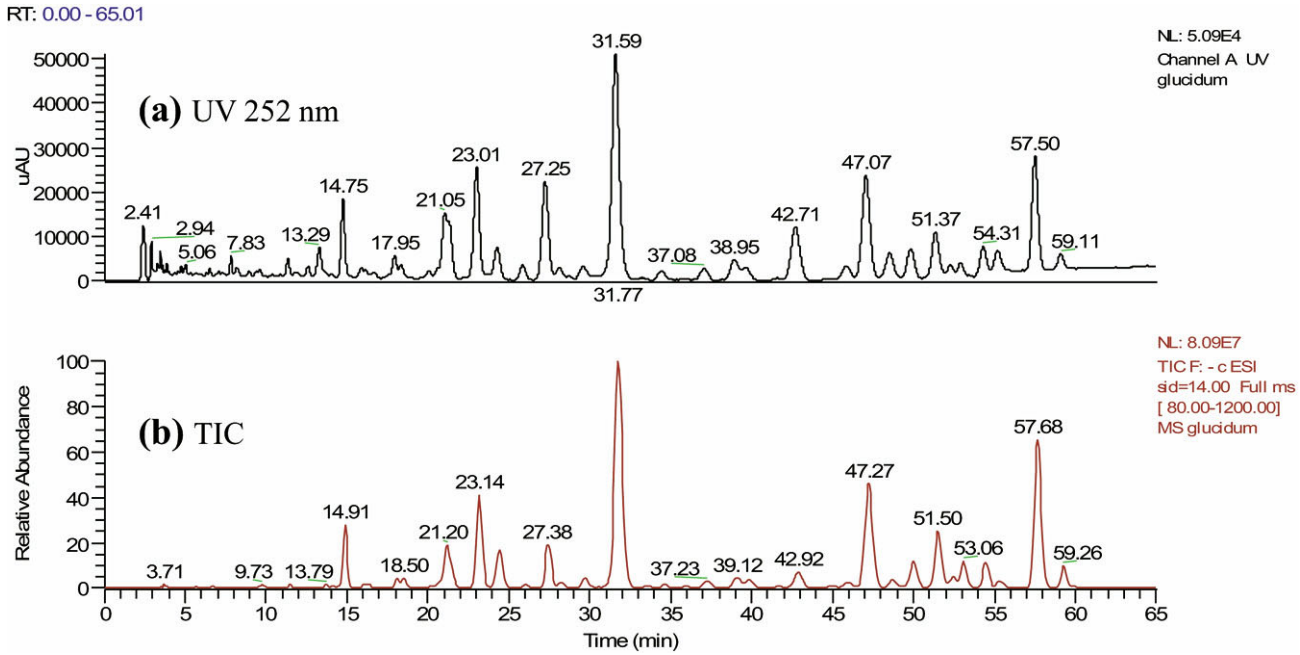


Figure 6. HPLC-DAD-ESI-MS^n analysis of the $CHCl_3$ extract of *Ganoderma lucidum*. (**a**) HPLC-UV chromatogram monitored at 252 nm. (**b**) LC-negative ion ESI-MS total ion current (TIC) profile.

11, 15-trioxochol-8-en-24-oic acid. The most prominent ion was *m/z* 397, originating from loss of a molecule of acetic acid (CH_3COOH, $\Delta m = 60$) through the β-cleavage of the carboxy group. In addition, $[M - H - H_2O]^-$ (*m/z* 439), $[M - H - 2H_2O]^-$ (*m/z* 421), and $[M - H - CO_2]^-$ (*m/z* 413) ions were also observed. The appearance of **a** + 2 (*m/z* 249) and **b** + 2 (*m/z* 303) ions demonstrated characteristic fragmentation feature for structures with 7-carbonyl.

HPLC-DAD-ESI-MS^n Analysis of the Crude Extract of Ganoderma lucidum

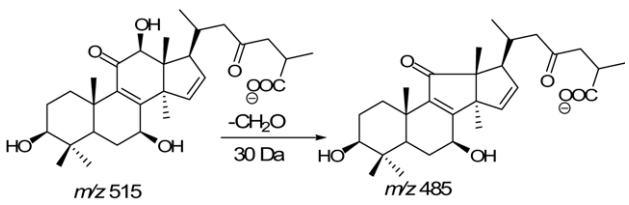
Figure 6 shows the HPLC-UV and TIC profiles of the extract of *Ganoderma lucidum*. To obtain optimal extraction efficiency and good separation, we optimized the extraction and chromatographic conditions [34]. Chloroform, methanol, and chloroform-methanol solutions were attempted as the extraction solvent. At last, chloroform was chosen as the extraction solvent since the triterpenoids could not only efficiently be extracted but also well resolved from background. Ultrasonic extraction was compared with refluxing. It was found that both extraction methods have the similar extraction efficiency but ultrasonic extraction was simpler, hence the ultrasonic bath extraction was chosen as the preferred method.

Different mobile phase compositions were optimized and acetic acid was added in the mobile phase to obtain desired separation and acceptable tailing factor. As a result, acetonitrile and water containing 0.2% acetic acid was chosen as the eluting solvent system. The UV detector was monitored at 252 nm to make sure that all triterpenoids gave good responses.

A total of 32 triterpenoids were characterized from the chloroform extract of *G. lucidum* (Table 1), and 11 of them were unambiguously identified by comparing their retention times and mass spectra with those of reference standards. For unknown peaks, the structures were tentatively established on the base of their ESI-MS and MS^n (n = 2 to 4) spectra, according to the general fragmentation rules of triterpenoids summarized above.

Identification of Type I Triterpenoids

In addition to ganoderic acid A (t_R = 31.84 min, **16**) and C_{2° (t_{R° = 14.91 min, **2**), another peak at 6.74 min (Table 1, Compound **1**) was plausibly identified. A group of signals at *m/z* 319 (**b**), 318 (**b** – 1), 317 (**b** – 2), and 303 (**b** – 16) demonstrated the structure of type I triterpenoid. The loss of 30 Da in the MS^3 spectrum was attributed to the elimination of a molecule of formaldehyde (HCHO) at C-12 (Scheme 2). Thus, Compound **1** was tentatively



Scheme 2. Proposed fragmentation mechanism for the neutral loss of 30 Da from ion at *m/z* 515 of Compound **1**.

Table 3. Characteristic ESI-MS Spectral Information for Type IV Triterpenoids

	Full scan (*m/z*, %)				
No.	$[M - H]^-$	$[M - H - H_2O]^-$	b	b – 16	b – 18
5	529 (11)	511 (100)	319	303	301
15	527 (91)	509 (100)	317	301	299
17	571 (17)	553 (100)	319	303	301
18	615 (15)	597 (100)	–	–	–
20	527 (8)	509 (100)	317	301	299
29	613 (56)	595 (100)	361	345	343
31	569 (33)	551 (100)	317	301	299

identified as 12-hydroxyganoderic acid C_2, which was reported for the first time.

Identification of Type II Triterpenoids

Ganoderic acid AM_1 (t_R = 24.47 min, **11**) and ganoderic acid J (t_R = 59.26 min, **32**) were identified by comparison with the standards. Compound **27** (t_R = 51.50 min) gave the $[M - H]^-$ ion at *m/z* 511, which gave almost identical MS^n (*n* = 2 to 4) spectra to those of ganoderic acid AM_1, except that the corresponding ions were lighter by two mass units. Thus, it is presumed to be a 3-oxo derivative, and was assigned as ganoderic acid F, just bearing a different substitute at C-3 compared with ganoderic acid AM_1.

Identification of Type III Triterpenoids

Ganoderic acid B (t_R = 23.14 min, **9**), D (t_R = 47.20 min, **25**), G (t_R = 21.20 min, **7**), K (t_R = 26.03 min, **12**), and ganoderenic acid B (t_R = 21.27 min, **8**) were identified by comparison with the standards. According to the data shown in Table 2, Compounds **12** (t_R = 26.03 min), **22** (t_R = 39.77 min), **23** (t_R = 42.92 min), and **28** (t_R = 52.60 min) could also be easily characterized. It was clear that Compounds **12** and **23** were ganoderenic acids (20, 22-double-bond) because their $[M - H]^-$ ions had similar abundance (>80%) to that of $[M - H - H_2O]^-$ ions. Thus, Compound **12** must be a C-20, 22 unsaturated derivative of Compound **13** (ganoderic acid K), and hence assigned as ganoderenic acid K, which is reported for the first time. Similarly, Compound **23** was tentatively characterized as ganoderenic acid D.

Compounds **22** and **28** showed very similar $ESI-MS^n$ spectra to those of Compounds **7** and **13**, respectively, except that the according ions were lighter by 2 Da, indicating the different substitution at C-3. Therefore, Compound **22** was assigned as 12-hydroxyganoderic acid D, and Compound **28** was tentatively identified as 12-acetoxyganoderic acid D.

Identification of Type IV Triterpenoids

Table 3 shows the characteristic ions of type IV triterpenoids characterized from extract of *G. lucidum*. These constituents have the same structural fragment bearing a hydroxyl (acetoxy) at C-12 and a keto group at C-7. Compound **17** (t_R = 31.91 min) was unambiguously identified as ganoderic acid H by comparison with the standard. Obviously, the peak at 18.50 min (**5**) gave identical **b**, **b** – 16, and **b** – 18 ions to those of ganoderic acid H. Thus, it was assumed to be a deacetylated derivative and assigned as the known compound ganoderic acid C_6.

Compound **31** (t_R = 57.68 min) should be an acetylated derivative of Compound **20** (t_R = 36.75 min). Their fragmentation behaviors were very similar to those of Compounds **17** and **5**, respectively, except that the corresponding ions were lighter by 2 Da. We assumed that they might be the derivatives of Compound **17** and **5** by oxidizing the secondary alcohol at C-3. Thus, Compound **20** was assigned as 12-hydroxy-3, 7, 11, 15, 23-pentaoxo-lanost-8-en-26-oic acid, and Compound **31** was tentatively characterized as 12-acetoxyganoderic acid F. Similarly, Compound **29** was plausibly characterized as the new compound named 3-acetylganoderic acid H.

The peak at 29.70 min (**15**) was an isomer of **20** and showed the characteristics of ganoderenic acid ($[M - H]^-$, *m/z* 527 (91%); $[M - H - H_2O]^-$, *m/z* 509 (100%)), indicating one of the carbonyl was hydrogenized when compared with **20**. The same **b**, **b** – 16, and **b** – 18 ions demonstrated identical structure of A-, B-, and C-rings to those of **20**; therefore it was suggested that the carbonyl at D-ring should be hydrogenized. Thus, Compound **15** was tentatively identified as elfvingic acid A, a known compound isolated from *Elfvingia applanata* [35].

The ESI-MS spectra of Compound **18** (t_R = 33.55 min) were somewhat different from other type IV triterpenoids (Table 3). But there was no doubt that the skeleton was still similar and there were two acetoxys in the structure; one was presumably attached at C-12. Since no signals for **a** or **b** ion were observed, another acetoxyl group should not be attached at C-3 but C-15. Hence, the peak was tentatively characterized as 12, 15-bis(acetoxy)-3-hydroxyl-7, 11, 23-trioxo-lanost-8-en-26-oic acid, which is a new compound.

Identification of Type V Triterpenoids

Compounds of this group have a C_{27} lanostane skeleton. The peak at 20.16 min (**6**) was identified as 3β-hydroxy-4, 4, 14-trimethyl-7, 11, 15-trioxochol-8-en-24-oic acid by comparison with the standard. The ESI-MS spectrum of Compound **24** (t_R = 45.92 min) gave $[M - H]^-$ ion at *m/z* 455, which was subjected to MS/MS analysis and showed very similar fragmentation pathway to that of **6** (Table 4), and the corresponding ions were lighter by two mass units. It was presumed to be an oxidized derivative of **6** at C-3 and, hence, assigned as lucidenic acid F.

The peak at 39.12 min (**21**) was an isomer of **6**. The ESI-MS/MS spectrum gave abundant **a** (*m/z* 247), **a** + 14

Table 4. Characteristic ESI-MS Spectral Information for Type V Triterpenoids

No.	$[M - H - H_2O]^-$	a	a + 14	b	b – 14
4	441 (100)	249	263	303	289
21	439 (100)	247	261	301	287
No.	$[M - H - 60]^-$	a + 2		b + 2	
6	397 (100)	249		303	
24	395 (100)	247		301	
No.	$[M - H - 42 - 30]^-$	b + 2 – HCHO		b + 2 – CH_4	e – OH
10	443 (100)	289		303	330
26	441 (100)	287		301	328
No.	$[M - H - H_2O]^-$	b	b – 1	b – 2	b – 16
14	441	301	300	299	285

(*m/z* 261), **b** (*m/z* 301), and **b** – 14 (*m/z* 287) ions, which were somewhat similar to those of type III triterpenoids discussed above. We assumed that there should be a hydroxyl at C-7 and the C-15 should be a carbonyl, just like those of ganoderic acid D. Thus, Compound **21** was tentatively characterized as lucidenic acid A. Similarly, Compound **4** (t_R = 18.13 min) was assigned as lucidenic acid N.

Compounds **10** (t_R = 24.09 min) and **26** (t_R = 48.72 min) were another couple of derivates, which just possessed the different substitutes at C-3. Therefore, they showed very similar fragmentation behaviors. The $[M - H]^-$ ion at *m/z* 513 of **26** produced the prominent ion at *m/z* 471 in the MS/MS spectrum. The obtained ion was subjected to MS^3 fragmentation, in which abundant ions at *m/z* 287 (**b** + 2 – HCHO), 301 (**b** + 2 – CH_4), and 328 (**e** – 17) were observed along with the base peak at *m/z* 441 ($[M - H - 42 - HCHO]^-$). The listed ions on **b** + 2 indicated that C-7 of **26** was oxidized to be a carbonyl. Moreover, according to the $[M - H]^-$ ion, **26** might be an acetoxylated derivative of lucidenic acid F (**24**). Thus, Compound **26** was tentatively identified as lucidenic acid D. Similarly, Compound **10** was plausibly characterized as lucidenic acid E.

Compound **14** (t_R = 24.09 min) was an isomer of lucidenic acid N (**4**). Its ESI-MS/MS spectrum gave a list of abundant ions at *m/z* 301 (**b**), 300 (**b** – 1), 299 (**b** – 2), and 285 (**b** – 16), which were obviously the characteristic ions of ganoderic acid A. Thus, there should be hydroxyls attached at C-7 and C-15. Hence, Compound **14** was tentatively identified as 7, 15- dihydroxy-4, 4, 14-trimethyl-3, 11-dioxochol-8-en-24-oic acid, a compound reported for the first time.

Identification of Other Compounds

Compounds **3** (t_R = 16.63 min), **19** (t_R = 34.61 min), and **29** (t_R = 54.47 min) gave very similar ESI-MS/MS spectra. The $[M - H]^-$ ion of Compound **3** was at *m/z* 501, which was 16 Da lighter than that of ganoderic acid C_2 (Table 5). The base peak in its MS/MS spectrum was $[M - H - H_2O]^-$ ion (*m/z* 483), and the $[M - H - H_2O - CO_2]^-$ ion (*m/z* 439) was abundant. Thus, this compound appeared to be a dehydroxylated derivative of ganoderic acid C_2. The known compound ganolucidic acid B could match this information; therefore, its structure was plausibly characterized and the ions at *m/z* 287 and 289 were presumed to be **b** and **b** + 2, respectively. Similarly, Compound **19** was tentatively characterized as ganolucidic acid A.

Compound **29** was an isomer of **19**. $[M - H - H_2O]^-$ (*m/z* 481), $[M - H - H_2O - CO_2]^-$ (*m/z* 437), **b** (*m/z* 285), and **b** + 2 (*m/z* 287) ions were observed in its MS/MS spectrum. Interestingly, $[M - H - H_2O - CO_2]^-$ ion was the base peak. We assumed it might be ganolucidic acid D, which had a different side chain at C-17 compared with ganolucidic acid A.

Conclusions

In this study, the fragmentation behavior of triterpenoids, which shared the same core triterpene structure as C_{30} or C_{27} lanostane, in electrospray ion trap mass spectrometer was studied. The predominant fragmentation pathways were losses of H_2O and CO_2, but their characteristic behaviors were cleavage of C- and D-rings. Different substitution at C-7, 12, 15 resulted in different fragmentation and the double-bond at C-20, 22 would also affect the behaviors of mass spectra.

Using HPLC-DAD-ESI-MS and MS^n (n = 2 to 4), we have demonstrated the assignment of 26 known triterpenoids along with six previously unidentified compounds of *Ganoderma lucidum*. For analysis of triterpenoids in crude extract, we recommended the initial application of negative ion ESI-MS to obtain the molecular mass information for the components via the $[M - H]^-$ ions, and to determine the structural information by acquiring MS^n spectra. The observed $[2M - H]^-$ ions were found very useful for peak confirmation. Construction of a library containing MS^n spectra for known triterpenoids would greatly facilitate the identification

Table 5. Characteristic ESI-MS Spectral Information for Compounds 3, 19, and 30

No.	$[M - H - H_2O]^-$	$[M - H - H_2O - CO_2]^-$	b	b + 2
3	483 (100)	439 (49)	287	289
19	481 (100)	437 (77)	285	287
30	481 (60)	437 (100)	285	287

of these compounds in real samples and would permit even more complete fingerprinting of triterpenoids that arise from species differences of different *Ganoderma* extracts.

Acknowledgments

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