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Liquid chromatographic characterization of the triterpenoid patterns in *Ganoderma lucidum* and related species

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ABSTRACT

Reversed-phase high-performance liquid chromatography was applied to the determination of the triterpenoids compositions in the Chinese medicinal fungus *Ganoderma lucidum* (Fr.) Karst and several taxonomically related species. Twenty-five well-characterized reference triterpenoids were selected for compositional comparison. It was found that characterization based on triterpenoid profile mapping was compatible with the morphological classification. The very unique oxygenated triterpenoids, which up to now have only been identified in the genus *Genoderma*, provided supporting evidence for classification. The triterpenoid profiles also gave characteristic information for distinguishing this medicinal fungus from other taxonomically related species. The method was also applicable to screening of specific triterpenoids with particular biological activities.

INTRODUCTION

Many secondary metabolites, such as terpenoids, flavonoids and phenolic natural products, have been used as marker compounds for the characterization and classification of taxonomically related fungi and higher plants [1,2]. These metabolites can provide useful information for chemical taxonomy provided that their occurrence and compositions are characteristic of a single, unique genus or are restricted to only a few closely related species. Common natural products or secondary metabolites which are greatly subjected to seasonal variations or subtle changes in culture conditions are not suitable for this purpose. In addition, the determination of these marker metabolites is essential for elucidating their compositions and abundance. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are nowadays the most commonly employed methods for this purpose [3,4].

Ganoderma lucidum (Fr.) Karst (Polyporaceae) and related species are fungi used in traditional Chinese medicine [5]. Recent studies on this fungus have demonstrated many interesting biological activities, inlcuding antitumour, anti-inflammatory and cytotoxicity to hepatoma cells [6,7]. Inhibitory activities of histamine release, platelet aggregation, cholesterol biosynthesis and angiotensin-converting enzyme (ACE) have also been reported [8–10]. Accumulated evidence supported the suggestion that these biological activities are mainly associated with oxygenated triterpenoids [11–21].

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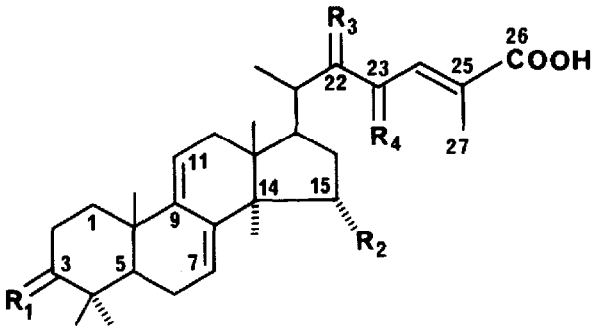
Particularly interesting from the taxonomical point of view is the natural occurrence of over 100 species of oxygenated triterpenoids, which have been identified recently from the culture mycelia and fruiting bodies of *G. lucidum*. These triterpenoids have several structural features of particular note. Their common lanostanoid skeleton contains highly oxygenated functionalities, namely hydroxyl, acetoxyl and oxo groups at C-3, C-15, C-22, C-23, and many other positions [12–16]. The C-26 carbon is also functionalized to the hydroxyl or carboxyl status in many triterpenoids of *G. lucidum* (Fig. 1). Most of these oxygenated triterpenoids have not been reported in other genera of Polyporaceae, although extensive screening has not yet been carried out for detailed comparison. To pave the way for the later application of these triterpenoids in the chemical taxonomy of the genus *Ganoderma*, the structural determination of many triterpenoids in this fungus has been accomplished recently. Screening of better producing strains for those biologically active triterpenoids is also desirable in pharmacological studies.

Both these two aspects rely heavily on the determination of these triterpenoids by chromatographic methods. We have previously elucidated the chromatographic behaviour of these oxygenated triterpenoids using silica gel thin-layer chromatography and normal-phase HPLC [22]. As multiple and different polar functional groups can occur, the volatilities of these triterpenoids are low. GC is less practical for direct measurement. It is also worth mentioning that many pairs of stereo- and positional isomers can appear simultaneously in the culture mycelia and fruiting bodies of *G. lucidum*. We have determined the contributions to molecular polarity of the hydroxy (OH) and acetoxy (OAc) substituents at the C-3 and C-15 positions in governing the retention behaviour (capacity factors) by using reversed-phase HPLC [23]. The trend of their weighing factors is as follows: 3β-OH > 3α-OH, 15α-OH > 3α-OAc > 3β-OAc. The chromatographic behaviour of these oxygenated triterpenoids allowed us to apply a binary solvent system of acetonitrile and water with gradient elution for optimum resolution of many pairs of stereo- and positional isomers [23]. The aim of this study was to explore the applicability of triterpenoid patterns, based on reversed-phase HPLC, to the chemical taxonomy of the fungus *G. lucidum*. The determination and comparison of production yields of several desired triterpenoids in the genus *Ganoderma* were also tested. The triterpenoid profiles of *G. lucidum* and several taxonomically related species are reported.

EXPERIMENTAL

Cultures of Ganoderma and related species

Two strains of *G. lucidum* (ATCC 32471 and 32472) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Strains CCRC 36111, 36114, 36143, 36144, 36110, 36125 and 36021 were obtained from the Culture Collection and Research Centre (CCRC), Food Industry Research and Development Institute (Hsinchu, Taiwain). Strains TP-1 and G.l.-chen were collected locally. Strains ATCC 64251 and CJ-3 were obtained from the Institute of Botany, Academia Sinica (Taiwan). All strains were maintained on potato–dextrose–agar slants. For mycelial culture, fungi were inoculated in 1-l culture flasks containing 300 ml of medium consisting of 30 g of dextrose, 20 g of malt extract, and 1 g of peptone per litre of distilled water at pH 6.5. Each strain was inoculated into three culture flasks and stationary cultures were maintained at 28 ± 1.5°C for 30 days [12,24].



	R_1	R_2	R_3	R_4
1	α-OAc	OAc	H_2	H_2
2	β-OAc	OAc	H_2	H_2
3	α-OH	OAc	H_2	H_2
4	α-OAc	OH	H_2	H_2
5	β-OH	OAc	H_2	H_2
6	β-OAc	OH	H_2	H_2
7	=O	OAc	H_2	H_2
8	α-OH	OH	H_2	H_2
9	β-OH	OH	H_2	H_2
10	α-OAc	OH	β-OAc	H_2
11	β-OH	OAc	β-OAc	H_2
12	α-OAc	OAc	β-OAc	H_2
13	β-OAc	OAc	β-OAc	H_2
14	α-OH	OAc	H_2	O
15	α-OAc	OAc	H_2	O
16	α-OAc	OH	H_2	O
17	α-OAc	OH	α-OH	H_2
18	α-OAc	OAc	α-OH	H_2
19	β-OAc	OAc	α-OH	H_2
20	β-OH	OH	β-OAc	H_2
21	α-OH	OH	β-OAc	H_2
22	β-OH	OH	β-OH	H_2
23	α-OH	OH	α-OH	H_2
24	α-OH	H	H_2	H_2
25	α-OAc	H	H_2	H_2

Fig. 1. Structures of marker triterpenoids (**1**–**25**) isolated from *Ganoderma lucidum* (strains ATCC 32471 and TP-1).

General procedure for the isolation of oxygenated triterpenoids

Mycelia were harvested by filtration through four layers of cheesecloth and were gently washed with water. The dry weight was obtained by heating the mycelia at 45°C in darkness for 48 h. About 1 g of mycelia from each sample was precisely weighed, ground into powder and extracted three times with 15 ml of methanol. The pooled extracts were concentrated on a rotary evaporator. Each concentrated preparation was suspended in 1.5 ml of methanol and passed through glass-wool in a disposable pipette to remove insoluble matter. To remove lipophilic metabolites, the filtrate was passed slowly through a Sep-Pak cartridge column [Waters Assoc. (Milford, MA, U.S.A.), C_{18} reversed phase]. Columns was eluted with acetonitrile and 5-ml volumes of eluates were collected. The solvents were evaporated to dryness at 45°C with a stream of nitrogen in darkness. The concentrated residues were weighed and dissolved in 1 ml of acetonitrile for the determination by HPLC.

Determination of triterpenoids by reversed-phase HPLC

A Model 1084B solvent-delivery system (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a Rheodyne Model 7125 sample injector, a Model 440 variable-wavelength UV detector (Waters Assoc.) and a Model 79850B integrator (Hewlett-Packard) was used in the HPLC analysis. Separation was performed on a prepacked reversed-phase column (Cosmosil 5 C_{18}, 5 μm, 25 × 0.46 cm I.D.; Nacalai Tesque, Kyoto, Japan). Multi-step linear gradient elution with a binary solvent system of acetonitrile and water was employed [23]. To achieve better resolution in the separation of triterpenoids containing a C-26 carboxyl group, a constant concentration of acetic acid (0.5%, v/v) was used to suppress the ionization. These oxygenated triterpenoids all contain a transoid diene skeleton and showed very strong and almost identical UV absorption at 235 nm ($\log \varepsilon = 4.14$), 243 nm ($\log \varepsilon = 4.16$) and 251 nm ($\log \varepsilon = 3.97$) in methanol [12]. Routinely, post-column UV detection at 243 nm was used. Quantification was based on the peak areas of the corresponding triterpenoids. At least ten reference triterpenoids (compounds **1–10** in Table I) were used to construct the calibration graphs.

Identification of oxygenated triterpenoids

Identification of triterpenoids in the HPLC profiles was based on their co-migration with the authentic triterpenoid standards. The reference standards were purified from *G. lucidum* (strains ATCC 32471 and TP-1). Their structures were completely identified by the spectroscopic methods reported previously [12–16]. To confirm the identity of these oxygenated triterpenoids produced in species which were not *G. lucidum*, several representative peaks in HPLC were also collected for UV and mass spectrometric identification.

RESULTS AND DISCUSSION

The compositional study of oxygenated triterpenoids in *G. lucidum* and related species was conducted in a comparable manner. We cultured these fungi under identical conditions for 30 days. Based on a previous study of strains TP-1 and ATCC 32471, this culture period was long enough to reach the stationary phase as judged by the mycelial biomass [24]. Under these conditions, the concentrations of the two most

abundant triterpenoid metabolites, namely lanosta-7,9(11),24-trien-3α,15α-diacetoxy-26-oic acid (compound **1** in Fig. 1) and lanosta-7,9(11),24-trien-3β,15α-diacetoxy-26-oic acid (compound **2**), which are epimers at C-3 in pairs, reached their plateau on day 28. The triterpenoid patterns remained unchanged in the reference strains (ATCC 32471 and TP-1) at least until day 50 [12]. In the culture of *G. lucidum*, the yellowish to brownish yellow pigments appeared between days 14 and 20, which was also the vigorous production period of these two major oxygenated triterpenoids. Ergosterol and other lipophilic metabolites were also produced in significant amounts by these fungi.

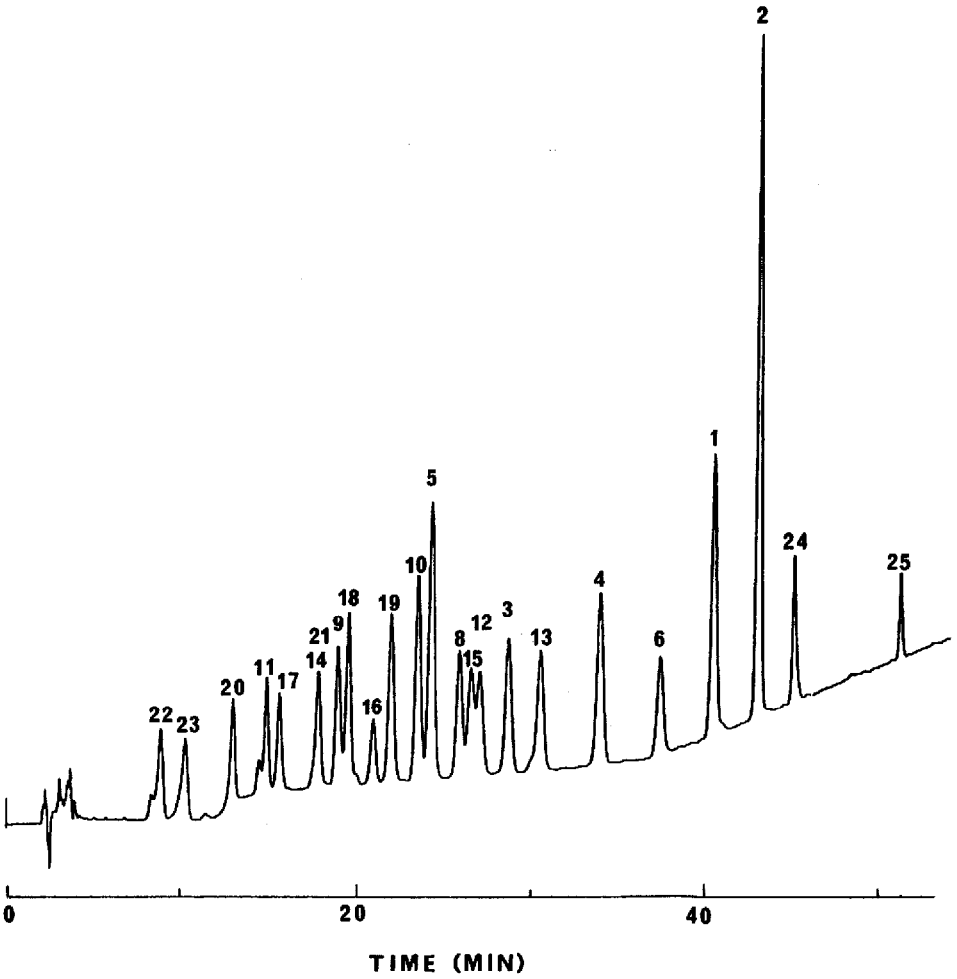


Fig. 2. Reversed-phase HPLC trace of reference triterpenoids (compounds **1–6** and **8–25**). Column, Cosmosil 5 C_{18}, 25 × 0.46 cm I.D. Eluent A, methanol–acetic acid (100:0.5, v/v); eluent B, methanol–water–acetic acid (80:20:0.5, v/v/v); flow-rate, 1.0 ml/min; UV detection at 243 nm. Gradient elution was started with 80% methanol, increased linearly to 84% in 15 min, to 86% in a further 15 min, to 88% in 10 min, to 94% in a further 10 min and finally to 100% in 20 min.

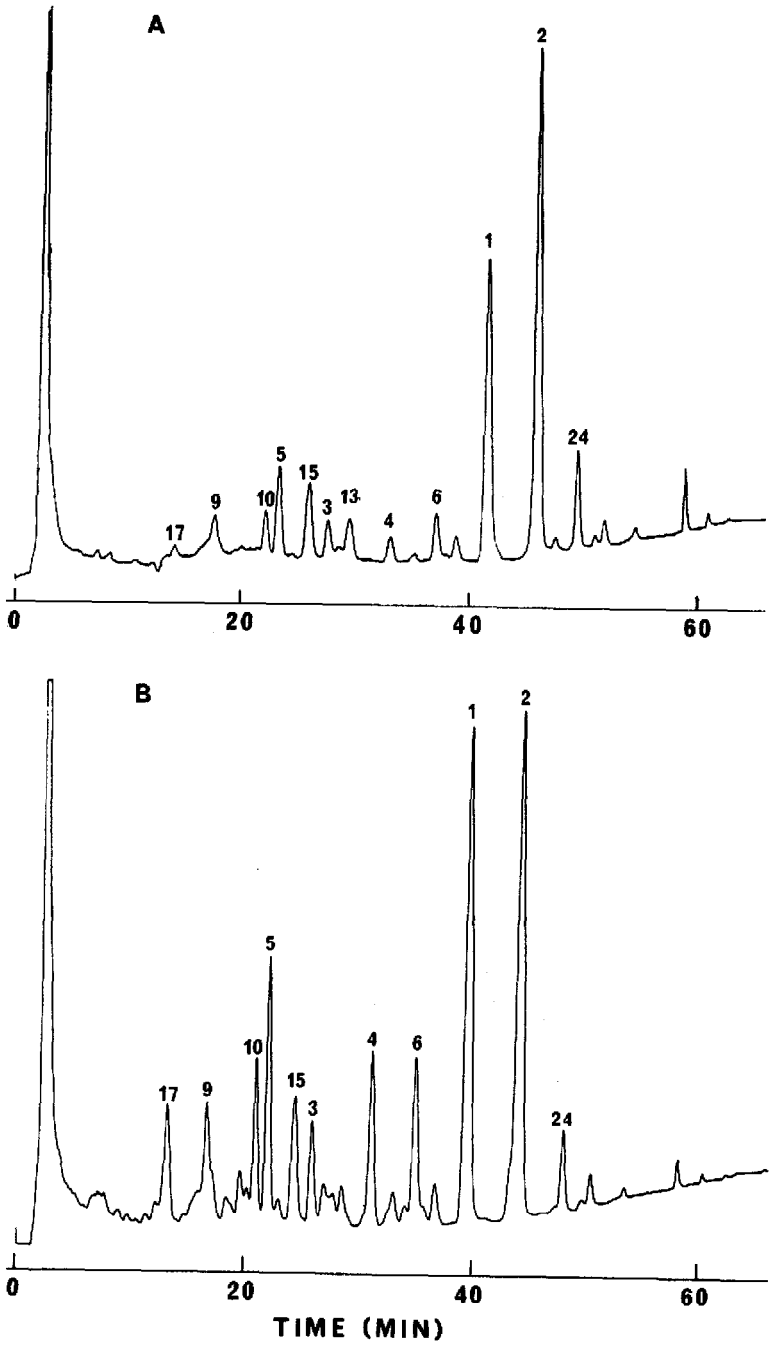


Fig. 3. Reversed-phase HPLC of triterpenoids of *G. lucidum*. (A) strain TP-1; (B) CCRC 36021. Conditions as in Fig. 2.

TABLE I

RELATIVE MOLAR PERCENTAGES OF MAJOR TRITERPENOIDS IN *GANODERMA* SPECIES AS DETERMINED BY HPLC

Strain	Triterpenoid[a] (mol%)[b]											
	17	**9**	**10**	**5**	**15**	**3**	**13**	**4**	**6**	**1**	**2**	**24**
TP-1	1.7	3.1	2.8	6.2	5.6	2.8	3.8	1.6	3.3	23.8	40.7	4.8
CCRC 36021	5.7	4.5	6.1	9.8	6.1	3.8	ND[c]	5.3	4.0	25.2	26.1	3.3
ATCC 64251	3.4	7.0	4.5	2.4	19.0	6.8	15.0	7.6	ND	18.6	3.3	12.4
CCRC 36111	10.1	6.0	9.1	0.7	5.3	5.7	3.9	28.0	ND	15.5	3.6	11.8
CCRC 36144	2.2	2.3	2.4	0.6	1.0	ND	4.1	31.8	ND	3.1	12.6	40.1
CCRC 36143	13.2	8.7	25.9	ND	20.7	ND	7.7	9.0	3.1	6.0	4.0	1.7
G.l.-chen	5.3	8.0	13.3	ND	35.3	ND	35.2	0.6	ND	1.5	0.4	0.5

[a] For structures, see Fig. 1.

[b] The total number of moles of these twelve triterpenoids was assigned a value of 100%. Results are mean values of three HPLC determinations.

[c] ND, not detectable.

The methanolic extracts of mycelia were passed through reversed-phase cartridge columns to reduce the interferences. This clean-up procedure was tested to ensure quantitative recoveries of the oxygenated triterpenoids listed in Fig. 1, which are all more polar than ergosterol (data not shown). Twenty-five triterpenoids were isolated from two reference strains of *G. lucidum* for this study (Fig. 1). Compound **7** is a minor metabolite and was therefore not used in the HPLC analysis.

The resolution of these triterpenoids by reversed-phase HPLC and their elution sequence are illustrated in Fig. 2. The triterpenoid profiles of these two reference strains of *G. lucidum* (TP-1 and ATCC 32471) were well characterized previously in our laboratory [23]. Their profiles were very similar to each other. It was found that many strains of *G. lucidum*, exemplified by CCRC 36021, gave triterpenoid patterns very close to those of TP-1 and ATCC 32471 (Fig. 3). The profiles of both CCRC 36021 and TP-1 showed that compounds **1** and **2** were predominant (Fig. 1). More than twelve major triterpenoid components in strians TP-1 and ATCC 32471 also appeared in strain CCRC 36021 (Table I). Strain CCRC 36021 was recently also confirmed to be *G. lucidum* by detailed morphological study at CCRC (data not shown). This fingerprint mapping of triterpenoid patterns determined by reversed-phase HPLC was in agreement with the morphological characterization.

The triterpenoid profiles were also determined in several taxonomically related species. For example, strain CCRC 36114 was a species of *Ganoderma*. This strain did not show the typical ovate double-walled basidiospores of *G. lucidum*. We found that strain CCRC 36114 also gave a completely distinct HPLC profile. The triterpenoid patterns of strains CJ-3 and CCRC 36110 were almost identical with each other (data not shown). However, their patterns were also completely different from those of characterized strains of *G. lucidum* (TP-1 and ATCC 32471). The strain CJ-3 has been classified as *G. tsugae* by other investigators [25]. It was therefore suspected that strain CCRC 36110 was also a species of *G. tsugae*. However, more detailed morphological information is needed to clarify this point. Strain CCRC 36143 and strain

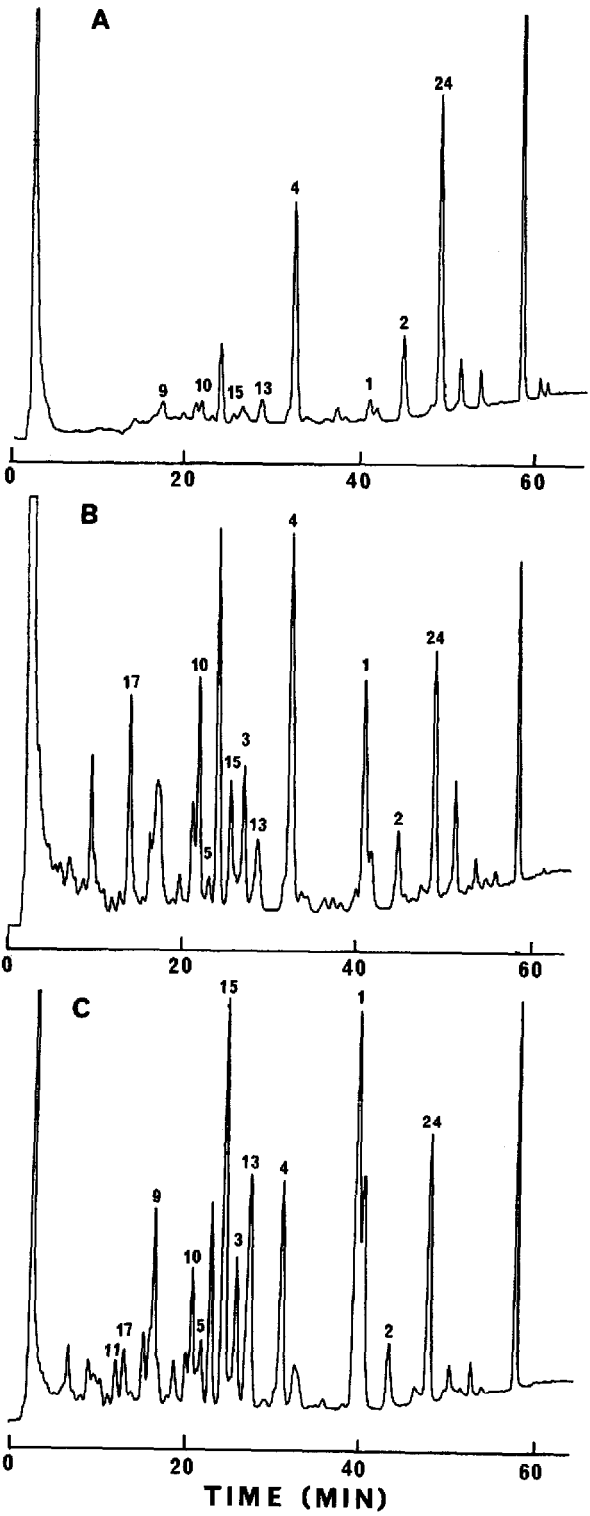


Fig. 4. Reversed-phase HPLC of triterpenoids of *Ganoderma* species. (A) CCRC 36144; (B) CCRC 36111; (C) ATCC 64251. Conditions as in Fig. 2.

G.l.-chen gave very similar triterpenoid patterns (Table I). These two strains also produced many common triterpenoids which also appeared in the reference strains of *G. lucidum* (TP-1 and ATCC-32471). The major difference between these two groups of *Ganoderma* species was that strains CCRC 36143 and G.l.-chen produced more polar triterpenoids listed in Fig. 1, which were eluted in front of triterpenoids **1** and **2** in reversed-phase HPLC. We confirmed that their major metabolites were compounds **10**, **13** and **15**. However, compounds **1** and **2** were still detectable in strains CCRC 36143 and G.l.-chen (Table I). The strains CCRC 36111 and 36144 were listed as *G. lucidum* by CCRC. They produced many triterpenoids which were common to those of reference strains ATCC 32471 and TP-1 (Fig. 4). However, their relative abundances were different (Table I). Interestingly, strain ATCC 64251, which was originally collected in Taiwan, also showed a triterpenoid pattern almost identical with that of CCRC 36111 (Fig. 4). The taxonomic classification of these strains remained to be clarified.

This study has illustrated the potential application of triterpenoid patterns in the chemical taxonomy of the genus *Ganoderma*. The information about triterpenoid patterns provided by HPLC and morphological characterization was generally in good agreement. This profile analysis should also be useful for the screening of particular triterpenoids with known biological activities. Both applications rely heavily on the precise determination of marker triterpenoids, which has been achieved.

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