Determination of stereo- and positional isomers of oxygenated triterpenoids by reversed phase high performance liquid chromatography

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Summary Twenty four oxygenated triterpenoids, including eight pairs of stereoisomers and five pairs of positional isomers, could be separated by reversed phase HPLC. The capacity factors obtained in methanol–water and acetonitrile–water solvent systems made it possible to correlate the molecular polarities due to the presence of multiple oxygenated functional groups in these compounds. It was found that the number and position of functional groups as well as the sterochemistry of these functional groups played important roles in governing the polarity of these lanostanoid acids. The polarity weighting factors were in the following order: 3β-OH > 3α-OH > 3α-OAc > 3β-OAc. The contribution to polarity due to 15α-OAc and 22β-OAc was probably very similar. The unique stereochemical character and eluting sequences of the lanostanoid acids provide information to generate empirical rules for predicting the role of individual polar functional groups in the chromatographic behavior in reversed phase HPLC.—**Shiao, M-S., L-J. Lin, and C-S. Chen.** Determination of stereo- and positional isomers of oxygenated triterpenoids by reversed phase high performance liquid chromatography. *J. Lipid Res.* 1989. **30:** 287–291.

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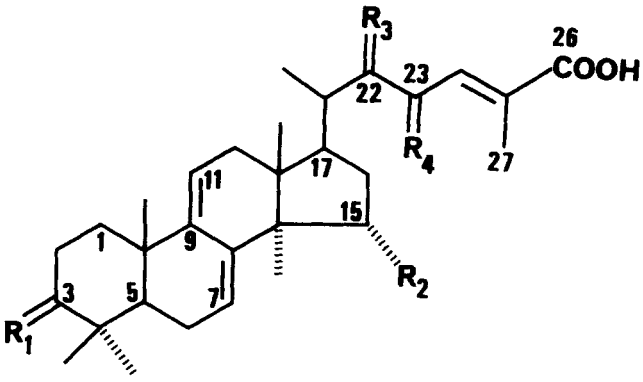
There is presently considerable interest in oxygenated triterpenoids from *Ganoderma lucidum*, a Polyporaceae used in traditional Chinese medicine (1–4). This fungus has been known to produce more than 100 species of oxygenated triterpenoids and sterols (2–3, 5–18) with various biological functions, such as cytotoxicity against hepatoma cells (5), antiinflammation and antihistamine activities (6). Several triterpenoids were also reported to be inhibitors of platelet aggregation and angiotensin converting enzyme systems (7). Structurally, the majority of these oxygenated triterpenoids belong to lanosta-7,9(11),24-trien-26-oic acid series with oxygenated functionalities at C-3, C-15, C-22, and C-23 positions (**Fig. 1**).

Most interestingly, many of these triterpenoids were identified as C-3 stereoisomers and C-3/C-15/C-22 positional isomers in pairs (14, 19, 20). Acetylating modifications of the C-3, C-15α, and C-22β hydroxy functional groups were also commonly observed (14, 15). In continuation of our search for hypolipidemic oxygenated sterols and triterpenoids from *G. lucidum* and related fungi, we have identified many pairs of stereo- and positional isomers containing multiple oxygenated functionalities. Complete separation of these fungal metabolites by traditional column chromatography and silica gel high performance TLC have been quite difficult (21). Their unique stereochemical character and rigid lanostanoid skeleton provide an excellent system to elucidate the role of individual polar functional groups on the eluting behavior in reversed phase HPLC. The present work was undertaken to generate empirical rules for the interpretation of their chromatographic behavior. The goal of this study was to delineate the role of oxygenated functionalities, -OH, -OAc, and -oxo, in governing the elution sequence of the lanostanoid acid-type positional isomers and stereoisomers in reversed phase HPLC.

EXPERIMENTAL PROCEDURES

Compounds 1–25 were obtained from the cultured mycelia of *G. lucidum* (strain TP-1, collected locally and also deposited at American Type Culture Collection as ATCC 64251). The isolation procedure and structure determination of these triterpenoids have been reported previously (19, 20). The methanolic extract of *G. lucidum* was prepared from freshly harvested mycelia of 30-day-old liquid cultures. The concentrated samples were passed through Sep-pak C_{18} cartridges (Waters Associates, Milford, MA) and subjected to reversed phase HPLC at ambient temperature.

Abbreviations: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.



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

Fig. 1. Structures of compounds 1-25 isolated from *G. lucidum*.

The HPLC system consisted of a Model 1084B (Hewlett-Packard, Avondale) or Model 590 (Waters) solvent delivery system equipped with a Model 7125 sample injector (Rheodyne, Berkeley, CA), an UVIDEC-100-V variable wavelength UV detector (Jasco, Tokyo, Japan), and a RC-250 recorder (Jasco). A prepacked Cosmosil $5C_{18}$ reversed phase column (5 μm; 25 × 0.46 cm I.D., Nakarai, Kyoto, Japan) was used for this investigation.

For determination of capacity factors of 1-6, 8-25, two isocratic solvent systems, namely 80% aqueous methanol (methanol-water-acetic acid 80:20:0.5, v/v/v) and 66% aqueous acetonitrile (acetonitrile-water-acetic acid 66:34:0.5, v/v/v) were used. In separation of at least 24 components isolated from *G. lucidum*, the gradient elution was started with 80% aqueous methanol and increased linearly to 84% methanol in 15 min. It then increased to 86% in another 15 min. In the next 10 min, the gradient was changed from 86% to 88% methanol. It was again increased to 94% and finally to 100% in another 10 and 20 min, respectively. Due to the presence of transoid conjugated diene skeleton in these oxygenated triterpenoids, they can be monitored by a UV detector at a wavelength of 243 nm.

RESULTS AND DISCUSSION

We initially used HPLC of C_{18} bonded stationary phase to correlate between polarity of these highly functionalized triterpenoids and capacity factors. The isocratic capacity factors of 24 triterpenoids with methanol-water-acetic acid 80:20:0.5 and acetonitrile-water-acetic acid 66:34:0.5 as mobile phases are listed in **Table 1**. The trend of elution sequence of these triterpenoids served as a measure to estimate qualitatively the contribution of the number of hydroxy and acetoxy groups as well as their configuration to the gross molecular polarity which affected the retention mechanism. Increase in number of hydroxy groups, such as in compounds 24, 8, 9, 22, and 23, reduced k' values drastically. It was also true for acetoxy groups but to a lesser extent. This was demonstrated by the eluting sequences of compounds 12 > 1 > 25[1] as well as 13 > 2. The 3β-hydroxy isomers were found to be more polar than the corresponding 3α isomers. This tendency was illustrated in the eluting order of the following pairs of diastereoisomers: 22 > 23; 9 > 8; 20 > 21; 5 > 3. By correlating positional isomerism and chromatographic behavior, we found that the hydroxy group at C-3 contributed more to the gross molecular polarity than the C-15 and C-22 hydroxy groups. The eluting sequences of at least five

[1]The notation a > b means that a is eluted before b.

TABLE 1. Capacity factors of compounds 1–6 and 8–25

Compound No.	$k'^{a,c}$	Compound No.	$k'^{b,c}$
22	2.8 (1.0)	22	1.5 (1.0)
23	3.1 (1.1)	23	1.9 (1.3)
20	4.3 (1.5)	20	3.7 (2.5)
11	5.8 (2.1)	17	4.2 (2.8)
17	6.9 (2.5)	9	5.6 (3.7)
14	8.3 (3.0)	21	6.2 (4.1)
21	8.3 (3.0)	11	6.8 (4.5)
9	8.6 (3.1)	14	8.7 (5.8)
18	9.7 (3.5)	18	9.4 (6.3)
19	12.3 (4.4)	16	9.6 (6.4)
16	14.2 (5.1)	8	10.6 (7.1)
10	14.5 (5.2)	19	11.5 (7.7)
5	15.2 (5.4)	5	13.8 (9.2)
15	15.4 (5.5)	10	14.1 (9.5)
8	15.9 (5.7)	3	18.4 (12.3)
12	17.9 (6.4)	15	22.6 (15.1)
3	21.0 (7.5)	4	23.8 (15.9)
13	22.8 (8.1)	12	28.6 (19.1)
4	29.5 (10.5)	6	29.7 (19.8)
6	37.3 (13.3)	13	36.1 (24.1)
1	49.1 (17.5)	1	48.1 (32.1)
2	62.8 (22.4)	2	55.4 (36.9)
24	63.6 (22.7)	24	62.8 (41.9)
25	141.1 (50.4)	25	147.3 (98.2)

[a]Capacity factors obtained in methanol–water–acetic acid 80:20:0.5 (v/v).
[b]Capacity factors obtained in acetonitrile–water–acetic acid 66:34:0.5 (v/v).
[c]Figures in parentheses indicate relative k' to the most polar compound 22.

pairs of positional isomers, namely, 14 > 16, 11 > 19, 18 > 10, 5 > 6, 3 > 4, confirmed this point of view. It is most interesting that upon acetylation of C-3 hydroxy groups, the resulting 3α-OAc-containing triterpenoids turned out to be more polar and were eluted faster than their 3β-OAc counterparts. This empirical rule was found to be valid whether additional C-15 and C-22 functionalities were present or not. We observed consistent eluting sequences in the C-3 acetoxy epimeric pairs such as 1 > 2; 12 > 13; 4 > 6; 18 > 19. We could empirically assign the polarity weighting factors to C-3 functional groups in the following order: 3β-OH > 3α-OH > 3α-OAc > 3β-OAc. Triterpenoids with more exposed polar groups had less affinity to the octadecylsilica stationary phase. The contribution to polarity due to 15α-OAc and 22β-OAc functional groups was probably of very similar magnitude. The capacity factors of these triterpenoids varied in a comparable way in the two solvent systems. The above mentioned empirical rules were valid in both methanol–water–acetic acid and acetonitrile–water–acetic acid solvent systems. They also guided the selection of gradient eluting conditions for separation of multiple pairs of isomers. Resolution of these multiple pairs of stereoisomers, which is almost impossible by normal phase high performance TLC (HPTLC), was readily achieved here by reversed phase HPLC. As demonstrated in **Fig. 2**, the separation of 24 triterpenoids could be achieved within 1 hr by gradient elution. Due to very close chromatographic behavior of 14 and 21 in 80% aqueous methanol, 23 peaks were observed. Compounds 14 and 21 were readily separated in acetonitrile–water–acetic acid 66:34:0.5 (v/v/v) with isocratic elution. It was found that the elution order of these triterpenoids in Fig. 2 was parallel to their corresponding capacity factors shown in column A of Table 1 in general, except for 19 and 16, and 15 and 8, owing to variation of the retention parameters of these compounds to different magnitude in different solvent systems.

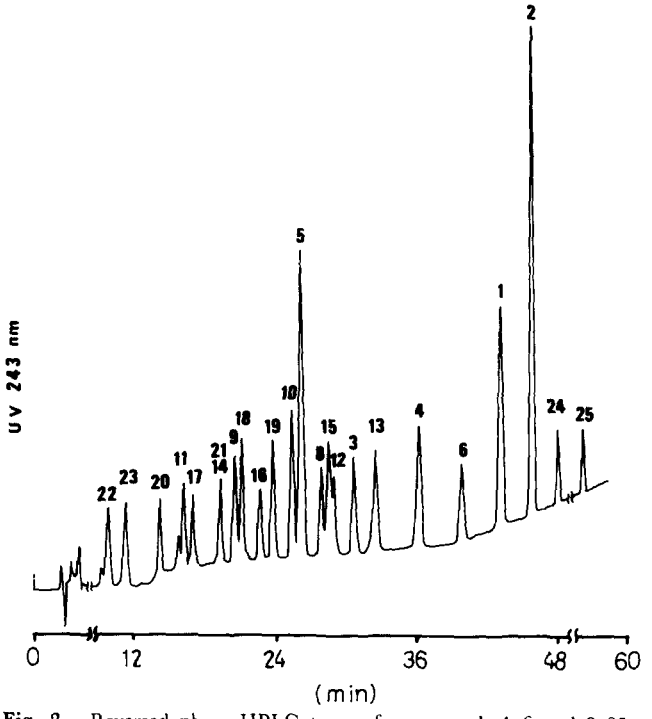


Fig. 2. Reversed phase HPLC trace of compounds 1–6 and 8–25. Eluent A: methanol–acetic acid 100:0.5 (v/v); eluent B: methanol–water–acetic acid 80:20:0.5 (v/v); flow rate, 1.0 ml/min; UV detection, 243 nm. The gradient profile was indicated in the Experimental section.

We also applied this HPLC method for quantitative analysis of these triterpenoids in liquid culture of *G. lucidum* (**Fig. 3**). The complete separation of three pairs of stereoisomers, namely, 18/19, 20/21, 22/23, is demonstrated in **Fig. 4** by isocratic elution in 66% aqueous acetonitrile. This illustrated the resolution of multiple pairs of stereoisomers by HPLC. It is therefore applicable to use the capacity factor to correlate the polarity of this series of compounds with their C-3 configuration in the reversed phase HPLC system.

The application of a dynamic chromatographic system, particularly reversed phase liquid chromatography, for the assessment of molecular hydrophobicity of structurally related compounds was recently extensively investigated (22-26). It was hindered by the lack of standardized methods for comparison and strong dependence of sample capacity factors on the particular chromatographic condition employed (27-29). We suggest that these paired stereoisomers and positional isomers, containing multiple functional groups, could act as reference compounds and could probably also serve as test compounds to evaluate the performance of commercially available reversed phase liquid chromatographic columns. This potential deserves further attention.

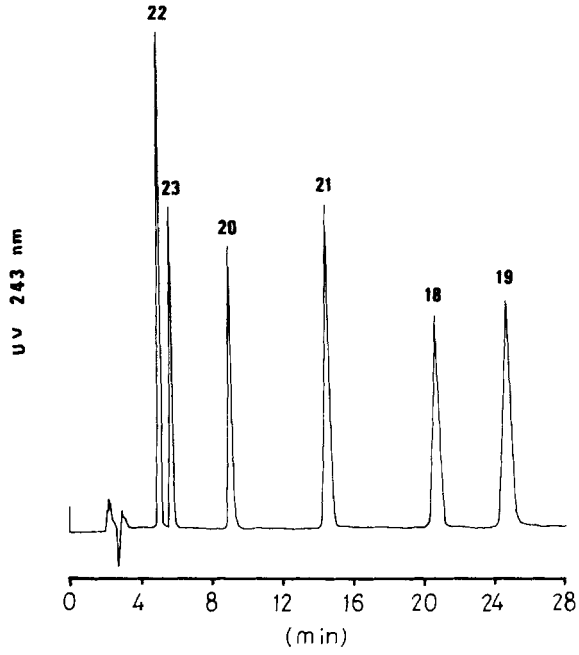


Fig. 4. Reversed phase HPLC trace of three pairs of C-3 epimers (compounds 18-23) in acetonitrile-water-acetic acid 66:34:0.5 (v/v) eluting system.

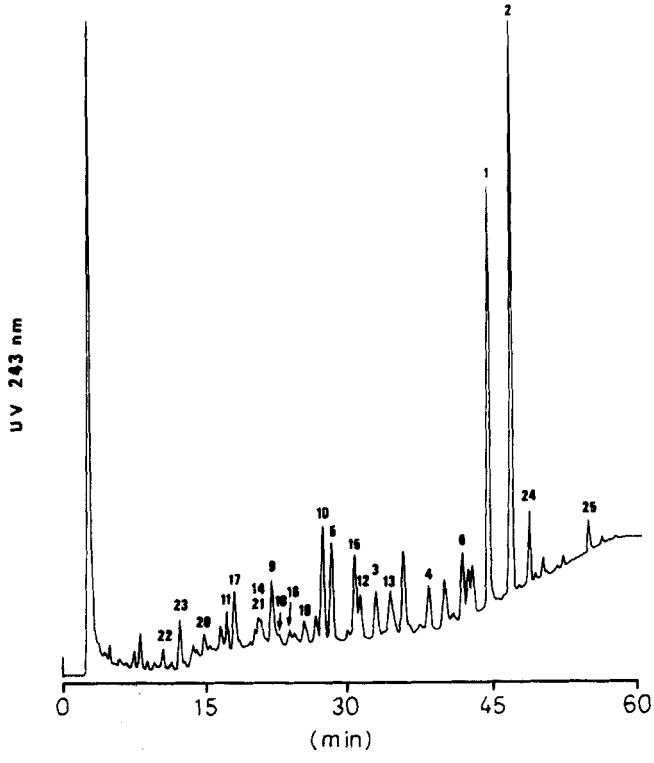


Fig. 3. Reversed phase HPLC trace of a methanolic extract of *G. lucidum* (strain TP-1). Conditions as in Fig. 2.

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