STUDIES ON THE INDEX COMPOUNDS FOR HPLC ANALYSIS OF *GLYCYRRHIZA URALENSIS*

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Abstract — HPLC of the AcOEt extracts of subterranean (roots or stolons) samples of *Glycyrrhiza uralensis* showed two types of the reproducible chromatograms distinct from those of other species (*G. glabra, G. pallidiflora, G. echinata, G. macedonica*). As index compounds for HPLC of *G. uralensis*, two new compounds, named glyurallins A and B, were isolated and their structures were defined, in addition to identification of nine compounds to glycycoumarin, semilicoisoflavone B, dehydroglyasperin C, licoisoflavanone, glycyrol, glycyrin, gancaonin N, licoricidine and 8-γ ,γ -dimethylallylwighteone.

Licorice, dried roots and stolons of *Glycyrrhiza uralensis, G. glabra* and some other species are an important source of traditional Chinese medicines, materials for glycyrrhizin preparation, and food and tobacco ingredients.[1]

We report in this paper that two new compounds, named glyurallins A and B were obtained along with nine known compounds from the AcOEt extracts of subterranean samples of *G. uralensis* and those of commercially available samples of Glycyrrhizae Radix. These 11 compounds were found

to be useful as HPLC indexes of this species. The AcOEt extracts of the subterranean parts (34 samples) of *G. uralensis* yielded two types of reproducible and characteristic chromatograms, which were distinct from those of four other analyzed *Glycyrrhiza* species (*G. glabra, G. echinata, G. macedonica, G. pallidiflora*) (Figure 1). The HPLC profiles showed 11 peaks caused by index compounds. Roots and stolons provided almost the same HPLC profiles.

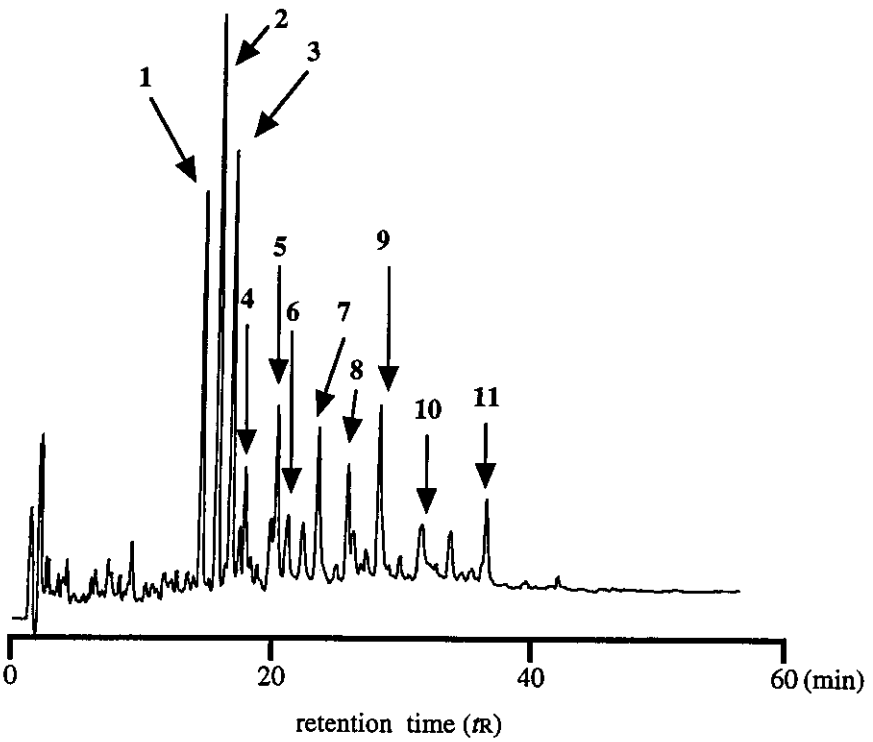
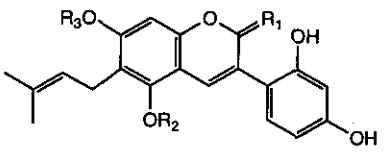
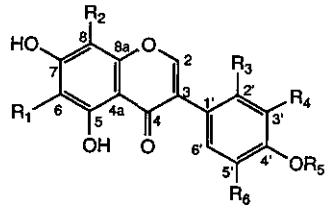


Figure 1

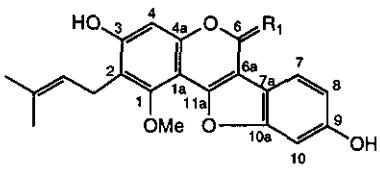
Index compound (**1**) was identified as glycycoumarin,[2] **2** as semilicoisoflavone B,[3] **4** as licoisoflavanone,[4, 5] **5** as glycyrol,[6, 7] **7** as glycyrin,[8] **8** as gancaonin N,[9] **9** as licoricidine[10] and **11** as 8-γ,γ-dimethylallylwighteone,[11] on the basis of MS, ^{1}H-NMR and ^{13}C-NMR spectroscopic data.



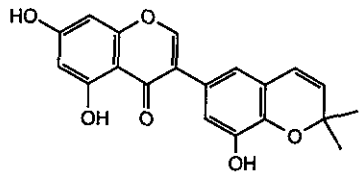
R_1=O, R_2=CH_3, R_3=H glycycoumarin (1)
R_1=H_2, R_2=CH_3, R_3=H dehydroglyasperin C (3)
R_1=O, R_2=R_3=CH_3 glycyrin (7)



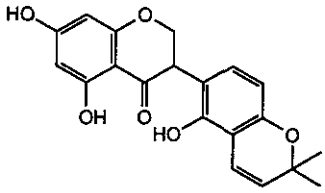
R_1=prenyl, R_2=R_4=R_6=H, R_3=OH, R_5=CH_3 gancaonin N(8)
R_1=R_2=prenyl, R_3=R_4=R_5=R_6=H 8-γ,γ-dimethylallylwighteone (11)
R_1=R_3=R_5=H, R_2=R_4=prenyl, R_6=OH glyurallin B (10)



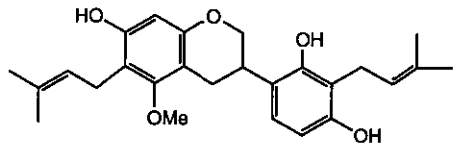
R_1=O glycyrol (5)
R_1=H_2 glyurallin A (6)



semilicoisoflavone B (2)



licoisoflavanone (4)



licoricidine (9)

Compound (**3**) (t_R=16.6 min) was obtained as a yellowish oil, and its identification as to dehydroglyasperin C was obtained as follows. The molecular formula was determined to be $C_{21}H_{22}O_5$ by the high-resolution electron impact mass spectrum (HR-EIMS) (M+, 354.1470, error 0.4 m.m.u.). The ^{1}H-NMR spectrum of **3** showed the signal (δ 4.93, 2H, s, 2-H) characteristic of isoflavene along with signals caused by the following moieties: a 3-methyl-2-butenyl (prenyl) group, a methoxy group, a 1, 2, 3, 4, 5 - pentasubstituted benzene ring and a 1, 2, 4 - trisubstituted benzene ring. The position of the methoxy and the prenyl group was determined by NOE experiment and heteronuclear multiple bond correlation (HMBC) spectrums (Figure 2). Dehydroglyasperin C (**3**) was isolated for the first time from *G. aspera* and the structure has been proposed by I. Kitagawa, W.Z. Cheng *et al.*[12]

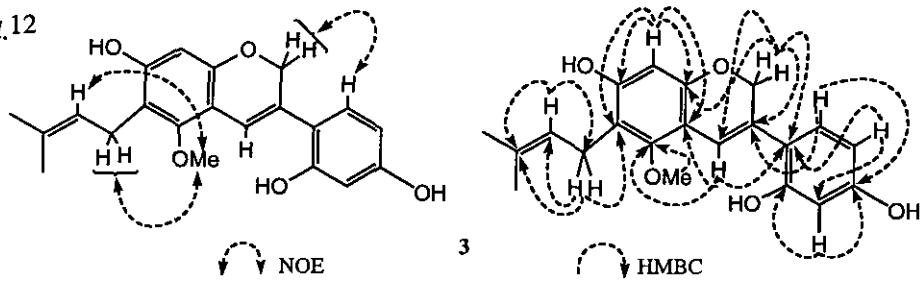


Figure 2

Table 1

	dehydroglyasperin C(3)		glyurallin B (10)			glyurallin A (6)	
t_R (min)	16.6		33.0			22.2	
mp (℃)	yellow oil		yellow oil			orange yellow oil	
EI-MS	m/z 354 M^+		m/z 422 M^+			m/z 352 M^+	
UV(MeOH) λ max nm (log ϵ)	220 (4.41) 330 (4.25)		218sh (4.60) 269 (4.46)			230 (4.30) 336 (4.29) 249 (4.14) 348 (4.17) 290sh(3.92) 350 (4.17)	
IR (KBr) (cm^{-1})	3372 1704 2922 1615 2360		3358 1649			3405 2365 2982 1610	
NMR	^1H*	^{13}C*	^1H**	^{13}C**		^1H***	^{13}C***
2	4.93s	68.6		154.3	1	3.88s(OMe)	157.6 62.3(OMe)
3		128.9		124.9	2		116.6
4	6.76s	116.4		182.3	3		154.9
5	3.67s(OMe)	156.8 62.3(OMe)		163.5	4	6.31s	100.9
6		115.2	6.35s	93.9	6	5.44s	65.2
7		156.8		160.4	7	7.20d(8.5)	119.5
8	6.20s	99.6		113.0	8	6.78dd(8.5,2.0)	113.1
4a		110.6		106.1	9		156.2
8a		154.1		157.4			
					10	7.18d(2.0)	99.1
1'		118.8		123.1			
					1a		104.4
2'		156.9	6.86d(2.2)	114.8	4a		159.2
					6a		107.3
3'	6.46d(1.8)	103.9		144.5	7a		118.9
					10a		157.5
4'		159.1		145.7	11a		154.5
5'	6.40dd (8.5,1.8)	108.2		129.6	1'	3.42br d(7.5)	23.0
6'	7.14d(8.5)	130.0	6.69d(2.2)	122.1	2'	5.24br t(7.5)	124.6
1"	3.24br d(7.2)	23.2	3.35br d(7.5)	22.3, 29.2	3'		130.9
2"	5.14br t(7.2)	125.0	5.24, 5.33br d(7.0)	123.3,123.9			
3"		130.6		132.0,132.9	(Me)2	1.78s, 1.84s	17.9, 25.8
(Me)2	1.63s, 1.73s	17.9, 25.8	1.64s, 1.71s	17.9, 17.9			
			1.71s, 1.78s	26.0, 26.0			

*: measured in aceton-d_6 **: measured in CD_3OD ***: measured in $CDCl_3$

Compound (**6**) (t_R=21.0 min) was isolated as an orange yellow oil and named glyurallin A. The molecular formula was defined as $C_{21}H_{20}O_5$ from the HR-EIMS (M+, 352.1305, error -0.5 m.m.u.). The UV spectrum [λ max (log ϵ) : 230 nm (4.30), 336 nm (4.29), 348 nm (4.17), 350 nm (4.17)] was similar to that of pterocarpenes.[13, 14] The ^{1}H-NMR spectrum of **6** showed the signal (δ 5.44, 2H, s, 2-H) characteristic of pterocarpene. The ^{1}H-NMR spectrum also indicated the presence of a prenyl group, a methoxy group, a 1, 2, 3, 4, 5 - pentasubstituted benzene ring and a 1, 2, 4 - trisubstituted benzene ring. The position of the methoxy and the prenyl groups was determined by NOE experiment and HMBC spectrum as for **3** (Figure 3). Thus, the structure of **6** (glyurallinA) was formulated as 1-methoxy-2-prenyl-3, 9-dihydroxypterocarpene.

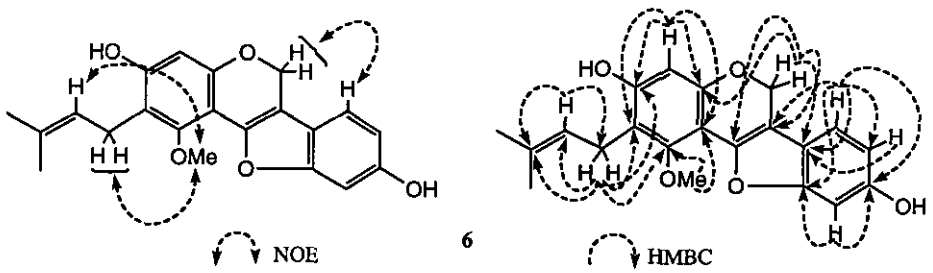


Figure 3

Compound (**10**) (t_R=31.4 min) was isolated as a yellowish oil and named glyurallin B. The molecular formula was determined as $C_{25}H_{26}O_6$ by the HR-EIMS (M+, 422.1734, error 0.6 m.m.u.). The UV absorption maximum at 265 nm (log ϵ 4.46) and the IR absorption at 1650 cm^{-1} suggested that it was an isoflavone derivative. The ^{1}H-NMR indicated the presence of a 1, 2, 3, 4, 5-pentasubstituted benzene ring (δ 6.35, s), a 1, 3, 4, 5-tetrasubstituted benzene ring (δ 6.69 and 6.86, d, J=2.2 Hz), two prenyl groups and a γ -pyrone ring (δ 7.91, s). The MS showed a characteristic fragment ion at m/z 221 (**10a**, A-ring), while NOE was observed as shown in Figure 4. Moreover, on methylation with diazomethane, **10** yielded tetramethyl ether (**10c**), and its ^{13}C-NMR spectrum showed one di-*ortho*-substituted methoxy carbon (δ 60.8) and three mono-*ortho*-substituted methoxy carbons (δ 57.0, 56.8 and 56.5) , revealing the presence of 8-prenyl group.[15] Thus, the structure of **10** (glyurallin B) was formulated as 5, 7, 3', 4'-tetrahydroxy-8, 5'-diprenylisoflavone.

Compounds (**3**) and (**6**) are so unstable that the HPLC peaks caused by them were high in fresh materials, whereas those caused by commercially available crude drugs were reduced. Especially, the peak of **3** is predominant in the HPLC profile (Figure 1) resulting from the new materials. Compounds (**3**) and (**6**) were detected in neither the MeOH nor the $CHCl_3$ extracts. These results

suggest that the extraction of the subterranean parts of *G. uralensis*, licorice (crude drugs), commercial licorice extracts or licorice preparations with AcOEt and HPLC analysis of the extracts are useful for discriminating species by using the 11 compounds including the two unstable compounds.

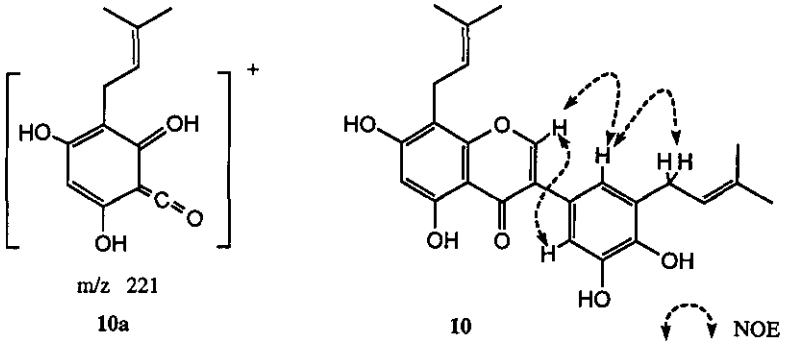


Figure 4

EXPERIMENTAL

HPLC pattern analysis

Materials : Thirty-four subterranean (roots or stolons) samples of *G. uralensis* were obtained from medicinal plant research gardens of Japan. [1. Medicinal Plant Gardens, Nayoro Medicinal Plant Research Station, National Institute of Health Sciences; 2. Medicinal Plant Gardens, Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences; 3. Medicinal Plant Gardens, Izu Medicinal Plant Research Station, National Institute of Health Sciences; 4. Faculty of Pharmaceutical Sciences, Health Science University of Hokkaido; 5. The Aizu Medicinal Plant Garden; 6. Tokyo Metropolitan Medicinal Plant Garden; 7. The Naito Museum of Pharmaceutical Science and Industry Medicinal Plant Garden; 8. Kyoto Herbal Garden, Takeda Chemical Industries; 9. Faculty of Pharmaceutical Sciences Tokyo University; 10. Kanzou Yashiki; 11. Faculty of Pharmaceutical Sciences, Hokkaido University; 12. Osaka University of Pharmaceutical Sciences; 13. Faculty of Pharmaceutical Sciences, Fukuyama University; 14. Samples (19) of commercially available Glycyrrhizae Radix were provided by Shinwabussan & Co. (Osaka) and Mikuni & Co.(Osaka)] .

Preparation of subterranean samples for HPLC pattern analysis : One gram of the sample (

dried at 40℃ overnight) was extracted two times with AcOEt (20 mL each) in a water bath with reflux for 60 min. The AcOEt solution (40 mL) was concentrated under reduced pressure, and then this residue was dissolved in MeOH (10 mL). One mL of this MeOH solution was the chromatographed on Sep pak C-18 (Waters), and eluted with MeOH (3 mL). An aliquot (50 μ L) was analyzed as follows with the HPLC method.

HPLC analysis : HPLC pattern analysis was carried out on a GULLIVER gradient system (JASCO). The HPLC conditions were as follows : column : Crestpak C-18 s (*i.d.* 4.6×150 mm, JASCO); column temperature : 40℃ ; flow rate : 1 mL/min ; detection : UV 254 nm. The mobile phases were flowed gradiently with 1% acetic acid in water (A) and 1% acetic acid in acetonitrile (B), from A/B=70 /30 to A/B=20/80 in 55 min.

Isolation of index components on HPLC pattern

Plant materials : Roots and stolons of *G. uralensis* cultivated at the Hokkaido Experimental Station for Medicinal Plant, National Institute of Health Sciences, were harvested in November, 1996.

Extraction and isolation : The dried roots and stolons of *G. uralensis* (500 g) were extracted three times with AcOEt (500 mL each) in a water bath with reflux for 1 h. The AcOEt extract was concentrated *in vacuo* and the residue was subjected to silica gel column chromatography using hexane-AcOEt to yield 25 fractions. Each fraction was monitored by HPLC. The 20th fraction was purified by semi-preparative HPLC (column : Crestpak C-18 *i.d.* 7.8×250 mm ; mobile phase : 40% MeCN ; flow rato: 2.0 mL/min) to yield compounds (**1**) (30 mg) and (**3**) (40 mg). The 10th fraction yielded compound (**2**) (30 mg) by preparative TLC ($CHCl_3$: MeOH = 15:1), and 5th and 6th fractions yielded compounds (**4**) (10 mg), (**5**) (10 mg), (**6**) (15 mg), (**7**) (13 mg), (**8**) (12 mg), and (**9**) (6 mg) by semi-preparative HPLC (column : Crestpak C-18. *i.d.* 7.8×250 mm ; mobile phase: 50% MeCN ; flow rato : 2.0 mL/min). 3rd and 4th fraction yielded compounds (**10**) (15 mg) and (**11**) (8 mg) by preparative TLC (hexane: AcOEt = 1: 1).

General procedures : The following instruments used for this study : Yanagimoto melting point apparatus (melting point, uncorrected), JASCO gradient system (for preparative HPLC), Hitachi M-

4000 spectrometer (for MS spectra), Perkin-Elmer 1720X-FTIR spectrophotometer (for IR spectra), and Varian Gemini-200, XL-300, General Electric GN-400 (for NMR spectra). NMR spectra were measured in acetone-d_6, $CDCl_3$ and CD_3OD on the δ scale by using tetramethylsilane as an external standard.

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Received, 30th June, 1997