Glucosylsterols in extracts of *Euryale ferox* identified by high resolution NMR and mass spectrometry

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Abstract The stuctures of three glucosylsterols in a glycolipid mixture from the product of the naturally dried medicinal plant *Euryale ferox* Salisb. have been elucidated by 500 MHz nuclear magnetic resonance and mass spectroscopic methods and characterized as follows: 24-methylcholest-5-enyl-3β-O-pyranoglucoside, 24-ethylcholest-5-enyl-3β-O-pyranoglucoside, and 24-ethylcholesta-5,22E-dienyl-3β-O-pyranoglucoside. These constituents may be the active substances of the medicinal plant. —**Zhao, H., S. Zhao, C. Sun, and D. Guillaume.** Glucosylsterols in extracts of *Euryale ferox* identified by high resolution NMR and mass spectrometry. *J. Lipid Res.* 1989. **30:** 1633–1637.

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Sterylglycosides have previously been identified in most membrane fractions of plant cells (1) and also have been found in animal sources as unusual glycolipids (2). These glycolipids may play important roles in the structure of cells or have certain medicinal activity. Since the similarity and complexity of these lipids make the isolation more difficult, the isolated glycolipids tend to be mixtures. High resolution NMR and MS are of value in the characterization of the structures of these lipids (3).

In the investigation of the active constituents of the Chinese medicinal plant *Euryale ferox* Salisb., a coarse aquatic herb, we have isolated and identified a glycosylsterol mixture, an acylglucosylsterol mixture, and other compounds from the product of the naturally dried rhizomes and their adventitious roots. The herb was considered to be a tonic and was also recommended for the treatment of pyodermas, hernia, and leucorrhea (4). Here we report the study of the structure of the glucosylsterol mixture by NMR and MS.

MATERIALS AND METHODS

Plant

The experimental material was the product of the naturally dried rhizomes with adventitious roots obtained from the water-lily family plant *Euryale ferox* Salisb.

Isolation of glycolipids

The sample (1.9 kg) was successively extracted with 70–95% ethanol at room temperature. The extracts were combined and evaporated under reduced pressure. The air-dried residue (20 g) was chromatographed on a silica gel column with dichloromethane, dichloromethane-ethanol >95: <5 azeotrope, and dichloromethane-methanol 92.7:7.3 azeotrope as eluants and afforded lipid B and other compounds. Lipid B (0.25 g) was obtained from the dichloromethane–methanol eluants, and was purified by recrystallization in chloroform–methanol. TLC was performed on silica gel plates (Huangyan, China) in the solvent systems $CHCl_3$–MeOH (9:1 and 8.5:1.5). The spots were visualized with iodine vapor. Lipid B showed only one spot on the chromatograms.

NMR spectra

Proton NMR, carbon-13 NMR, and H–H COSY spectra were measured on a Bruker AM-500 spectrometer with tetramethylsilane or $CDCl_3$ as internal standards. The chemical shifts are given in ppm values. About 5 mg of each sample was dissolved in $CDCl_3$ or pyridine-d_5.

MS

The MS of the aglycones hydrolyzed from lipid B was recorded in the EI mode on a Finnigan 4000 mass spectrometer.

Acetylation of glycolipid

A solution of lipid B in mixture of dichloromethane and 4-dimethylaminopyridine was treated with excess acetic

Abbreviations: NMR, nuclear magnetic resonance; MS, mass spectrometry; EI, electron impact; TLC, thin-layer chromatography; COSY, correlated spectroscopy; TMS, tetramethylsilane; s, singlet; d, doublet; t, triplet; q, quartet; br, broad; dd, doublet of doublets; qq, quartet of quartets; Ac, acetate.

anhydride at room temperature for ca. 4 h, and then diluted with dichloromethane and washed with saturated sodium chloride solution. The dried dichloromethane layer was evaporated to dryness under reduced pressure to give acetylated Ac-B.

Hydrolysis of lipid

Lipid B was hydrolyzed by boiling with excess 10% H_2SO_4 (v/v) for 7 h. The aglycones were extracted into chloroform.

RESULTS AND DISCUSSION

Lipid B is a white amorphous powder and was shown to be sterylglycoside by its diagnostic color change upon Liebermann-Burchard reaction and Molisch reaction. The EI-MS of the aglycones of lipid B (**Fig. 1**) shows three molecular ions at *m/z* 414, 412, and 400, suggesting that lipid B is a mixture. The main ion peaks can be divided into three groups. Each of them agrees with the regular fragmentations of 24-methylcholest-5-en-3β-ol (a), 24-ethylcholest-5-en-3β-ol (b), and 24-ethylcholesta-5,22E-dien-3β-ol (c), respectively (5–7). The parent ring structures of the three sterols are the same, giving ions at *m/z* 273, 271, 255, 246, and 213. The other ions displayed in **Table 1** correspond to the different side-chains linked to the ring structure at C-17. The peak at *m/z* 369 (M–43) indicates a double bond at 22, 23 positions in a sterol (M^+ 412) producing a β-cleavage.

Proton chemical shifts for C-21, 26, 27, 28, and C-29 methyl groups of NMR were also used to detect phytosterols (8). **Table 2** shows the proton chemical shift assignments of the methyl groups from the steryl aglycones of lipid B, based on the comparison with those of corresponding C-24 ethyl and methyl sterols (8). The signals between 0.68 ppm and 1.04 ppm for C-18, 19, 26, 27, 28, and C-29 methyl protons from the aglycones (**Fig. 2**) are much closer to the methyl resonances of a, b, and c or their acetates, respectively. The chemical shifts only for C-19 methyl groups were influenced significantly by C-3 substituents. The peak at 1.02 ppm arising from C-19 methyl protons overlapped the signal for a C-21 methyl group in the NMR spectra of the aglycone, but it was well upfield at 0.99 ppm in the spectra of the acetylated lipid B (Ac-B) (**Fig. 3**). Though the 500 MHz spectra can clearly reveal the characteristics of the lipid, the configurations at the 24 position of the sterols cannot be recognized unless the spectra of reference standards are measured accurately under the same conditions as the spectra of lipid B for a fine comparison between the epimers.

Acetylation of lipid B improved the quality of the NMR spectra. In the proton NMR spectrum of Ac-B, the four acetoxy peaks at 1.98 ppm (s), 2.02 (s), 2.06 (s), 2.09 (s) suggest that there are only four types of acetoxy groups in compound Ac-B. Each of the integrals for the acetoxy peaks is equal to those for all C-18 methyl peaks at 0.68–0.70 ppm, suggesting that each steryl aglycone is attached to a sugar containing four free hydroxy groups

TABLE 1. Characteristic ions in mass spectrometry of aglycones of lipid B

Aglycone[a]	*m/z*						
1	400	385	382	367	315	289	261
2	414	399	396	381	329	303	275
3	412	397	379	369	301	300	

[a]Aglycones 1, 2, and 3 correspond to sterols a, b, and c, respectively.

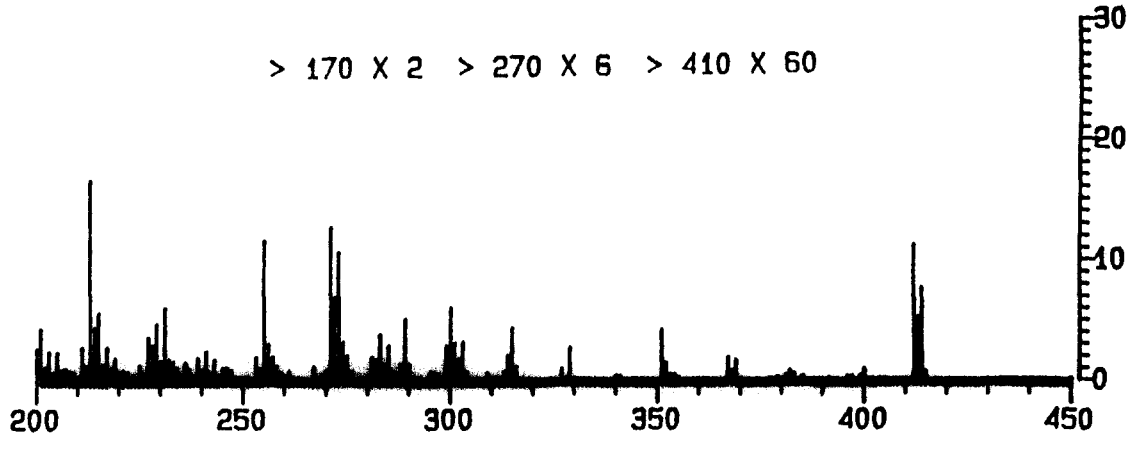

Fig. 1. The EI-MS of the aglycones of lipid B (conditions: 40 eV, 1.1 KV). The numbers at the top of the figure indicate the enlargement factors of intensity in the different mass ranges.

TABLE 2. Proton chemical shift assignments of methyl groups from aglycones of lipid B[a]

Aglycone[b]	C-18[c]	C-19[c]	C-21[d]	C-26[d]	C-27[d]	C-28[d]	C-29[c]
				ppm			
1	0.68	1.02	0.92	0.86	0.79	0.78	
2	0.68	1.02	0.93	0.84	0.82		0.85
3	0.70	1.02	1.04	0.85	0.80		0.81

[a]$CDCl_3$ was used as solvent and internal reference at 7.26 ppm.
[b]Aglycones 1, 2, and 3 correspond to sterols a, b, and c, respectively.
[c]s.
[d]d, J 7 Hz.
[e]t, J 7-8 Hz.

in lipid B. The resonances between 5.20 ppm and 3.68 ppm arise from sugar protons. The signals are clear and assigned by a H-H COSY in **Table 3**. The coupling constants (J1'2', J2'3', J3'4', J4'5') ranged from 8 to 10 Hz, indicating that these axial protons must be on a β-glucosyl ring. The α-H on C-3 attaching to the sugar resonated at 3.49 ppm (heptad, Jaa 11 Hz, 11 Hz; Jae 6 Hz, 6 Hz). Six peaks from the sugar carbons at 99.6 (C-1'), 71.5 (CH), 71.7 (CH), 68.6 (CH), 73.0 (CH), and 62.1 (C-6') ppm are well resolved in carbon-13 spectrum of Ac-B, and in which the C-3 resonated at 80.1 ppm. The fact that the resonances of the protons and the carbons from the glucose moiety showed only one glucose linked to the sterols, was proof that there must be three glucosylsterols in lipid B whose aglycones are similar.

The carbon-13 spectrum of Ac-B displayed four peaks from vinyl carbons: 140.3 (quaternary carbon), 122.1 (CH), 138.4 (CH), 129.3 (CH), corresponding to C-5, 6, 22, and 23. These were compared with literature values for related C-24 ethyl and methyl phytosterols (9–11), which also revealed that the differences between the side-chains did not produce any significant chemical shift change for the carbons on the A, B rings in $CDCl_3$.

The *trans* coupling vinyl protons, from the side-chain of the 24-ethylcholesta-5,22E-dien-3β-ol moiety, at the 22, 23 positions were confirmed by two signals at 5.15 ppm (dd, J 9.4, 15.4 Hz) and 5.02 ppm (dd, J 9.4, 15.4 Hz) in the H-H COSY of Ac-B. The residual vinyl proton signal resonated at 5.36 ppm (br, d, J 3.1 Hz), for which the integral is about double that for the signal at either 5.15 ppm or 5.02 ppm and equal to that for a glucosyl proton. These indicate that all of the three glucosylsterols contain a double bond at C-5, 6, and about half of the glucosylsterols contain two double bonds at C-5, 6 and C-22, 23.

From the above observations, it was concluded that lipid B is composed of 24-methylcholest-5-enyl-3β-O-pyranoglucoside, 24-ethylcholest-5-enyl-3β-O-pyranoglucoside, and 24-ethylcholesta-5,22E-dienyl-3β-O-pyranoglucoside. The latter two compounds are in approximately a 1:1 ratio and the first was a trace according to the proton NMR integral.

In 1983, Okuyama and Yamazaki isolated a sterylglucoside mixture containing, among others, campesterol,

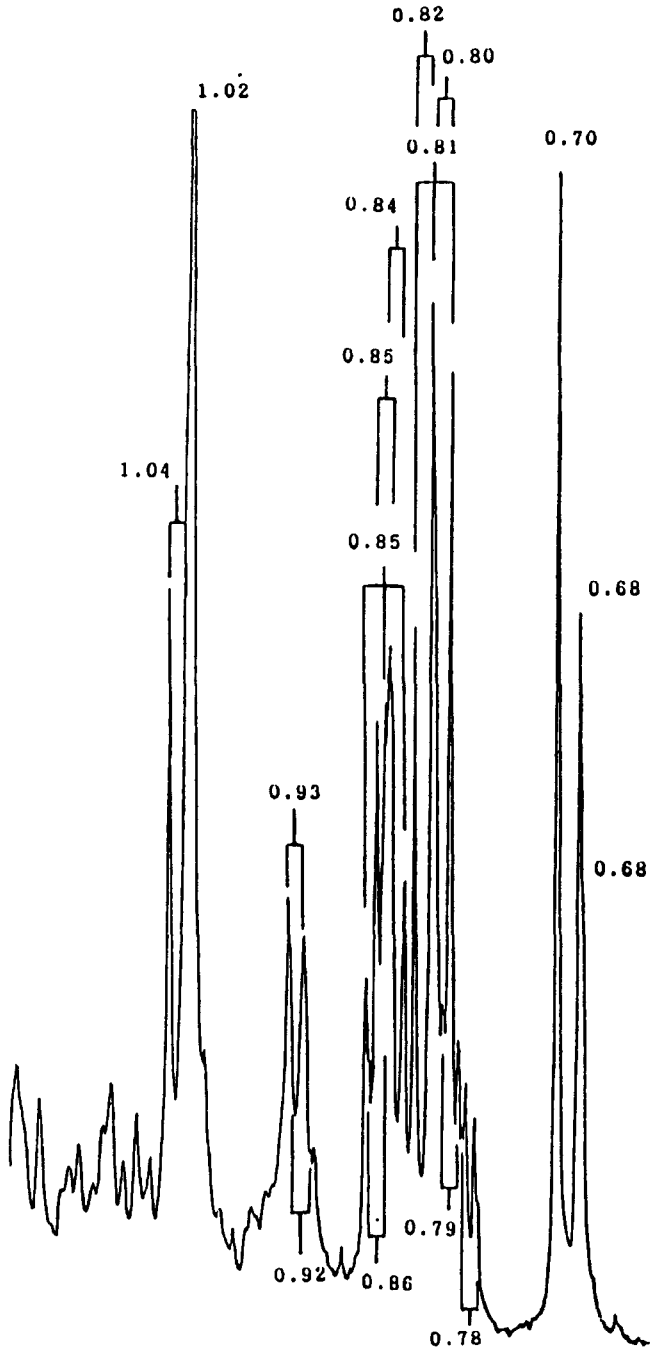

Fig. 2. Expansion of the methyl signals in the proton NMR (500 MHz) of the aglycones of lipid B. $CDCl_3$ was used as a solvent and an internal standard at 7.26 ppm. The chemical shifts are given in ppm values.

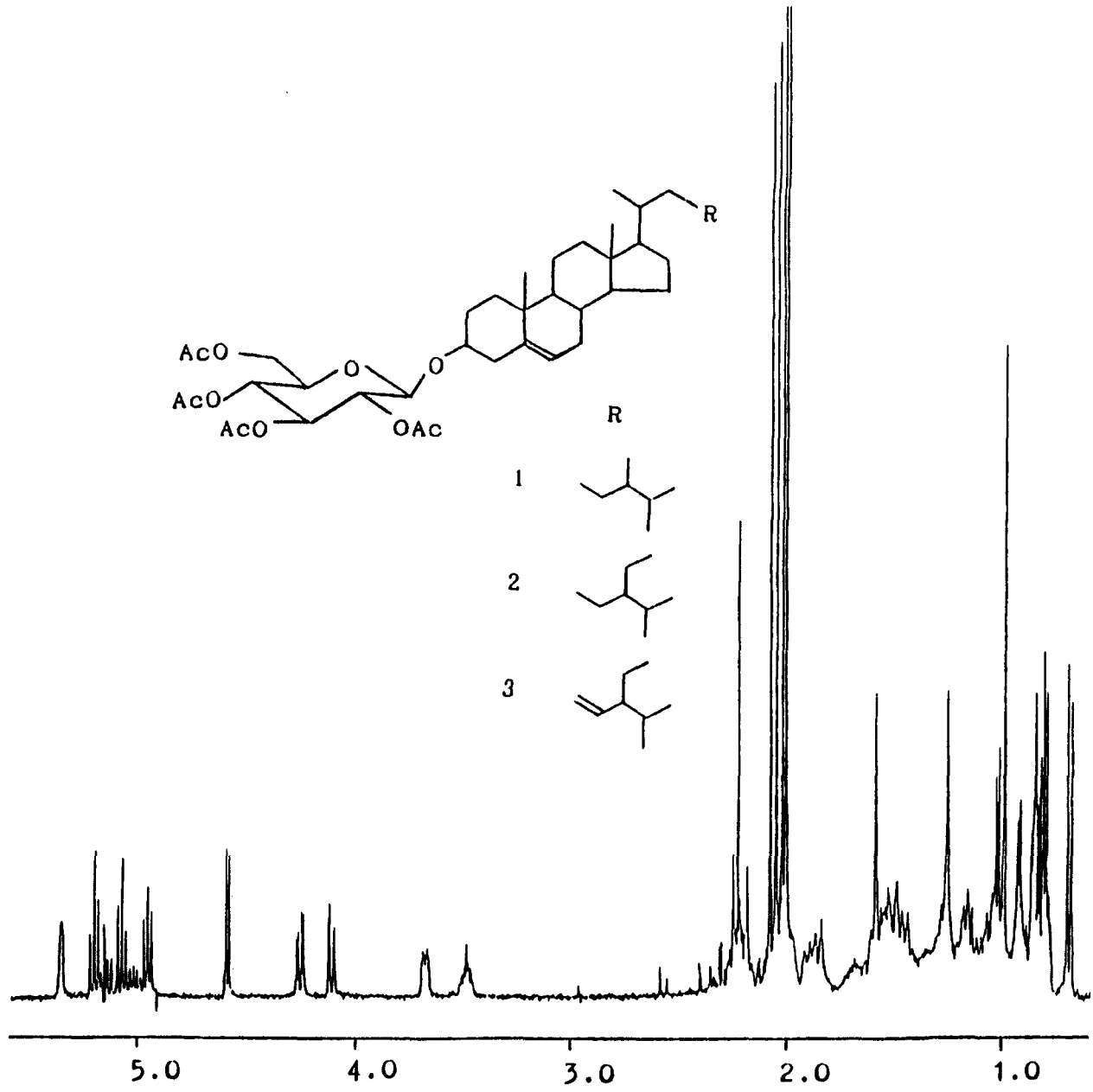

Fig. 3. The proton NMR spectrum (500 MHz) of Ac-B, the acetylate of lipid B, measured in $CDCl_3$ with TMS as internal standard. The chemical shifts are given in ppm values.

TABLE 3. NMR assignment for sugar protons from Ac-B

Proton	(ppm)		J (Hz)	
1'	4.60	d	J 1'-2'	= 8
2'	4.97	dd	J 2'-3'	= 10
3'	5.20	t	J 3'-4'	= 10
4'	5.08	t	J 4'-5'	= 10
5'	3.68	qq	J 5'-6'a	= 5
6'a	4.26	dd	J 5'-6'b	= 2
6'b	4.12	dd	J 6'a-6'b	= 12

β-sitosterol, and stigmasterol as aglycones from a medicinal herb *Tetragonia tetragonoides*. This mixture was said to prevent ulcer formation in mice under restrained and water-immersion conditions (12). This herb was prescribed to "cleanse toxic heat, expel wind, and disperse swelling" (13). The roots of *Euryale ferox* were said to have similar effects. We suggest that the lipid B may be one of the active principles.

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REFERENCES

1. Stumpf, P. K., and E. E. Conn. 1980. The Biochemistry of Plants. Vol. 4. Academic Press, New York. 15–17.
2. Abraham, W., P. W. Wertz, R. R. Burken, and D. T. Downing. 1987. Glucosylsterol and acylglucosylsterol of snake epidermis: structure determination. *J. Lipid Res.* **28:** 446–449.
3. Wertz, P. W., P. M. Stover, W. Abraham, and D. T. Downing. 1986. Lipids of chicken epidermis. *J. Lipid Res.* **27:** 427–435.
4. Jiansu College of New Medicine. 1977. Dictionary of Chinese Medicine (Zhongyao Da zidian). Shanghai Science and Technology Publishing House, Shanghai. 1074–1075.
5. Hopkins, B. J., and F. Scheinmann. 1971. Triterpenes in the seed oil of evening primrose, *Oenothera lamarckiana*. *Phytochemistry.* **10:** 1956.
6. Knightts, B. A. 1967. Identification of plant sterols using combined GLC-mass spectrometry. *J. Gas Chromatogr.* **5:** 273.
7. Sheikh, Y. H., and C. Djerassi. 1974. Steroids from sponges. *Tetrahedron.* **30:** 4095.
8. Rubinstein, I., L. J. Goad, A. D. H. Glague, and L. J. Mulheirn. 1976. The 220 MHz NMR spectra of phytosterols. *Phytochemistry.* **15:** 195–200.
9. Holland, H. L., P. R. P. Diakow, and G. J. Taylor. 1978. C nuclear magnetic resonance spectra of some C-19-hydroxy, C-5,6 epoxy, C-24 ethyl, and C-19-norsteroids. *Can. J. Chem.* **56:** 3121.
10. Lou, F., C. Ma, and F. Du. 1989. Phytochemical study of *Lysimachia foenumgraecum* I. *J. China. Pharm. Univ.* **20:** 37–39.
11. Koizumi, N., Y. Fujimoto, T. Takeshita, and N. Ikekawa. 1979. Carbon-13 nuclear magnetic resonance of 24-substituted steroids. *Chem. Pharm. Bull.* **27:** 38–42.
12. Okuyama, E., and M. Yamasaki. 1983. The principles of *Tetragonia tetragonoides* having an antiulcerogenic activity. I. Isolation and identification of sterylglucoside mixture (compound A). *J. Pharm. Soc. Japan.* **103:** 43–48.
13. Hsu, H-Y. 1986. Oriental Materia Medica, a Concise Guide. Oriental Healing Arts Institute, Long Beach, CA. 839.