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Breynins, New Sulfur-containing Glycosides with Hypocholesterolemic Activity

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Breynins are new sulfur-containing glycosides extracted from *Breynia officinalis* Hemsl (Family Euphorbiaceae). Two major components, breynins A ($C_{40}H_{56}O_{23}S$) and B ($C_{40}H_{56}O_{24}S$), were isolated and characterized. Breynins A and B showed significant hypocholesterolemic activity in rats at a daily dose of 0.005 mg/kg and 0.025 mg/kg, respectively.

In the course of our pharmacological screening program of natural products, an alcoholic extract[2] of *Breynia officinalis* Hemsl (Family Euphorbiaceae) collected in Taiwan, showed remarkable activity in the hypocholesterolemic test. The crude extract contained three closely related active components designated breynins A, B and C. This paper reports the isolation and characterization of the two major components, breynins A and B.

Isolation of Breynins A and B

Dried whole plant of *Breynia officinalis* Hemsl was treated with chloroform to remove dark-colored inactive materials and the hypocholesterolemic activity was extracted with 50% aqueous methanol. Further fractionation and purification of the bioactive components was followed by silica gel thin-layer chromatography (TLC) (system N-101),[3] UV assay at 258 nm and hypocholesterolemic testing in rats.

The methanolic extract was evaporated and the residual solid was partitioned between *n*-butanol and water. The solvent layer was concentrated to a small volume to which ethyl acetate was added to induce precipitation of the active components. The crude active material was shaken with a solvent mixture of *n*-butanol–methanol–water (4:1:3), and the upper layer was separated and concentrated to dryness. The solid thus obtained was purified in a 200-plate counter current distribution (CCD) apparatus using a solvent system of *n*-butanol–methanol–3% aqueous NaCl solution (4:1:3) for 450 transfers. The distribution as monitored by UV assay at 258 nm showed two peaks centrered at tube No. 170 and No. 205 in the distribution curve. Tube Nos. 195 through 215 were combined and further purified by silica gel column chromatography with chloroform–methanol (8:2). Crystallization from ethanol-acetone gave a white crystalline powder of breynin A. Likewise, breynin B was isolated as a white crystalline powder from tube Nos. 160 through 180 of the above CCD by essentially the same purification procedures as used for breynin A.

Physico-chemical Properties of Breynins A and B

Breynins A and B are quite similar to each other. They are readily soluble in water and methanol, less soluble in ethanol and *n*-butanol, and practically insoluble in ethyl acetate, chloroform and other common organic solvents. They gave positive reactions with Tollens, anthrone and Gibbs reagents.

An analytical sample of breynin A was isolated as a monohydrate, $C_{40}H_{56}O_{23}S \cdot H_2O$, which melted at 195—197°. It is optically active: $[\alpha]_D^{22.2}$ +13° (c=0.5, water). The ultra-

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2) Formerly given a code number of TW-678A in our screening program.
3) Solvent system N-101: *n*-butanol–acetic acid–water (63:10:27).

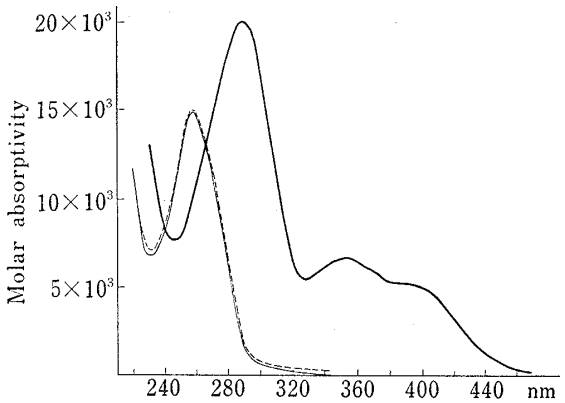

Fig. 1. Ultraviolet Spectrum of Breynin A
———: H_2O, pH 7.0 ----------: 1/10N HCl
———: 1/10N NaOH

violet (UV) absorption spectrum of breynin A is shown in Fig. 1. An absorption maximum at 258 nm is seen both in distilled water and in 0.1N HCl solution, and three maxima at 290, 350 and 395 nm in 0.1 N NaOH solution. The infrared (IR) spectrum of breynin A in KBr pellet as shown in Fig. 2 includes a characteristic absorption band at 1780 cm^{-1}. The nuclear magnetic resonance (NMR) spectrum (100 MHz, 10% in DMSO-d_6) of breynin A is shown in Fig. 3.

Breynin B was obtained as a monohydrate, $C_{40}H_{56}O_{24}S \cdot H_2O$, indicating the presence of an additional oxygen atom as compared with breynin A. It melted at 208—210°, $[\alpha]_D^{22.2}+2°$ (c=0.5, water). The UV, IR and NMR spectra of breynin B are similar to those of breynin A. The components are differentiated by silica gel TLC (system N-101) with an Rf of 0.24 for breynin A and 0.20 for breynin B.

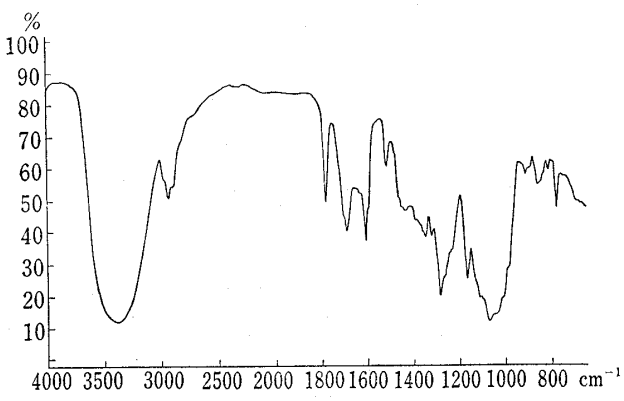

Fig. 2. IR Spectrum of Breynin A (in KBr pellet)

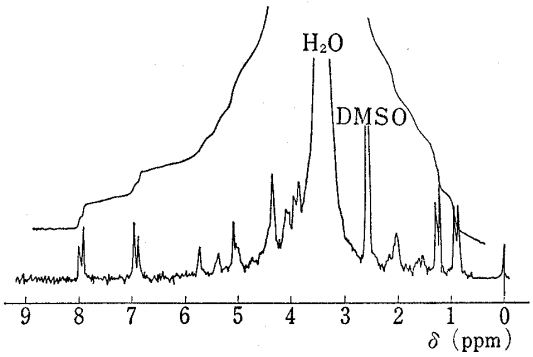

Fig. 3. NMR Spectrum of Breynin A (100 Mc, in DMSO-d_6)

Acid hydrolysis of breynin A gave glucose, rhamnose and an aglycone designated breynogenin ($C_{22}H_{26}O_9S$), which was further hydrolyzed to p-hydroxybenzoic acid and a sulfur-containing compound, breynolide ($C_{15}H_{22}O_7S$).[4] Detailed chemical studies on these compounds will be reported in a separate paper.[5]

Hypocholesterolemic Activity of Breynins A and B

The effects of breynins A and B on serum total cholesterol levels were determined in male Wister rats (3 rats/group) weighing 150—200 grams. The compounds were dissolved in water and administered at graded dose levels by intraperitoneal injection once daily for 4 days. All animals were fasted starting the evening of the 4th day and sacrified on the 5th day by decapitation. Each animal was allowed to bleed into a test tube, the serum sample collected and analyzed for cholesterol by a Technicon Autoanalyzer according to the method of Block, *et al.*[6] Three control rats were included in every experiment and the control serum cholesterol levels determined.

4) K. Sasaki and Y. Hirata, *Tetrahedron Letters*, **1973**, 2439.
5) F. Sakai, H. Ohkuma, H. Koshiyama, T. Naito, and H. Kawaguchi, *Chem. Pharm. Bull.* (Tokyo), **24**, 114 (1976).
6) W.D. Block, K.J. Jarrett, Jr. and J.B. Levine, *Clin. Chem. Acta.*, **12**, 681 (1966).

The value for each sample was obtained from a standard curve plotted from O.D. readings obtained from cholesterol standards ranging between 2.5 and 10 mg%. The mean serum cholesterol concentration of the 3 control samples was determined and compared to the mean values obtained for the breynin-treated animals. The percentage decrease of serum cholesterol was calculated by the equation shown below and a decrease of 15% or more was considered to be significant:

$$\frac{C-T}{C} \times 100 = \% \text{ decrease}$$

where C is an average control value (mg %) and T is an average value for a treated group (mg %).

The results obtained with breynins A and B are summarized and shown in Table I. In five independent experiments, breynin A showed significant hypocholesterolemic activity at 0.02 and 0.005 mg/kg/day but no effect was seen at 0.001 mg/kg/day. It was noted that the serum samples of rats given higher doses of breynin A (0.1 mg/kg/day or greater) were apt to assume yellowish color, which resulted in misreading of the serum cholesterol determination. Breynin B was less active than breynin A, showing significant hypocholesterolemic activity at 0.1 and 0.025 mg/kg/day. The activity of breynin B is estimated as about one-fifth that of breynin A.

Table I. Hypocholesterolemic Activity of Breynins

	No. of test	No. of rats used	Dose (mg/kg/day)	Serum total cholesterol	
				mg %	% decrease
Breynin A	5	15	0.02	34.8±0.76[a]	25.5[b]
	5	15	0.005	37.4±0.84	19.9[b]
	5	15	0.001	42.7±0.97	8.6
Breynin B	1	3	0.1	30.8±3.71	34.0[b]
	1	3	0.025	31.8±2.52	32.0[b]
	1	3	0.006	44.2±3.16	5.4
Control	7	21	—	46.7±1.33	—

a) mean ± S.E.
b) t: $p < 0.01$

The acute intraperitoneal LD_{50}'s of breynins A and B were determined by the method of van der Wärden[7] in male mice weighing 18—20 g, the LD_{50} being 0.25 (0.20—0.31) mg/kg for breynin A and 0.41 (0.34—0.49) mg/kg for breynin B.

Experimental

Crude Extract of Breynins——Air-dried whole plant of *Breynia officinalis* Hemsl, 5 kg, was cut into pieces and heated with 3 × 10 liters of chloroform. The solvent was removed and the plant was then extracted with three 10-liters portion of methanol under refluxing temperature. The methanolic extracts were combined and concentrated under reduced pressure to give 414 g of dark brown sticky solid. The solid was dissolved in 500 ml of water, and the solution was filtered and then extracted with three 500 ml portions of *n*-butanol. The *n*-butanol extracts were combined, concentrated to about 200 ml and mixed with 2 liters of ethyl acetate to precipitate 52 g of yellowish brown solid which contained breynins A and B (*Rf* 0.24 and 0.20 by TLC N-101).

The aqueous layer obtained after the above *n*-butanol extraction contained a minor active component, breynin C, which showed an *Rf* of 0.02 by TLC N-101.

Isolation of Breynins A and B——The crude extract of breynins A and B, 50 g, was shaken with three 500-ml portions of a 4: 1: 3 solvent mixture of *n*-butanol–methanol–water. The upper layers were combined and evaporated *in vacuo* to give 24 g of light yellowish brown solid. The solid was subjected to a 200-plate

7) B.L. van der Wärden, *Arch. Exp. Pharmacodyn.*, **143**, 240 (1940).

counter current distribution apparatus using a solvent system of *n*-butanol–methanol–3% aqueous NaCl solution (4: 1: 3) in 450 transfers. Distribution peaks determined by UV assay at 258 nm were found at tube No. 170 and No. 205.

Tube Nos. 195 through 215 which contained breynin A as shown by TLC (N-101, *Rf* 0.24) were combined and concentrated *in vacuo*. The concentrate was applied to a column (24 mm × 650 mm) of silica gel (Mallinckrodt 100 mesh, 120 g). The column was developed with 2 liters of chloroform followed by 4 liters of chloroform–methanol (8: 2). Fractions showing *Rf* 0.24 by TLC were collected and concentrated *in vacuo* to dryness. The resultant solid, 5.8 g, was repeatedly crystallized from ethanol–acetone (8: 2) to yield 1.9 g white crystalline breynin A. *Anal.* Calcd. for $C_{40}H_{56}O_{23}S \cdot H_2O$: C, 50.30; H, 6.13; S, 3.36. Found: C, 50.12; H, 6.55; S, 3.67.

Tube Nos. 160 through 180 which contained breynin B (TLC N-101, *Rf* 0.20) were combined and purified by silica gel column chromatography in the same manner as described above. Breynin B, 1.4 g, was isolated as a white crystalline powder. *Anal.* Calcd. for $C_{40}H_{56}O_{24}S \cdot H_2O$: C, 49.48; H, 5.81; S, 3.30. Found: C, 48.99; H, 6.09; S, 3.69.

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