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Chemical and Toxicological Studies on Bracken Fern, *Pteridium aquilinum* var. *latiusculum*. I. Introduction, Extraction and Fractionation of Constituents, and Toxicological Studies including Carcinogenicity Tests

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Toxicity tests to experimental animals and cultured HeLa cells were performed on the extracts of young fronds of bracken, *Pteridium aquilinum* var. *latiusculum*, aiming the isolation and characterization of the carcinogen(s) and the cattle poison(s). The separation of the extracts monitored by cytotoxicity tests resulted in the isolation of more than twenty kinds of sesquiterpenes having 1-indanone nucleus, named pterosins, and their glucosides, pterosides, and several known phenolics and steroids.

Although the dried fronds and rhizomes of the fern exhibited carcinogenicity by feeding to rats, none of the extracts and the fractions along with pterosin B and pteroside B, major indanones, induced tumors under the employed conditions.

Keywords——*Pteridium aquilinum* var. *latiusculum*; cytotoxicity test; HeLa cell; carcinogenicity test; cattle poison; pterosins; pterosides; 1-indanone derivatives

The bracken fern (*Pteridium aquilinum* Kuhn var. *latiusculum* Underwood, Pteridaceae; Japanese name, warabi)[2] is one of the oldest and commonest plants in the world, but up to the end of the last century neither its biological activity nor especial secondary metabolites of the plant had been known. In 1893 the lethal property to cattles was first reported[3] and since then tremendous numbers of works have been conducted in the field of veterinary sciences. In Japan poisoning of cattles in meadows by bracken had been known from old times and the recent epidemiological survey confirmed bracken as the causative.[4] The main symptom of poisoning of cattles is chronic enzootic hematuria probably due to bone-marrow damage. The causative was once alleged to be antithiamine factors such as thiaminase[5] and polyphenols, 3,4-dihydroxycinnamic acid[6] and flavonoids (astragalin and isoquercitrin).[7] However the syndrome known as "Cattle Bracken Poisoning" is quite dramatic and not explicable as avitaminosis, being characterized by generalised hemorrhage, anorexia and extensive intestinal damage and ulceration. Such syndrome quite similar to the effect of radiation[8]

1) Location: *a*) *Kamiyoga-1-chome, Setagaya-ku, Tokyo*; *b*) *Shirokanedai, Minato-ku, Tokyo*; *c*) *Urafune-cho, Minami-ku, Yokohama*; *d*) *Sugao, Takatsu-ku, Kawasaki*; *e*) *Hatanodai, Shinagawa-ku, Tokyo*; *f*) the author to whom the inquiry on this paper should be addressed.
2) Although the plant growing in Japan is identified as the variety but it is a sort of geographical race and the constituents and biological activity are assumed to be essentially same as the original species (*cf.* G. Cooper-Driver, *Bot. J. Linn. Soc.*, **73**, 35 (1977)).
3) D.N. Storrer, *J. Compt. Path.*, **6**, 279 (1893): J. Penberthy, *ibid.*, **6**, 266 (1893).
4) Agriculture, Forestry and Fishery Research Council Secretariat, Ministry of Agriculture and Forestry, Japan, (Ed.), Research Rev., No. 70, "Studies on the Bovine Panmyelolitis in Japanese Meadows," 1973.
5) W.C. Evans, N.J. Jones, and I.A. Evans, *Biochem. J.*, **47**, 12 (1950).
6) J. Berüter and J.C. Somogy, *Experientia*, **23**, 996 (1967).
7) T. Nakabayashi, *Bull. Agr. Chem. Soc. Japan*, **19**, 104 (1955).
8) I.A. Evans, *Cancer Research*, **28**, 2252 (1968).

(radiomimetic nature) attracted the attention of many pathologists. Following the first report on experimental proof of polypus nature in the urinary bladder mucosa of cattle feeded bracken,[9] it has been demonstrated by feeding that the plant induces urinary bladder tumors in cows,[10,11] rats,[12,13] and guinea pigs, intestinal adenocarcinomas and sarcomas especially at illeum region in rats[8,11–15] and Japanese quails,[8] and pulmonary adenomas and leukemia in mice.[16] Thus it is now quite evident that the fern contains rather strong carcinogenic chemical(s), though whether the same chemical(s) is responsible for the acute toxicity in cattle remains obscure.

As for the chemical constituents besides the polyphenolic antithiamine factors,[6,7] prunasin[17] which may have some connection to the acute toxicity, carotenoids, amino acids, and phytoecdysons[18] had been reported when we commenced this study in 1970 but none of these were assumed to be carcinogenic. All known about the nature of carcinogenic principle (s) and poisoning(s) was that they are thermostable and removable by alcohol extraction.

Our works in last seven years attempting for characterization and isolation of the carcinogenic principle(s) have so far been unsuccessful but in this paper and the following papers the results so far obtained for the chemical constituents, especially on the characteristic sesquiterpenes of 1-indanone derivatives, named pterosins, and their glucosides, pterosides, and some toxicological observations will be reported. The preliminary communications on the chemistry[19–23] and toxicology[24] have been published before. In this first report introduction, preliminary examination, extraction and isolation of the constituents, acute toxicity, cytotoxicity and carcinogenicity tests using the extracts will be described.

Materials and Methods

Plant Materials——Since outbreak of bracken poisoning of cattle has been confined to some area,[4] we tried to use the bracken collected at the places where the poisoning had occurred nearby as shown in Table I. The edible part used in Japan is young curling tops of the plant and the bracken poisoning of cattles occurs by eating the young fronds.[4,8] Thus we used for present study chiefly young fronds collected in May–July. Since it was shown later that the rhizomes showed stronger carcinogenicity[25] and toxicity to cattles[26] than young fronds, rhizomes were also used for the study. The methods of drying actually influence the toxicity;

9) Von G. Rosenberger and W. Heeschen, *Deut. Tierärzt. Woch.*, **67**, 201 (1960).
10) A.M. Pamukcu, S.D. Göksoy, and J.M. Price, *Cancer Res.*, **27**, 917 (1967).
11) J.M. Price and A.M. Pamukcu, *Cancer Res.*, **28**, 2247 (1968).
12) A.M. Pamukcu and J.M. Price, *J. Natl. Cancer Inst.*, **43**, 275 (1969).
13) A.M. Pamukcu, S. Yalçiner, and J.M. Price, *Cancer Res.*, **30**, 2671 (1970).
14) I.A. Evans and J. Mason, *Nature* (London), **208**, 913 (1965).
15) I. Hirono, C. Shibuya, K. Fushimi, and M. Haga, *J. Natl. Cancer Inst.*, **45**, 179 (1970); I. Hirono, I. Sasaoka, C. Shibuya, M. Shimizu, K. Fushimi, H. Mori, K. Kato, and M. Haga, *Gann, Monographs on Cancer Research*, **17**, 205 (1975).
16) A.M. Pamukcu, E. Ertük, J.M. Price, and G.T. Bryan, *Cancer Res.*, **32**, 1442 (1972).
17) H. Kofod and R. Eyjolfsson, *Tetrahedron Lett.*, **1966**, 1289; W.D. Bennett, *Phytochemistry*, **7**, 151 (1968).
18) J.N. Kaplanis, M.J. Thompson, W.E. Robbins, and B.M. Bryce, *Science*, **157**, 1436 (1967); H. Hikino, S. Arihara, and T. Takemoto, *Tetrahedron*, **25**, 3909 (1969).
19) K. Yoshihira, M. Fukuoka, M. Kuroyanagi, and S. Natori, *Chem. Pharm. Bull.* (Tokyo), **19**, 1491 (1971).
20) K. Yoshihira, M. Fukuoka, M. Kuroyanagi, and S. Natori, *Chem. Pharm. Bull.* (Tokyo), **20**, 426 (1972).
21) M. Fukuoka, M. Kuroyanagi, M. Tōyama, K. Yoshihira, and S. Natori, *Chem. Pharm. Bull.* (Tokyo), **20**, 2282 (1972).
22) M. Kuroyanagi, M. Fukuoka, K. Yoshihira, and S. Natori, *Chem. Pharm. Bull.* (Tokyo), **22**, 723 (1974).
23) M. Kuroyanagi, M. Fukuoka, K. Yoshihira, and S. Natori, *Chem. Pharm. Bull.* (Tokyo), **22**, 2762 (1974).
24) M. Saito, M. Umeda, M. Enomoto, Y. Hatanaka, S. Natori, K. Yoshihira, M. Fukuoka, and M. Kuroyanagi, *Experientia*, **31**, 829 (1975).
25) I. Hirono, K. Fushimi, H. Mori, T. Miwa, and M. Haga, *J. Natl. Cancer Inst.*, **50**, 1367 (1973).
26) J. Iwata and K. Yamaguchi, The paper presented at Annual Meeting of Japanese Society of Veterinary Sciences, 1974.

it has been reported that the materials dried by heating under blowing retain carcinogenicity,[27] while drying under sunshine diminishes the toxicity to cattles.[4] For preliminary chemical and toxicological works we have employed several methods of drying; *e.g.* drying in sunshine and in shade, heating under blowing, and freeze-drying. As for the materials for carcinogenicity tests and large-scale extraction those dried at 50—70° under blowing were chiefly used. The materials used are summarized in Table I.

Table I. Materials

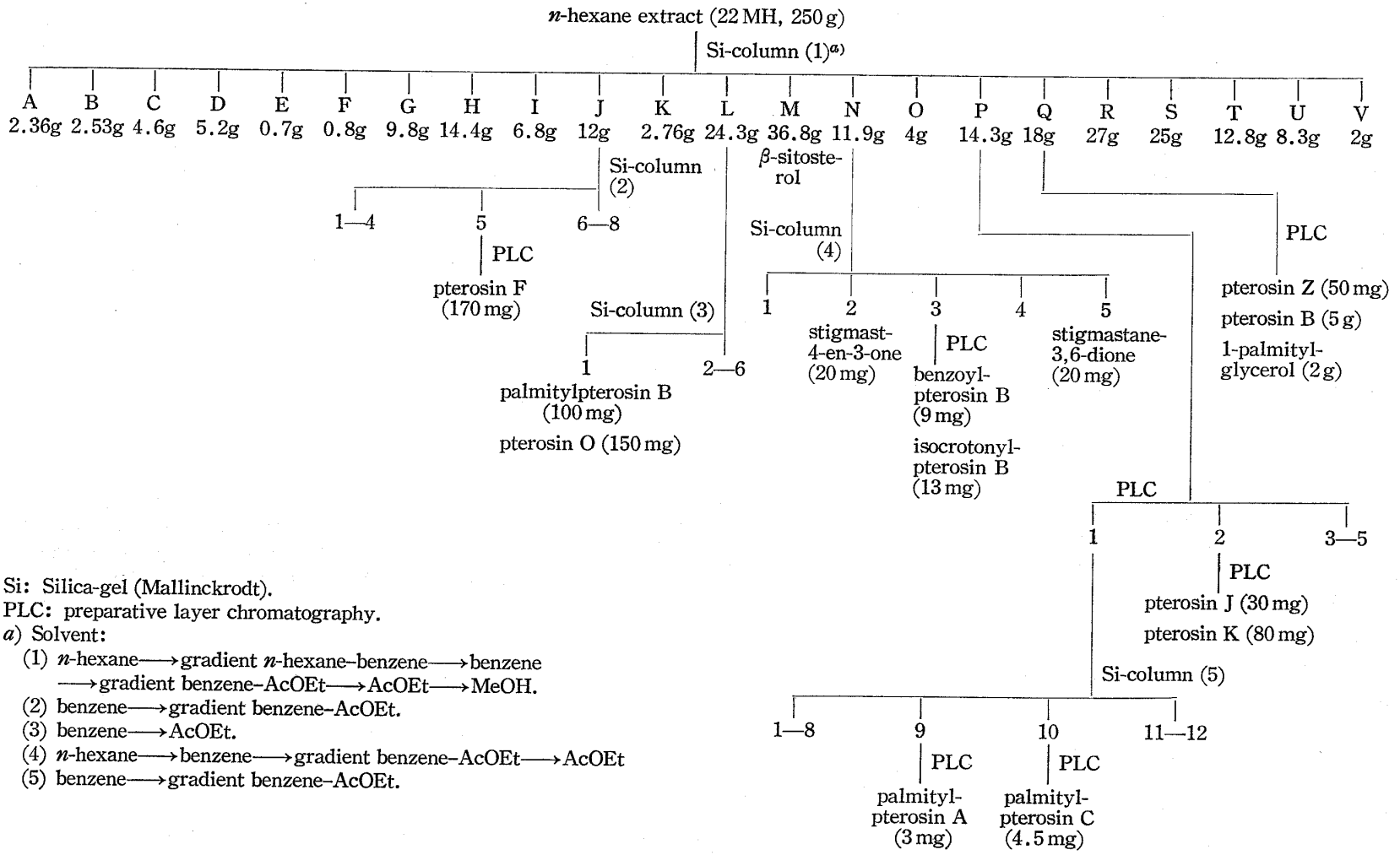
Sample number	Part used	Date and place of collection	Experiment performed
1	Fronds, stalks	April 1970 Minamiizu, Shizuoka	Preliminary chemical and toxicological tests
2	Fronds (curling tops)	June 1970 Nayoro, Hokkaido	Extraction and fractionation with toxicity tests
3	Fronds, stalks, rhizomes	May 1971 Minamizu, Shizuoka	Comparison of the parts and methods of drying
4	Fronds (curling tops)	June 1971 Nayoro, Hokkaido	Preparation of extracts for carcinogenicity test (Exp. 1)
5	Fronds, stalks, rhizomes	August 1971 Minamiizu, Shizuoka	Same as 3
6	Fronds,	August 1971 Nayoro, Hokkaido	Same as 3
	rhizomes		Extraction and fractionation, carcinogenicity test (Exp. 1)
7	Fronds, (curling tops)	Ditto	Carcinogenicity test (Exp. 1)
8	Fronds	June 1972 Nayoro, Hokkaido	Separation of indanones
9	Rhizomes	Unknown Ina, Nagano	Comparison of locality
10	Fronds	June 1972 Nayoro, Hokkaido	Preparation of extracts, carcinogenicity test (Exp. 1)
12	Fronds, stalks, rhizomes	June 1974 Yamakita, Kanagawa	Drying methods
13	Fronds	July 1974 Nayoro, Hokkaido	Preparation of extracts for toxicity tests including carcinogenicity tests (Exp. 2)
14	Fronds,	July 1975 Nayoro, Hokkaido	Preparation of extracts
	rhizomes		Separation of compounds, carcinogenicity test (Exp. 2)

Extraction of Plant Materials for Preliminary Tests——Conventional extraction methods using several kinds of organic solvents were employed for the preparation of extracts and fractions (see an example at the footnote *a*) of Table V).

Preparation of Extracts for Carcinogenicity Tests——a) Experiment 1 (Table VII): Young fronds (sample 4 in Table I, 15 kg) were powdered after drying and extracted with benzene, ethyl acetate, and methanol successively at a room temperature. The solvents were evaporated under reduced pressure to afford benzene extract (160 g), ethyl acetate extract (74 g), methanol extract (800 g), and the residue (*ca.* 14 kg) respectively.

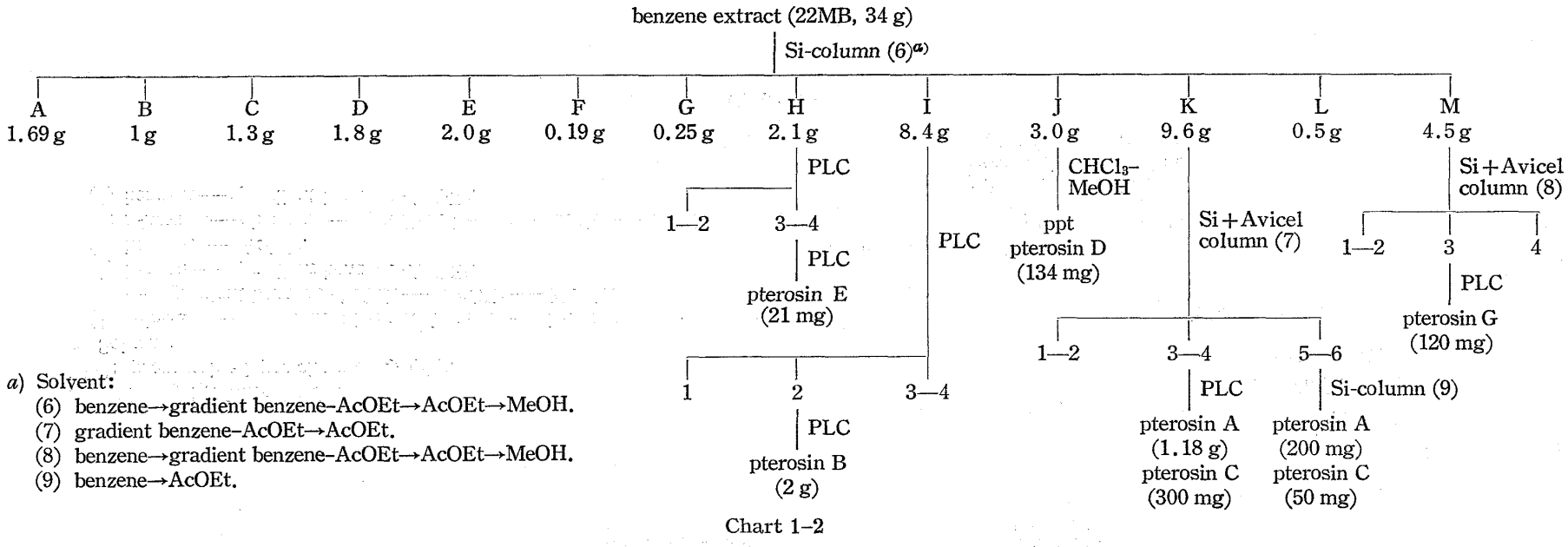
b) Experiments 2 (Table VII): The dried powder of the young fronds (sample 13 in Table I, 60 kg) was twice extracted with benzene (240 l) at room temperature for three days and the benzene extract, after evaporation *in vacuo*, was dried in a freezing drier to afford a solid (132PB, 950 g). A half of the benzene extract was treated with methanol (6 l) to afford a methanol insoluble-part (132PBN, 132 g) and a soluble-part, which was chromatographed on charcoal (100 g) to afford a methanol-eluate (132PBO, 222 g) and a mixture (132PBP, 80 g) of acetone, chloroform, and benzene eluates.

27) I. Hirono, H. Mori, K. Kato, and T. Kato, The paper presented at Annual Meeting of Japan Cancer Association, 1976.



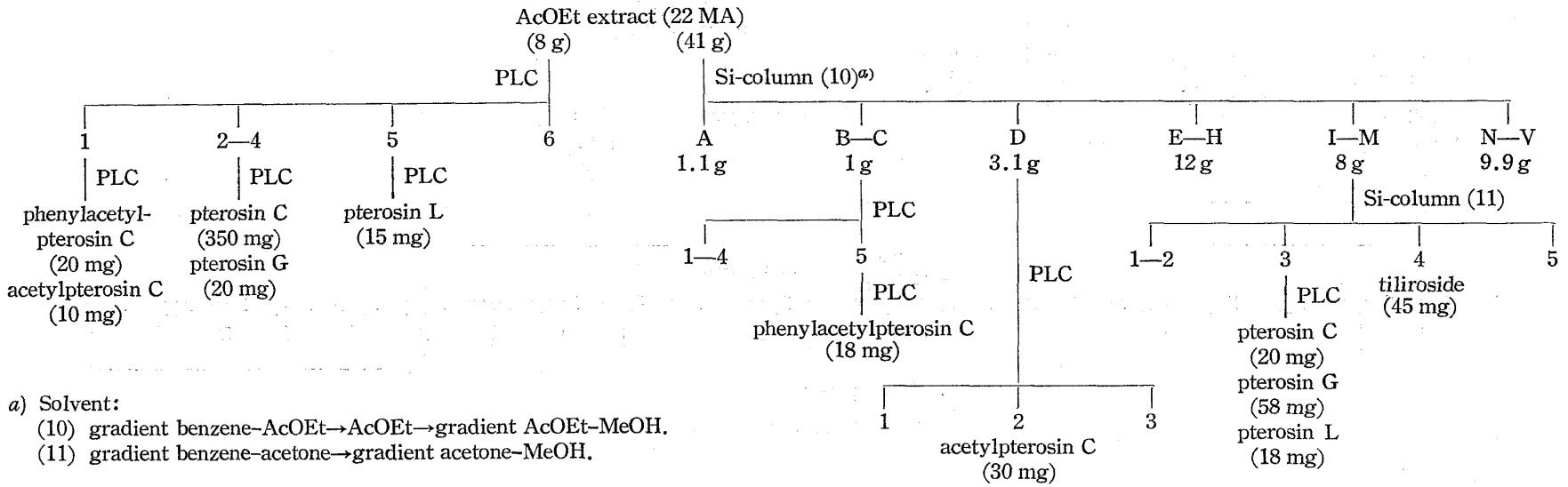
Si: Silica-gel (Mallinckrodt).
PLC: preparative layer chromatography.
a) Solvent:
(1) *n*-hexane⟶gradient *n*-hexane–benzene⟶benzene ⟶gradient benzene–AcOEt⟶AcOEt⟶MeOH.
(2) benzene⟶gradient benzene–AcOEt.
(3) benzene⟶AcOEt.
(4) *n*-hexane⟶benzene⟶gradient benzene–AcOEt⟶AcOEt
(5) benzene⟶gradient benzene–AcOEt.

Chart 1-1



a) Solvent:
(6) benzene→gradient benzene–AcOEt→AcOEt→MeOH.
(7) gradient benzene–AcOEt→AcOEt.
(8) benzene→gradient benzene–AcOEt→AcOEt→MeOH.
(9) benzene→AcOEt.

Chart 1–2



a) Solvent:
(10) gradient benzene–AcOEt→AcOEt→gradient AcOEt–MeOH.
(11) gradient benzene–acetone→gradient acetone–MeOH.

Chart 1–3

Table II. Results of Animal Toxicity Test (Mouse and Rat) and Cytotoxicity (HeLa Cell) of the Extracts and Fractions

Fraction[a]	Cytotoxicity IC_{50} (μg/ml)	Toxicity (grade) and toxic pattern[b]			
		Mouse		Rat	
		s.c.	*p.o.*	*s.c.*	*p.o.*
Dried young frond, 22.		/	‡[c] M	/	‡[c] M
22M	470	−	±K	±	/
22MH	320	−	−	−	/
22MHA[d]	Over 1000	+M, K	+M		
22MHB	195	+M	+M, K		
22MHC	690	±	±		
22MHD	220	±	±		
22MHE	30	±M	+M		
22MHE-1[e]	70				
22MHE-2	Over 100				
22MHE-3	Over 100				
22MHE-4	Over 100				
22MHF					
22MHF-1	690	+M	±		
22MHF-2	320	+M	+		
22MHG	50	−	+L		
22MHH	180	+M	±M		
22MHI	260	−	−		
22MHJ	250	‡M, L	±		
22MHJ-1	Over 100				
22MHJ-2	Over 100				
22MHJ-3	100				
22MHJ-4	60				
22MHJ-5	70	−	/		
22MHJ-6	Over 100				
22MHJ-7	Over 100				
22MHK	320	−	+L		
22MHK-1	60				
22MHK-2	50				
22MHK-3	20				
22MHL	180	−	−		
22MHM	580	−	−		
22MHN	470	+M	±		
22MHN-1	Over 100				
22MHN-2	Over 100				
22MHN-3	100				
22MHN-4	100				
22MHN-5	Over 100				
22MHN-6	60				
22MHN-7	Over 100				
22MHN-8	60				
22MHN-9	Over 100				
22MHN-10	Over 100				
22MHO	220	−	−		
22MHP	40	−	−		
22MHP-1	10				
22MHP-2	60				
22MHP-3	50				
22MHP-4	70				
22MHP-5	100				
22MHQ	100	+M	+M		
22MHR	180	±	−		
22MHR-1	60				
22MHR-2	Over 100				
22MHR-3	Over 100				
22MHR-4	Over 100				
22MHR-5	100				
22MHR-6	Over 100				
22MHR-7	100				

Fraction[a]	Cytotoxicity IC_{50} (μg/ml)	Toxicity (grade) and toxic pattern[b]			
		Mouse		Rat	
		s.c.	*p.o.*	*s.c.*	*p.o.*
22MHR-8	Over 100				
22MHS		+M	−		
cryst.	Over 1000				
filt.	570				
22MHT		+M	−		
ppt.	Over 1000				
filt.	1000				
22MHU		±	−		
ppt.	Over 1000				
filt.	530				
22MHV	530	−	−		
22MB	100	−	−	±	/
22MBA					
22MBA-1	640				
22MBA-2	320				
22MBA-3					
22MBA′					
22MBA′-1	470				
22MBA′-2	180				
22MBA′-3	195				
22MBB	220				
22MBC	220				
22MBC-1	150				
22MBC-2	470				
22MBC-3	470				
22MBD	320				
22MBD-1	180				
22MBD-2	470				
22MBD-3	220				
22MBE	470				
22MBE-1	180				
22MBE-2	470				
22MBE-3	690				
22MBE-4	470				
22MBE-5	470				
22MBF	50				
22MBF-1	10				
22MBF-2	40				
22MBF-3	100				
22MBF-4	20				
22MBG	50				
22MBG-1	30				
22MBG-2	100				
22MBG-3	100				
22MBH	70				
22MBH-1	100				
22MBH-2	30				
22MBH-3	10				
22MBH-4	30				
22MBI	100				
22MBI-1	10				
22MBI-2	60				
22MBI-3	30				
22MBI-4	Over 100				
22MBJ	60				
22MBJ-1	60				
22MBJ-2	30				
22MBJ-3	100				
22MBJ-4	Over 100				
22MBK	200				
22MBK-1					

Fraction[a]	Cytotoxicity IC_{50} (μg/ml)	Toxicity (grade) and toxic pattern[b]			
		Mouse		Rat	
		s.c.	*p.o.*	*s.c.*	*p.o.*
22MBK-2					
22MBK-3	60				
22MBK-4	Over 100				
22MBK-5	Over 100				
22MBK-6	Over 100				
22MBK-7	60				
22MBL	100				
22MBM	20				
22MBM-1	100				
22MBM-2	6				
22MBM-3	50				
22MBM-4	80				
22MBM-5, 6	Over 100				
22MBM-7	8				
22MBM-8	20				
22MA	260	−	−	+M	/
22MAA	60				
22MAB	Over 320				
22MAC	Over 320				
22MAD	60				
22MAE	180				
22MAF	180				
22MAG	180				
22MAH	180				
22MAI	180				
22MAJ	180				
22MAJ-1	100				
22MAJ-2, 3	Over 100				
22MAJ-4	70				
22MAJ-5					
22MAJ-6	30				
22MAJ-7	70				
22MAK	180				
22MAL	60				
22MAM					
22MAM-1	180				
22MAM-2	180				
22MAM-3	320				
22MAM-4 to 7	Over 320				
22MAN					
22MAO					
22MAO-1	180				
22MAO-2 to 10	Over 320				
22MBu	220	−	−	±	/
22MW	220	−	−	±	/
22W	Over 1000	/	+[c] M	/	+[c]
Control		/	−[c]	−	/

a) 22M indicates the methanol extract of the dried young fronds, No. 22. 22MH, 22MB, 22MA and 22MBu are the fractions obtained by the successive partition with hexane: water, benzene: water, ethyl acetate: water and butanol: water, respectively, and 22MW indicates the partitioned aqueous layer. 22W indicates the hot water extract of the methanol-extracted fronds.

b) M: damage on actively-dividing cells; L: hepatotoxic; K: nephrotoxic. ##: lethal toxicity, #: showing definite pathological lesions with decrease of body weight, +: either showing definite histological lesions or loss of body weight, ±: positive histological lesions only, −: no positive evidence for toxic effects.

c) Feeding experiment.

d) Fractions of column chromatography. See Chart 1.

e) Fractions of the thin-layer chromatography. See Chart 1.

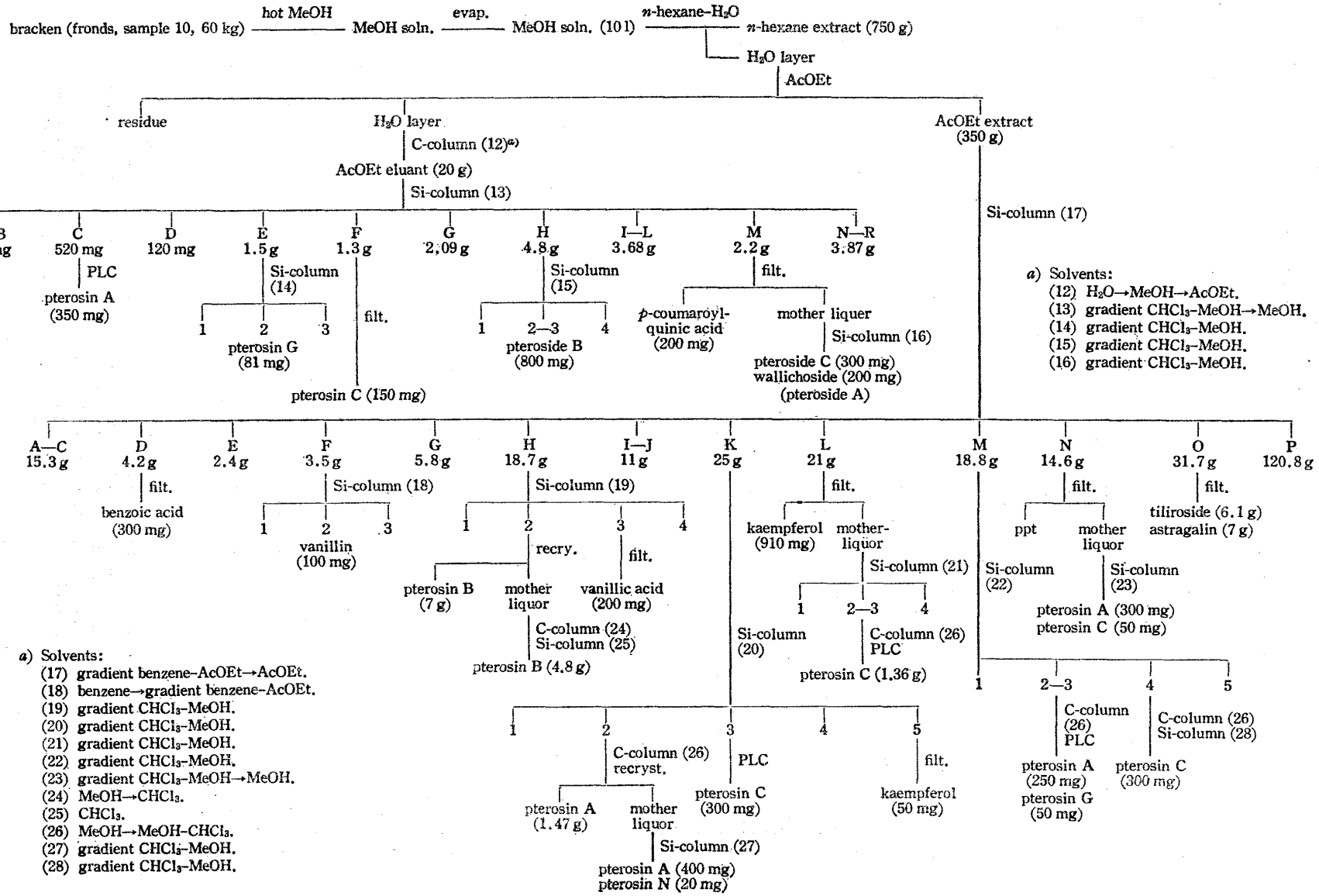


Chart 2

TABLE III. Pterosins and Pterosides, C_{14}- and C_{15}-Sesquiterpenoids from Bracken Fronds

Compound	mp (solvent of recrystallization)	$[\alpha]_D$ (solvent)	Structure R_1	R_2	R_3	R_4	R_5
Pterosin A (1)	125—127° (AcOEt)	−45.3° (MeOH)	$-CH_2OH$	$-CH_3$	H	H	$-CH_2OH$
Pterosin B (2)	109—110° ($CHCl_3$–hexane)	−31.5° (MeOH)	$-CH_3$	H	H	H	$-CH_2OH$
Pterosin C (3)	153—156° (AcOEt)	+93.4° (MeOH)	H	$-CH_3$	OH	H	$-CH_2OH$
Pterosin D (4)	189—190° (MeOH–$CHCl_3$)	+4.8 (EtOH)	$-CH_3$	$-CH_3$	OH	H	$-CH_2OH$
Pterosin E (5)	160—162° (CCl_4)	±0°	$-CH_3$	H	H	H	–COOH
Pterosin F (6)	66—67° (hexane)	−14.6° (MeOH)	$-CH_3$	H	H	H	$-CH_2Cl$
Pterosin G (7)	152—153° (CCl_4)	−14.6° (MeOH)	$-CH_2OH$	H	H	H	$-CH_2OH$
Pterosin J (8)	136—137° (benzene–hexane)	+83.5° ($CHCl_3$)	H	$-CH_3$	OH	H	$-CH_2Cl$
Pterosin K (9)	85—87° (hexane)	−37.5° (MeOH)	$-CH_2OH$	$-CH_3$	H	H	$-CH_2Cl$
Pterosin L (10)	139—141° (benzene)	+23.7° (MeOH)	$-CH_2OH$	$-CH_3$	OH	H	$-CH_2OH$
Pterosin N (11)	165—167° (acetone)	±0°	$-CH_3$ and OH		H	H	$-CH_2OH$
Pterosin O (12)	45—46° (hexane)	±0°	$-CH_3$	H	H	H	$-CH_2OMe$
Pterosin Z (13)	86—88° (hexane)	±0°	$-CH_3$	$-CH_3$	H	H	$-CH_2OH$
Acetylpterosin C (14)	115—118° (benzene–hexane)	+86.1° ($CHCl_3$)	H	$-CH_3$	OH	H	$-CH_2OAc$
Benzoylpterosin B (15)	68—70° (MeOH)	−20° ($CHCl_3$)	$-CH_3$	H	H	H	$-CH_2OBz$
Isocrotonyl-pterosin B (16)	Oil	−3.5° ($CHCl_3$)	$-CH_3$	H	H	H	$-CH_2O$ isocrotonyl
Palmitylpterosin A (17)	50—51° (hexane)	−37.8° (MeOH)	$-CH_2OH$	$-CH_3$	H	H	$-CH_2O$ palmityl
Palmitylpterosin B (18)	51—52° (hexane)	−3.3° (cyclohexane)	$-CH_3$	H	H	H	$-CH_2O$ palmityl
Palmitylpterosin C (19)	95—97° (hexane)	+52.8° ($CHCl_3$)	H	$-CH_3$	OH	H	$-CH_2O$ palmityl
Phenylacetyl-pterosin C (20)	67—68° (hexane)	+38.6° (MeOH)	H	$-CH_3$	OH	H	$-CH_2O$ phenylacetyl
Pteroside A (21)[a]	116—118° (acetone)	−49.6° (MeOH)	$-CH_2OH$	$-CH_3$	H	H	$-CH_2O$ glucose
Pteroside B (22)[b]	120—122° (MeOH)	−48.8° (MeOH)	$-CH_3$	H	H	H	$-CH_2O$ glucose
Pteroside C (23)	Amorphous		H	$-CH_3$	OH	H	$-CH_2O$ glucose
Wallichoside (24)[c]	216—218° (EtOH)	+24.1° (MeOH)	H	$-CH_3$	OGlu	H	$-CH_2OH$

a) H. Hikino, T. Takahashi, and T. Takemoto, *Chem. Pharm. Bull.* (Tokyo), **20**, 210 (1972).
b) H. Hikino, T. Takahashi, S. Arihara, and T. Takemoto, *Chem. Pharm. Bull.* (Tokyo), **18**, 1488 (1970).
c) P. Sengupta, M. Sen, S.K. Niyogi, S. Chandra, and E. Ali, *Phytochemistry*, **15**, 995 (1976).

The benzene-extracted bracken powder was then reextracted twice with methanol (240 l) at room temperature for three days and the methanol extract was evaporated *in vacuo* to afford a soup (132PM, about 3.4 kg), which was unable to be dissolved into methanol after perfect drying. The half of the methanol extract (1.7 kg) was treated with a mixture of butanol-butan-2-one and water (1 : 1) to afford an organic layer (132PMO, 310 g) and an aqueous layer (132PMP, 620 g).

Extraction and Fractionation for Acute Toxicity Tests and Cytotoxicity Tests and Isolation of Constituents——Dried young fronds of bracken (sample 2 in Table I, 22 kg) was extracted with hot MeOH and the extract (22 M, 2.22 kg) in water (10 l) was successively partitioned with hexane, benzene and ethyl acetate, giving extracts weighing 391 g (22MH), 35 g (22MB) and 89 g (22MA) respectively. A portion (1 l) of the remaining aqueous layer was extracted with BuOH to afford the BuOH extract (13 g, 22MBu). The other part of the aqueous layer left the residue (1.4 kg, 22MW) by evaporation.

The methanol-extracted bracken was then reextracted three times with hot water (50 l). The water soluble part was evaporated in a spray-drier to afford a mass (2.55 kg, 22W).

These extracts were examined by cytotoxicity and animal toxicity tests (Table II). The hexane extract (250 g), the benzene extract (34 g), and a part of the ethyl acetate extract (49 g) were then precisely separated by column and preparative thin-layer chromatographies as shown in Chart 1-1, 1-2, and 1-3. Each fractions of column chromatographies were examined by thin-layer chromatography (TLC) and used for acute toxicity tests to rats and mice (Table II) along with cytotoxicity tests to HeLa cells (Table II). Sub-fractions obtained by TLC were checked by cytotoxicity tests as shown in Table II.

A portion of the hexane extract (22MH, 90 g) was absorbed on a charcoal column and eluted with MeOH, acetone, and ethyl acetate. Each fraction was examined by TLC and further fractionation by silica-gel column and thin-layer chromatographies and fractional recrystallization afforded a mixture of fatty acids, palmitylpterosin B (**18**, 430 mg), pterosin F (**6**, 170 mg) and pterosin B (**2**, 500 mg).

The butanol fraction (22MBu) was examined by preparative TLC and the presence of pteroside B (**22**, *ca.* 10 mg from 0.1 g extract) was confirmed.

A portion of aqueous layer (*ca.* 100 g, 22MW) was passed through a charcoal column and eluted successively with water, MeOH and EtOAc, leaving the extracts weighing 50 g, 6.5 g and 2.7 g respectively. By preparative layer chromatography on silica-gel of the MeOH eluate, pterosin L (**10**, 20 mg), pteroside B (**22**, 100 mg) and pteroside A (**21**, 100 mg) were isolated and the presence of pterosin C (**3**), pterosin A (**1**) and pteroside C (**23**) was shown. From the EtOAc fraction, pterosins A and C and pterosides A, B and C in small amounts were isolated.

TABLE IV. Known Compounds isolated from Bracken Fronds

Substance	mp (solvent of recrystallization)	$[\alpha]_D$ (solvent)	IR spectrum (cm^{-1})
Glyceryl palmitate	64—70° (*n*-hexane)		3290, 2925, 2870, 1735, 1465, 1175
Benzoic acid	121—122° (*n*-hexane)		3100—2830, 1690, 1425, 1328, 1295, 935, 707
p-Hydroxybenzoic acid[a]	213—214° (water)		3400, 2560—2300, 1628, 1597, 1420, 1312, 1287, 1240
Vanillic acid[a]	211° (benzene)		3500, 2960, 1682, 1600, 1520, 1432, 1298, 1280, 1235, 1200
Vanillin	79—80° (*n*-hexane)		3200, 1670, 1595, 1510, 1460, 1428, 1297, 1260, 1198, 1170, 1150
Kaempferol	275—280° (methanol)		3260, 1660, 1610, 1570, 1500, 1380, 1308, 1245, 1218, 1170
Astragalin[b]	180—184° (methanol)	−12.2° (methanol)	3200, 1647, 1605, 1495, 1355, 1287, 1170, 1160, 1010
Tiliroside[c]	216—219° (methanol)	−53.1° (methanol)	3420, 3240—3120, 2900, 1685, 1655, 1610, 1500, 1355, 1295, 1180
p-Coumaroylquinic acid	220° (dec.) (methanol)	+22.2° (methanol)	3360—3280, 2800, 2610—2320, 1688, 1608, 1285, 1120, 1075
β-Sitosterol	136—137° (*n*-hexane)		3400, 2930, 1462, 1380
Stigmast-4-en-3-one	70—73° (methanol)	+78.7° (chloroform)	2925, 1680, 1620, 1463, 1380
5α-Stigmastane-3,6-dione	203—205° (methanol)	+5.5° (chloroform)	2930, 1713, 1458, 1422, 1384, 1254

a) A.D.M. Glass and B.A. Bohn, *Phytochemistry*, **8**, 629 (1969).
b) See Ref. 7.
c) See Ref. 37.

Conventional Procedure for the Isolation of Pterosins and Pterosides from Young Fronds——For toxicological tests especially for carcinogenicity test and also for chemical works further amounts of materials became necessary. For such purpose the separation shown in Chart 2 was performed to isolate pterosins and pterosides from young fronds.

By these methods about twenty kinds of 1-indanone derivatives named pterosins and pterosides were obtained as shown in Table III besides known compounds such as glyceryl palmitate, benzoic acid, *p*-hydroxybenzoic acid, vanillin, vanillic acid, *p*-coumaroylquinic acid, kaempferol, astragalin,[7] tiliroside, β-sitosterol, stigmast-4-en-3-one and stigmastane-3,6-dione. The physical properties of these compounds were shown in Table IV and they were identified by conventional methods comparing with the authentic specimens.

Extraction and Isolation of the Constituents of Rhizomes——Since rhizomes were proved to be more toxic the constituents of the rhizomes were also analyzed. The details will be shown in Part 3 of this series.[28]

Cytotoxicity Tests to HeLa Cells——HeLa cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum. For testing toxic concentration and morphological changes, modified panel method was employed.[29] Each cup of panels contained round cover-glasses, and the cells grown on them, were treated with test samples for 3 days. These cover-glasses were then fixed with Carnoy's fixative and stained with hematoxylin and eosin. Cell injuries were recorded as 0 through 4, where 0 means no appreciable effect, 4 lethal effect and 1, 2, 3, gradual injuries between 0 and 4.

In Table II the concentration exhibiting injuries of the grade 2 for each fraction is shown as 50% growth inhibition concentration (IC_{50}).

Acute Toxicity Assay Using Experimental Animals (Animal Tests)——Animals: For toxicity tests, 6 to 8 week-old male mice (DDD strain bred in the Institute of Medical Science, University of Tokyo) weighing between 19 and 26 g were used.

Male rats of Wistar strain 5-week-old, were purchased from Nippon Rat Co. Ltd. They were initially 68 to 82 g in body weight. Both mice and rats were fed a basal CE-2 pellet or powder except those mice and rats which were fed the dried samples of bracken fronds and rhizomes and the residue of extraction.

Two male guinea pigs of Hartley strains, weighing 710 and 750 g, were obtained from Nihon Clea Co. Ltd. and used for toxicity test.

Samples and Ways of Administration: All dried samples of bracken and residues of water extraction were mixed with the basal diet in the proportion by weight of 30%. These were molded by water into cakes and were fed to mice (5 g/day) and rats (15 g/day) for three days. Each extract and fraction were dissolved in olive oil to make 1—10% solution. For subcutaneous injection into a mouse 0.2 ml (or 0.3 ml)/20 g body weight was used and for gastric intubation into a mouse 0.4 ml/20 g body weight was used.

Mice were killed three days after subcutaneous injection or gastric intubation. In some cases, mice were injected subcutaneously for consecutive three days and killed on the fourth day. The control mice received 0.2 ml or 0.4 ml/20 g body weight solvent only.

Double concentrations of test samples in olive oil were made for rats and 0.4 ml/200 g body weight solution was injected subcutaneously into a rat. Control rats received 0.4 ml/200 g body weight olive oil only. Some of the isolated compounds were also tested individually. Rats were killed three days after injection.

Each 30 g of dried powder of bracken rhizomes mixed with the basal diet (CG-3) in the proportion by weight of 30% were given to guinea pigs daily for 5 days.

All animals were allowed to food and water *ad libitum*.

Animals were weighed every day, the general conditions of the animals were observed and the abnormal signs, if any, were recorded.

Animals killed or dying were carefully autopsied. Histological sections of heart, lung, liver, kidney, pancreas, thymus, lymph node, spleen, bone marrow, adrenal, stomach, small and large intestines, urinary bladder, and testis were prepared and stained with hematoxylin and eosin.

Carcinogenicity Tests——For Experiment 1, 8- to 10-week-old male rats and for Experiment 2, 7- to 8-week-old male rats were obtained from Nihon Clea Co., Ltd. and used for the feeding tests. Dried powder of young fronds or rhizomes of bracken and residues after extraction were mixed with the basal diet (CE-2 powder) in the proportion by weight of 30% and made into pellets. Other samples, benzene, ethyl acetate and methanol extracts, pterosin B and pteroside B in Experiment 1 and the fractions, 132PB, 132PBN, 132PBO, 132PBP, 132PM, 132PMP and 132PMO, in Experiment 2, were mixed with the basal diet at the various ratio, each equivalent to 1.6—3 times as much as concentration contained in dried bracken. These diets of 15 to 20 g per rat daily were administrated perorally for 35 to 205 days. After the termination of feeding each samples, the basal diet was given freely.

Animals were weighed every two weeks, intake of diet was checked periodically and general condition of the animals were observed.

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28) M. Kuroyanagi, M. Fukuoka, K. Yoshihira, and S. Natori, *Chem. Pharm. Bull.* (Tokyo), in the press.
29) M. Umeda, T. Yamashita, M. Saito, S. Sekita, C. Takahashi, K. Yoshihira, S. Natori, H. Kurata, and S. Udagawa, *Jap. J. Exp. Med.*, **44**, 83 (1974).

Complete autopsies were performed on the animals killed or dying. Histological examination was performed on the main organs including intestinal tissues showing macroscopic abnormalities. Paraffin sections prepared in the usual fashion were stained with hematoxylin and eosin.

Results and Discussions

Biological testing methods applicable as a monitor for fractionation and separation are prerequisite for the isolation of biologically active substances. When we commenced this work in 1970 mutagenicity tests using bacteria, now being widely used for preliminary tests for carcinogenicity,[30] had not developed. As a matter of convention cytotoxicity tests using HeLa cells and acute and subacute toxicity tests to rats and mice with pathological examination were employed for the monitoring. The preliminary tests to compare the parts of the

Table V. Results of Preliminary Animal Toxicity Test (Mouse) and Cytotoxicity (HeLa Cell)

Fraction[a]	Toxicity (grade) and toxic pattern[b]			Cytotoxicity IC_{50} (μg/ml)
	s.c.×1	*s.c.*×3	*p.o.*	
31E	−	−	−	Over 1000
32E	/	−	−	520
32PH	±M	−	−	690
32PB	/	−	−	520
32PA	±M	−	−	570
32PM	+L	−	−	Over 1000
33E	/	−	−	620
34E	/	/	−	570
34PH	/	/	−	470
34PB	/	/	−	520
34PA	/	/	+	470
34PM	−	/	−	620
35E	−	−	−	690
36E	−	−	−	520
36PH	−	−	−	620
36PB	−	−	−	470
36PA	−	−	−	570
36PM	−	−	−	Over 1000
51E	+M	/	/	520
52E	±	/	/	1000
52PB	−	/	/	570
52PA	−	/	/	570
52PM	±K	/	/	Over 1000
55E	±	/	/	570
56PB	±	/	/	520
56PA	−	/	/	170
56PM	−	/	/	470
Dried fronds (32)	/	/	+*	
Control	/	−	−*	

a) First numbers (3 and 5) indicate the sample number in Table I. Second numbesr (1—6) indicate as follows: 1: fresh fronds, 2: dried fronds, 3: fresh stalks, 4: dried stalks, 5: fresh rhizomes, 6: dried rhizomes. E, PH, PB, PA and PM indicate the solvents for extraction. E: ethanol extract; PH, PB, PA and PM are the eluates obtained by the successive percolation with hexane, benzene, ethyl acetate and methanol respectively.

b) M: damage on actively-dividing cells; L: hepatotoxic; K: nephrotoxic. ##: lethal toxicity, #: showing definite pathological lesions with decrease of body weight, +: either showing definite histological lesions or loss of body weight, ±: positive histological lesions only, −: no positive evidence for toxic effects.

30) T. Sugimura, "Fundamentals in Cancer Prevention," P.N. Magie, ed., University of Tokyo Press, Tokyo, 1976, p. 191.

plants, methods of drying, and the plants collected at different season of an year and locality were performed with these toxicity tests. Although there existed some difference in the constituents according to the part of the plants, other factors seemed to show no remarkable difference in these materials. Furthermore we could not observe any syndrome relating to carcinogenicity or the cattle poisoning in the experimental animals as shown in Table V.

However, since the carcinogen(s) and cattle poison(s) had been known to be extractable by alcohol and the cell culture tests indicated that the extracts obtained by non-polar solvents showed stronger cytotoxicity than those obtained by successive extraction by polar solvents (Table II), we initiated the work from methanol extraction, followed by successive partitioning with hexane, benzene, ethyl acetate, and butanol using young fronds of bracken collected at Nayoro, Hokkaido, where the cattle poisoning had occurred nearby. The hexane, benzene, and ethyl acetate extract were then extensively separated by the combination of column and preparative thin-layer chromatographies as shown in Chart 1-1, 1-2, and 1-3. Each fractions obtained by column chromatography were used for toxicity tests using rats and mice expecting concentration of the active principle may render some pathological observations. Whole subfractions obtained by further separation by TLC were checked by the cytotoxicity tests to HeLa cells, the available method for checking a large numbers of samples in small amounts.

The results of animal test using mice and rats for the extracts and for the fractions of the hexane extract were shown in Table II.

Table VI. Toxic Effects of Pterosins and Pterosides on HeLa Cells and on Mouse

Compound	Formula	Cytotoxicity IC_{50} (μg/ml)	Toxicity (grade) and toxic pattern[a]	
			s.c.	*p.o.*
Pterosin Z	**(13)**	10		
Pterosin I	Methyl ether of pterosin Z	60		
Pteroside Z	Glycoside of pterosin Z	195		
(3*R*) Pterosin D	**(4)**	Over 320		
(3*S*) Pteroside D	Glycoside of (3*S*) pterosin D	320		
(2*S*) Pterosin A	**(1)**	320	−	/
(2*S*) Pterosin K	**(9)**	Over 100		
(2*R*, 3*R*) Pterosin L	**(10)**	180		
(2*S*)Pteroside A	**(21)**	Over 320		
(2*R*) Pterosin B	**(2)**	100	+ L	−
(2*R*) Pterosin F	**(6)**	65		
(2*R*) Pterosin E	**(5)**	30	±	/
(±) Pterosin O	**(12)**	30		
(2R) Palmitylpterosin B	**(18)**	Over 100		
Acetyl-Δ^2-dehydropterosin B[b]		10		
(2*R*) Pteroside B	**(22)**	Over 100		
(2*S*, 3S) Pterosin C	**(3)**	320	−	/
(2*S*, 3*S*) Pterosin J	**(8)**	Over 100		
(2*S*, 3*S*) Acetylpterosin C	**(14)**	Over 100		
(2*R*, 3*R*) Pteroside C[c]		Over 320		
(2*S*) Pterosin G	**(7)**	180		
Pterosin N	**(11)**	220		
(2*S*) Pterosin P[d]		100		
(2*S*) Pteroside P[c]		Over 320		

a) See Table II and V.
b) See Part II.
c) Isolated from the rhizome, see Part III.[28]
d) See Part III.[28]

As shown in the table several fractions of the chromatographic separation exhibited noticeable cytotoxicity to HeLa cells but further separation of such fractions indicated that the amounts of the cytotoxic fractions such as those in MHP (*cf.* Chart 1-1 and Table II) are quite small in amounts and as a whole negligible.

In the course of such precise fractionation many fractions showing the presence of crystalline compounds or a single spot in TLC were obtained. These fractions were purified further to lead the isolation of more than thirty constituents of the bracken fronds. Most characteristic constituents in these are a group of C_{14} or C_{15} sesquiterpenoids with 1-indanone nucleus, named pterosins, and the glucosides, pterosides,[19–23] the total yield being about 0.1% and assumed as the major constituents of the plant fronds as a whole. They are cumulatively shown in Table III and the details of the structure elucidation of these compounds will be the subjects of the following papers.

Besides these indanone derivatives, several phenolic compounds and steroids shown in Table IV were also isolated and identified with authentic samples.

In these characterized compounds some of the indanone derivatives showed cytotoxicity to HeLa cells (Table VI). The morphology of HeLa cells injured by these indanone compounds (toxic degree 1—3) was more or less similar. The size of treated cells became more or less smaller. Some cells became spindle-shaped and the others retained polygonal shape. The cytoplasm was clear, sometimes with vacuoles. The nucleus stained deeper with hematoxylin and tiny chromatin condensates were scattered throughout the nucleus. The nucleolus became rounded. Mitotic figures were decreased. However, these morphological changes did not suggest any sign of alkylation which is one of the action of most carcinogenic compounds.

The animal toxicity tests were also performed on some of the pterosins as shown in Table VI with no obvious toxicity.

For such chemical and biological works especially for carcinogenicity tests for the constituents a large amount of materials was required and the extraction and isolation of pterosins were repeated; the typical example of such conventional procedures for the isolation of pterosins was shown in Chart 2. The constituents of the rhizomes were also analyzed and the details will be reported in Part 3 of this series.[28]

Recently, many screening methods for estimating the carcinogenic properties of chemicals have been explored, though the only method for ascertaining the carcinogenicity is to demonstrate cancer production in animals treated with chemicals. However, because of limitation of available amounts of the sample, we had to estimate carcinogenic property by combining the results of several biological assays. Since Evans disclosed the possible radiomimetic nature of the bracken toxin,[8] a variety of small scale biological assays have been tried for the isolation of carcinogenic agents from bracken. However, they were abandoned due to insufficient specificity of assays or difficulties in obtaining the biologically active fractions. Ushijima and Yuasa[31] recently demonstrated that appearance of hematuria, edema with inflammatory change of the bladder and activation of lysosomal enzymes in the bladder mucosa were charateristic signs of guinea pigs fed some fractions of bracken. Our assay using guinea pigs by feeding powder of rhizomes revealed marked edema and mucosal hemorrhage of their urinary bladder, but without macroscopic hematuria.

As for animal tests frequently observed findings were damages on the actively dividing cells including involution of thymus and cytotoxic changes in the lymph follicles and in the crypts of the small intestine.[32] However, these changes observed by the extracts, the frac-

31) J. Ushijima and R. Yuasa, The paper presented at Annual Meeting of Japan Society of Veterinary Sciences, 1974.

32) M. Enomoto, Y. Sodemoto, K. Miyata, M. Harada, and K. Sato, *The St. Marianna Medical J.*, 4, No. 2, 63 (1976); Y. Sodemoto and M. Enomoto, *ibid.*, 4, No. 2, 77 (1976); M. Enomoto, K. Miyata, K. Sato, and Y. Hatanaka, *ibid.*, 4, No. 3, 57 (1976).

TABLE VII. Carcinogenicity Tests

Sample	Initial No. of rats	Strain of rats	Approximate amount applied in diet; per day	Period of feeding; days	No. of survivors at the end of feeding	No. of rats with tumors: Ileum	No. of rats with tumors: Other tissues	Total No. of tumor (range of the tumor occurrence; days)
Experiment 1								
Young fronds (curling tops) (sample 7)[a]	16	Wistar	30%	120	12	2	1(colonal adenocarcinoma)	3(426—802)
Young fronds (sample 4)	16	Wistar	30%	120	12	3	0	3(456—650)
Young fronds (sample 10)	16	Fischer	30%	120	15	7	0	7(232—365)
Rhizomes (sample 6)	16	Wistar	30%	120	12	5	2(colonal adenocarcinoma and lymphoma)	7(120—508)
Benzene extract[b]	16	Wistar	55 mg	120	14	1	3(hepatic sarcoma, colonal adenocarcinoma and bladder carcinoma)	4(580—718)
Ethyl acetate extract[b]	16	Wistar	26 mg	120	14	0	1(mammary carcinoma)	1(713)
Methanol extract[b]	16	Wistar	277 mg	120	16	1	1(reticulum cell sarcoma)	2(460—626)
Residues after extraction[b]	16	Wistar	30%	120	10	0	1(hepatic sarcoma)	1(487)
Pterosin B	16	Wiatar	4 mg	120	14	0	0	0
Pteroside B	16	Wistar	10 mg	120	15	0	0	0
Experiment 2								
Young fronds (sample 13)	20	Fischer	30%	115	20	13	0	13(250—440)
132PB[b]	20	Fischer	167 mg	150	20	0	0	0
132PBN[b]	8	Fischer	60 mg	180	8	0	1(myeloid leukemia)	1(375)
132PBO[b]	8	Fischer	96 mg	170	8	0	1(adrenal adenoma)	1(410)
132PBP[b]	8	Fischer	30 mg	185	8	0	1(adenocarcinoma of neck)	1(415)
132PM[b]	20	Fischer	340 mg	160	16	0	1(myeloid leukemia)	1(365)
132PMP[b]	8	Fischer	220 mg	205	8	0	0	0
Rhizomes (sample 14)	20	Fischer	30%	35	11	4	2(myeloid leukemia and abdominal fibrosarcoma)	6(390—529)
	20	Fischer	7%	196	19	6	0(hyperplastic nodule, liver)	6(490)

a) Numbers are those in Table I.
b) See Materials and Methods (p. 2348).

tions and the indanone derivatives (Tables II, V, VI) were possibly reflecting the radiomimetic property of toxic principles of bracken, but they were not so remarkable as compared with those found in animals fed the original powder of bracken. Our attempts to concentrate these biological activity in the specific fraction have been unsuccessful.

The followings were noticed as other pathological findings: Damage of the hepatopoietic cells of the bone marrow or injury of spermatogenesis were scarcely observed. Hepatic changes were vacuolar degeneration of the liver cells. Renal changes were mostly vacuolar degeneration of the tubular epitheliums with appearance of casts. Increase in mitotic figures of the liver cells was found sporadically in some of mice including controls.

Since these observations did not suggest any syndrome relating to carcinogenicity we initiated long-term feeding experiments to test the carcinogenicity (Experiment 1 in Table VII), though the experiments need a lot of time and materials. The dried young fronds, the extracts obtained by successive extraction with benzene, ethyl acetate, and methanol, and the residual fronds after extraction were used for Experiment 1 in Table VII. Since the rhizomes were reported to exhibit stronger carcinogenicity[25] the rhizomes were also feeded. Although there existed no evidence to support the activity, pterosin B, the major indanone derivatives in the fronds, and pteroside B, the major indanone glucoside in the rhizomes,[28] were also tested after the isolation of these compounds in large amounts.

Since the benzene extract seemed to be more effective than other extracts in Experiment 1, further separation was carried out as shown in Materials and Methods (p. 2348) and the fractions thus obtained were tested in Experiment 2.

The results are shown in Table VII.

Mean body weight of rats administrated each sample, except benzene extract in Experiment 1 and 132 PMP in Experiment 2, showed slight decrease for the first month but showed gradual increase from the second month. Rats administrated benzene extract and 132 PMP showed increase of body weight from the first month.

Ileac tumors observed in the rats administrated powdered fronds and rhizomes in Experiments 1 and 2 were histopathologically epithelial tumor including adenoma and papillary adenocarcinoma and all were localized in the area of the ileum, mainly at 15 cm from the ileac end, as was reported by Hirono.[15] Metastatic lesions in the ileac subserosa were noticed in one case of rat with ileac adenocarcinoma administrated young fronds in Exp. 2.

The interesting findings observed in the carcinogenicity tests of bracken in the rats were strain difference in tumor incidence and target of their carcinogenic effect. Fischer rats showed higher susceptibility to carcinogenic effect than Wistar rats. As for target tissues a variety of tumors was induced in Wistar rats, though some of them seemed not attributable to bracken. When these observations were compared with those of carcinogenicity experiments of bracken in rats,[15] mice, hamster, guinea pigs, rabits, qualis, sheep,[3,8,33] and cattle,[10,12] difference in incidence and variety of tumors induced by bracken is likely to be influenced by many factors including species and strains differences of animals, source and parts of bracken administered, administration period, total amount of bracken fed and age of animals at the start of administration.

Although the fronds and rhizomes induced tumors as reported by many workers, all our trials using the extracts and the fractions were unsuccessful in inducing tumors probably due to insufficient amounts administered.

While we were spending several years in vain for the isolation of carcinogenic principle(s), several attempts were also conducted on the same line in other laboratories. Pamukcu and the coworkers attempted a comparison of the carcinogenicity of several fractions of the extracts by the pellet implantation to bladder lumen of mice[34] and finally reached to a conclusion

33) I.A. Evans, "Chemical Carcinogens," American Chemical Society Monograph No. 173, ed. by C.E. Seale, American Chemical Society, Washington, D.C., 1976, p. 690.
34) A.M. Pamukcu, J.M. Price, and G.T. Bryan, *Cancer Res.*, **30**, 902 (1970).

that a tannin fraction might be the carcinogenic principle.[35] However, the tannin does not seem to be carcinogenic when administered orally.[36] In the course of their works they isolated and identified organic acids, astragalin, isoquercitrin, and tiliroside as the constituents[37] and examined the effect by their pellet implantation method with no positive results.[35]

The report of Evans *et al.*[38] that they succeeded in the isolation of a carcinogenic and mutagenic principle, $C_7H_8O_4$, attracted much attention but later the compound was proved to be shikimic acid.[39] They claimed that shikimic acid,[40,41] the precursor of aromatic amino acids and other phenolics occurring ubiquitously in all plants, showed unexpectedly carcinogenicity.[39] However, reexamination by other workers since then has never proved their results.[42]

Hirono's report that the boiling-water extracts clearly showed carcinogenicity[43] was noticeable in suggesting the nature of the carcinogen(s). By the collaboration with Hirono's group Takatori[44] and Aoki[45] reported the isolation of pterolactam, the related compounds, organic acids, and indanones from water-soluble fractions but none of them were assumed to be carcinogenic and one of them, pterolactam, was failed to exhibit carcinogenicity.[46]

Iwata[47] reported the bracken poison for cattle exists in the fraction not precipitated by lead acetate but by basic lead acetate of methanol extract of the rhizomes using cattle and observing haematuria.

Regarding whole these works nobody has succeeded in obtaining a conclusive evidence for the carcinogen(s) and/or cattle poisoning(s).

In this period of time the testing methods for mutagenicity using bacteria, mammalian cells and others have developed rapidly and the correlation with carcinogenicity is much discussed.[30] Although there still exists several problems to correlate directly mutagens to carcinogens, the bacterial mutagen tests have some advantages in requiring small amounts of samples and getting results in a short period of time. We have tested several fractions obtained in the course of separation, those prepared newly in views of the recent works abovementioned, and pterosins and other isolated compounds by mutation tests using bacteria (*Salmonella typhimurium*) and mammalian cells (FM3A cells) and also by chromosome aberration tests using Chinese hamster cells. The results so far obtained were not promising and, as far as the pterosin derivatives concerned, none of them so far examined exhibited mutagenicity[48] and chromosome aberration.[49]

35) C.Y. Wang, C.W. Chiu, A.M. Pamukcu, and G.T. Bryan, *J. Natl. Cancer Inst.*, **56**, 33 (1976).
36) A.M. Pamukcu, private communication; *cf.* C.Y. Wang, A.M. Pamukcu, and G.T. Bryan, International Conference on Ecological Perspectives on Carcinogens and Cancer Control, Cremona, Italy, Sept. 1976.
37) C.Y. Wang, A.M. Pamukcu, and G.T. Bryan, *Phytochemistry*, **12**, 2298 (1973).
38) H. Leach, G.D. Barber, I.A. Evans, and W.C. Evans, *Biochem. J.*, **124**, 13p (1971).
39) I.A. Evans and M.A. Osman, *Nature* (London), **250**, 348 (1974).
40) B. Stavric and D.R. Stoltz, *Fd. Cosmet. Toxicol.*, **14**, 141 (1976).
41) Our reexamination of their procedure proved the presence of shikimic and quinic acids in the extract by paper chromatography.[24]
42) I. Hirono, K. Fushimi, and N. Matsubara, *Toxicol. Let.*, **1**, 9 (1977).
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44) K. Takatori, S. Nakano, S. Nagata, K. Okumura, I. Hirono, and M. Shimizu, *Chem. Pharm. Bull.* (Tokyo), **20**, 1087 (1972).
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Further attempts to approach the causative(s) are under progress in our laboratories.

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