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Chemical and Toxicological Studies on Bracken Fern, *Pteridium aquilinum* var. *latiusculum*. VI.[1] Isolation of 5-*O*-Caffeoylshikimic Acid as an Antithiamine Factor

Masamichi Fukuoka

National Institute of Hygienic Sciences, Kamiyoga-1-chome, Setagaya-ku, Tokyo 158, Japan

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5-*O*-Caffeoylshikimic acid (dactylifric acid) was isolated from bracken fern as a major constituent of its acutely toxic fraction, which causes depression of leucocytes and thrombocytes in calves. 5-*O*-Caffeoylshikimic acid exhibited an antithiamine effect *in vitro*, but had no hematuric effect in guinea pigs.

Keywords——*Pteridium aquilinum* var. *latiusculum*; antithiamine factor; hematuric effect; bracken toxin; 5-*O*-caffeoylshikimic acid; astragalin

Introduction

It is known that bracken fern (*Pteridium aquilinum* Kuhn var. *latiusculum* Underwood, Pteridaceae) contains an acutely toxic and a chronically toxic principle(s); the former causes a hemorrhagic syndrome in cattle[2] and an avitaminotic syndrome in homogastric animals such as horses and rats,[3] while the latter causes tumors to develop in cattle,[4] rats,[5] mice,[6] guinea pigs,[7] and Japanese quails.[7] However, it is not known whether or not the acute toxicity and the carcinogenicity are attributable to the same compound.

The acute toxicity in the case of cattle is known as "cattle bracken poisoning" which is characterized by hemorrhage, anorexia and destruction of the bone marrow, leading to leukopenia and thrombocytopenia.[8] The cattle bracken poisoning was suggested to be caused by antithiamine factors such as thiaminase,[9] caffeic acid,[10] astragalin[11] and isoquercitrin,[11] but is now considered not to be associated with thiamine deficiency,[12] because of the rumen flora-bacterial synthesis of thiamine in cattle.[13]

On the other hand, the acute toxicity in homogastric animals is a different syndrome, known as "bracken staggers" for horses,[14] "bracken rhizome poisoning" for pigs[15] and "avitaminosis B_1" for rats.[3] The syndrome is not caused by a thermostable antithiamine factor, caffeic acid, in rats[16,17] but is alleged to be caused by a thermolabile antithiamine enzyme, thiaminase I.[18] However there is no report of toxicity caused by an enzyme isolated[19] from the fern as far as I know. The fern exhibits a high thiaminase I activity, and hence it seemed desirable to examine whether or not the fern contains antithiamine factors other than thiaminase,[9] caffeic acid,[10] astragalin[11] and isoquercitrin.[11]

This paper deals with the isolation and some properties of antithiamine factors from bracken fern.

Experimental

Melting points were measured in a Yanaco micro apparatus and are uncorrected. Ultraviolet (UV) spectra were determined in methanol solution and infrared (IR) spectra in KBr discs unless otherwise specified. ^{1}H- and ^{13}C-Nuclear magnetic resonance (PMR and CMR, respectively) spectra were measured in CD_3OD with Me_4Si as an internal standard and chemical shifts, determined on a JEOL JNM-FX 200 Fourier transform NMR spectrometer, are given in δ value (ppm). Abbreviations are s=singlet, br s=broad singlet, dd=double-doublet, t=triplet and m=multiplet. Optical rotations were determined with a JASCO DIP-180 automatic polarimeter. Mass spectra were determined on a JEOL 01SG-2 high-resolution mass spectro-

meter with direct inlet system for electron impact (EI) mass spectroscopy (MS), and a JEOL JMS-DX 300 mass spectrometer and a Hitachi Model 80 gas chromatograph-double-focus mass spectrometer for field desorption (FD) mass spectroscopy. Intensities of fluorescence were measured with a Hitachi MPF-4 fluorescence spectrophotometer at 435 nm emission wavelength and at 365 nm excitation wavelength. Centrifugal partition chromatography was carried out with a Sanki Engineering centrifugal partition chromatograph, Model L-90; volume of one column: 20 ml; 18-hole, cassette column.

Plant Materials——Dried bracken leaves used were the same as those used in the previous paper and proved to induce tumor in rats.[20]

Decomposition of Thiamine by Thiochrome Negative Form——Thiamine HCl (17 μg/ml, 0.1 ml) in 0.1 N HCl solution[21] was mixed with 1/30 M phosphate buffer (pH 7.0, 0.2 ml) and samples (17 μg or its multiple/ml, 0.1 ml). The solutions were incubated at 37°C for 48 h. The solutions were analyzed by the thiochrome method[21,22] for the determination of remaining thiamine (Chart 1).

Isolation of Antithiamine Factors from Bracken Fern——Bracken powder (500 g) was twice extracted with boiling 70% ethanol (4 l) for 10 min and the ethanol extract thus obtained was subjected to separation, monitored by following the *in vitro* antithiamine activity, as shown in Chart 1. Two active fractions (Fr. II and Fr. III) were obtained and Fr. III was crystallized from aqueous methanol to give a crystalline compound, mp 175—178°C, which was identified as astragalin[20] by mixed mp determination and comparison of the IR

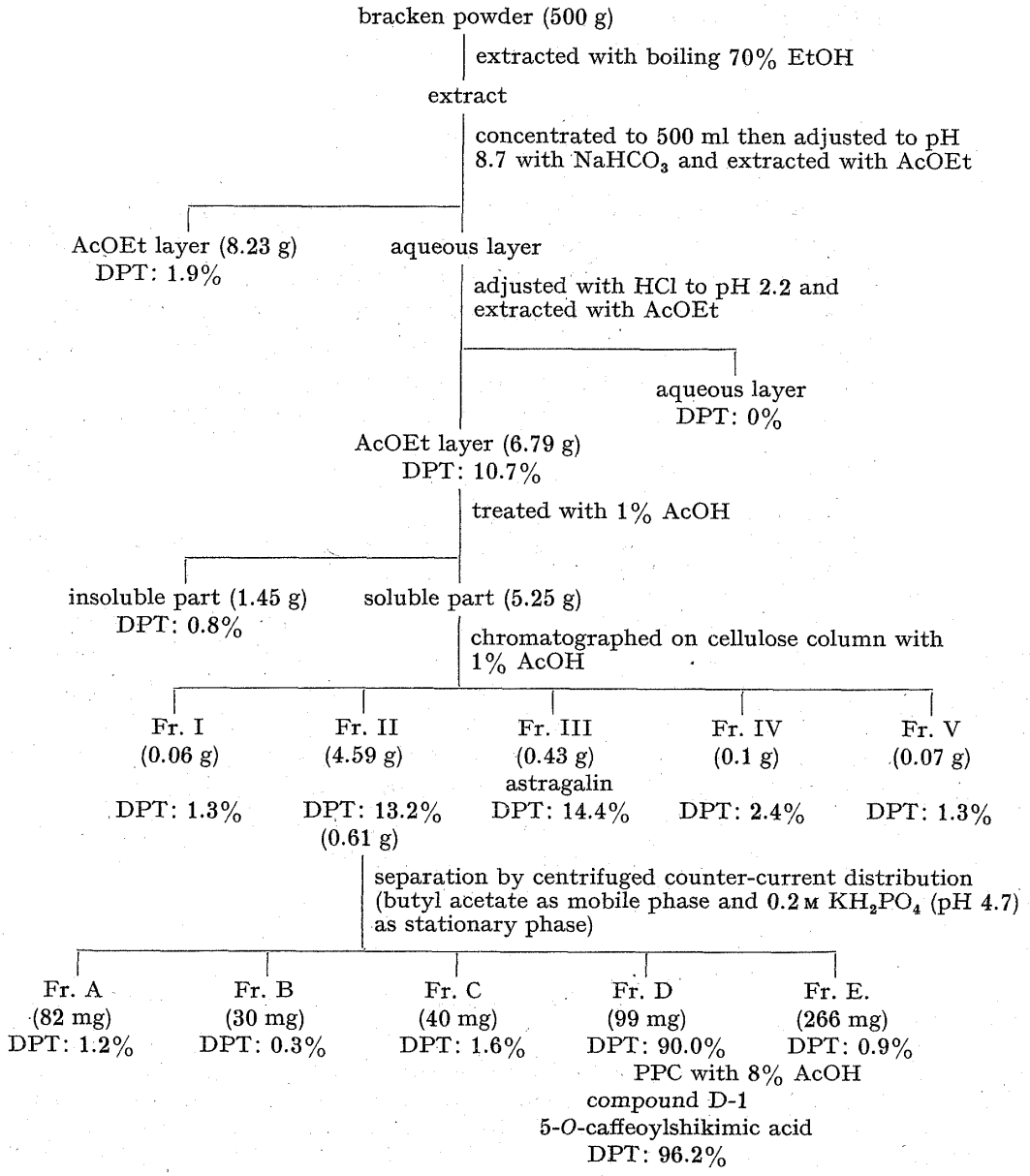


Chart 1. Isolation of Antithiamine Factors from Bracken Fern

Abbreviation: DPT=decomposition (%) of thiamine.

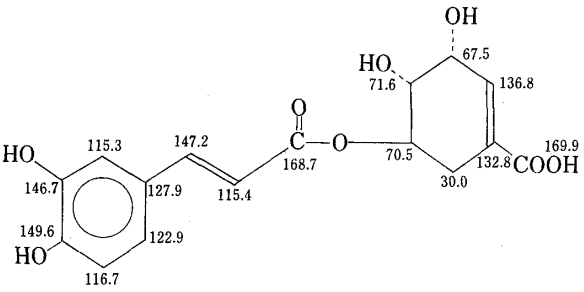
spectrum with that of an authentic sample. Fr. II (0.61 g) was separated by centrifugal partition chromatography to afford an active fraction (Fr. D, about 99 mg). Fr. D was chromatographed on paper (Toyo Roshi No. 514) (PPC) with 8% AcOH, and three compounds, D-1, D-2 and D-3, were eluted with water from the paper. However, the compound, D-2 and D-3, decomposed when water was completely removed, and compound D-1 (about (60 mg) was subjected to physico-chemical analysis in wet form with acetic acid.

Compound D-1 from an acutely Toxic Fraction——The acutely toxic fraction, 'methyl acetate-soluble part' (2.66 g)[18] (*vide post*) supplied by Evans, was chromatographed on a polyamide column (100 mesh, 50 g, ϕ=3.3 cm). Sixty-one fractions were obtained by elution with gradients from 33 to 100% methanol in water and then from 20 to 100% benzene in methanol. Fractions 1 to 5 (1.3 g, 50 ml per fraction), fractions 6 to 10 (0.87 g) and fractions 11 to 61 (0.97 g) were combined. The fractions 1 to 5, which exhibited a yellow color in the eluted solution, showed one spot on thin-layer chromatography (TLC) but changed into a black mass after removal of the solvent. The black mass was separated into a methanol-soluble part and an insoluble part (1.1 g). The soluble part was chromatographed on paper (Toyo Roshi No. 514) with 8% AcOH to give a compound unstable to drying. The compound was indistinguishable from compound D-1 (PPC and TLC, and UV, PMR and CMR spectra).

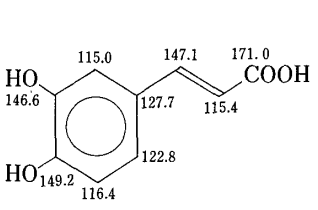
The fractions 6 to 10 were crystallized from aqueous methanol to afford a crystalline compound (0.8 g), mp 175—178°C, identified as astragalin[20] by mixed mp determination and IR spectral comparison with an authentic sample.

From fractions 11 to 61 isoquercitrin, tiliroside and pteroside B were identified by chromatographic comparison with authentic samples isolated from bracken fern.[20]

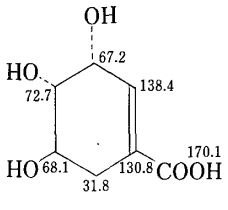
Compound D-1——This compound is a white crystalline powder when wet, but is unstable to drying and heating. The compound shows mp over 300°C after decomposition at near 200°C (depending on the heating rate) and $[\alpha]_D$ −135 (c=0.1 MeOH). UV: λ_{max} 328, 299 (inf.), 244 (inf.) and 217 nm; λ_{max}^{MeONa} 375 and 266 nm; $\lambda_{max}^{AlCl_3}$ 358 and 262 nm; $\lambda_{max}^{AlCl_3\text{-}HCl}$ 329, 299 (inf.) and 245 (inf.) nm. PMR δ: 2.30 (1H, dd, J=18.0, 4.0 Hz), 2.88 (1H, dd, J=18.0, 4.0 Hz), 3.90 (1H, dd, J=7.45, 3.90 Hz), 4.41 (1H), 5.25 (1H), 6.27 (1H, d, J=16.1 Hz), 6.76 (1H), 6.78 (1H, d, J=7.8 Hz), 6.96 (1H, dd, J=7.8, 1.5 Hz), 7.05 (1H, d, J=1.5 Hz) and 7.57 (1H, d, J=16.1 Hz). CMR; see Fig. 1. EI-mass (MS) m/e: 157.0506 (157.0501 for $C_7H_9O_4$), 139.0398 (139.0395 for $C_7H_7O_3$), 138.0335 (138.0333 for $C_7H_6O_3$) and 121.0262 (121.0265 for $C_7H_5O_2$). FD-mass m/z: 336 (M^+, $C_{16}H_{16}O_8$), 337 (M^++1), 359 (M^++Na) and 375 (M^++K).



5-*O*-caffeoylshikimic acid



caffeic acid



shikimic acid

Fig. 1. CMR Spectra of Caffeic, 5-*O*-Caffeoylshikimic and Shikimic Acids

The signal assignments were based on proton off-ressnance and selective proton decouplings and on the ^{13}C-^1H long range couplings of quaternary carbons.

Acid Hydrolysis of Compound D-1——Compound D-1 (about 10 mg) was heated with 2 N HCl (1 ml) for 30 min, then the reaction mixture was allowed to stand overnight. The mixture was extracted with ethyl acetate to afford the ethyl acetate and aqueous layers. Both layers gave caffeic and shikimic acids, which were indistinguishable from authentic specimens by PPC and TLC (*vide infra*).

Chromatographic Identification of Caffeic and Shikimic Acids, Compound D-1 and Flavonoids and Pteroside B——PPC: paper, Toyo Roshi No. 50 and 514, and Whatman No. 1; solvent, *n*-butanol saturated with water, *n*-butanol–acetic acid–water (4: 1: 5), *sec*-butanol–ethyl acetate–formic acid–water (35: 35: 8: 20) and 8% acetic acid for caffeic and shikimic acids[23,24] and compound D-1; detection, fluorescent color under UV light (364 nm) and Pauly's reagent for caffeic acid and Compound D-1, and periodate-aniline reagent[25] for shikimic acid and compound D-1.

TLC: plate, Merck Kieselgel 60 F-254, 5 cm × 20 cm × 0.25 mm; solvent, benzene–dioxane–acetic acid (90: 25: 4),[26] benzene–methanol–acetic acid (45: 8: 4), ethyl acetate–isopropanol–water (65: 24: 11),[27] and chloroform–methanol (8: 2); detection, fluorescent color and absorption under UV light and iodine vapor, periodate–aniline[25] and benzidine–metaperiodate[27] for caffeic acid, shikimic aicd, compound D-1, pteroside B, isoquercitrin, astragalin and tiliroside.

Preparation of 5-*O*-Caffeoylshikimic Acid from Caffeic and Shikimic Acids——An authentic sample of 5-*O*-caffeoylshikimic acid was not available and the synthetic procedure was reported without details of experimental conditions and physical data.[28] Therefore the preparation was performed as follows.

Caffeic acid (1.8 g) was heated at 175°C with dichlorodiphenylmethane (10 ml) for 30 min. The excess dichlorodiphenylmethane was removed *in vacuo* and the residual mass was treated with 10% NaOH to separate benzophenone. The alkaline solution was acidified and extracted with benzene to afford 3,4-diphenylmethylenedioxycinnamic acid (0.64 g, mp 229—230°C, from MeOH). IR ν_{max}: 3400, 1670, 1620 and 1600 cm^{-1}; UV λ_{max} (log ε): 320 (4.24) and 287 (4.15) nm; MS *m/z*: 344.1063 (M^+=344.1049 for $C_{22}H_{16}O_4$); PMR δ: 6.25 (1H, d, J=15.9 Hz), 6.88 (1H, d, J=8.1 Hz), 7.03 (1H, d, J=8.1 Hz), 7.13 (1H, s), 7.34 (5H), 7.55 (5H), and 7.59 (1H, d, J=15.9 Hz).

The cinnamic derivative (1.28 g) was treated with boiling thionyl chloride (20 ml) for 1 h to afford a chloride. The chloride, without purification, was reacted with shikimic acid acetonide (0.79 g)[29] in a mixture of chloroform (15 ml) and pyridine (5 ml) at room temperature for 2 d. The reaction solution was diluted with chloroform, washed with 5% HCl and dried on Na_2SO_4. The mixture was repeatedly chromatographed on silica gel to afford an amorphous product (0.60 g). IR ν_{max}: 3400, 1725, 1705, 1625 and 1600 cm^{-1}; UV λ_{max} (log ε): 326.5 (4.46), 294 (4.29) and 243 (4.33) nm; PMR δ: 1.38 (3H), 1.42 (3H), 2.37 (1H, br d, J=18 Hz), 2.87 (1H, br d, J=18 Hz), 4.30 (1H, br t, J=8 Hz), 4.77 (1H, br s), 5.27 (1H, br s), 6.24 (1H, d, J=15.9 Hz), 6.83 (1H, d, J=8.1 Hz), 7.01 (1H, d, J=8.3 Hz), 7.08 (2H, s), 7.34 (5H), 7.54 (5H) and 7.58 (1H, d, J=15.9 Hz); MS *m/z*: 540.1778 (M^+=540.1774 for $C_{32}H_{28}O_8$).

The amorphous product (0.6 g) was hydrolyzed with 80% acetic acid (16 ml) for 2 h on a steam bath. After concentration under a vacuum, the reaction mixture was extracted with chloroform and then ethyl acetate. The former layer gave benzophenone, indistinguishable from an authentic specimen by IR spectroscopy. The latter layer was chromatographed on paper (Toyo Roshi, No. 514) with 6% acetic acid to afford 5-*O*-caffeoylshikimic acid, which was identical with the natural product, compound D-1 (PPC, TLC, and UV, PMR and CMR spectra).

Results and Discussion

Dried bracken powder, which is carcinogenic to rats,[20] was extracted with aqueous ethanol and the extract was subjected to the separation monitored by following the *in vitro* antithiamine activity as shown in Chart 1. Two constituents, astragalin and a thermolabile compound, were isolated as antithiamine factors.

The structure of the labile compound was established as 5-*O*-caffeoylshikimic acid on the basis of its physico-chemical properties (EI and FD mass, UV, PMR and CMR spectra) and by synthesis from shikimic and caffeic acids for the reason already stated. The compound had already been isolated as an enzymic browning substrate from *Phoenix dactylifera* (Palmae) and named as dactylifric acid.[28]

5-*O*-Caffeoylshikimic acid and astragalin were also isolated as major constituents from the acutely toxic fraction, 'methyl acetate-soluble part' supplied by Evans *et al.*,[18] which caused depression of leucocytes and thrombocytes in calves.[18] The fraction was also proved to contain pteroside B, isoquercitrin and tiliroside as minor constituents.

Although there is no evidence of a correlation between hematuria for cattle and for guinea pigs,[30] it was expected that the constituents of the toxic fraction might exhibit acute toxicity with guinea pigs. Therefore 5-*O*-caffeoylshikimic acid and the known constituents[20] isolated from bracken fern, ptersins A, B, C and F, and pterosides A, B, C and Z, and flavonoids, astragalin and isoquercitrin, were subjected to the hematuria test on guinea pigs[18] by

Ushijima's group.[31] All the constituents exhibited negative results.[31] However, it is known that thiamine deficiency had been produced in lambs and calves[32] and that the deficiency was not observed in mature ruminants having rumen-bacterial synthesis[13] of thiamine, as already stated. Therefore antithiamine factors (thermolabile factor such as 5-*O*-caffeoylshikimic acid and thermostable factors such as astragalin and isoquercitrin[11]) were suspected to be one of the acute toxins for calves.

Leach *et al.*[32] and Evans[33] isolated a toxin from another acutely toxic fraction for both calves and mice. They reported that the toxin did not exhibit mutagenicity in the Ames test and the loss of toxicity took place when the material was dried and stored desiccated at low temperature under nitrogen. Moreover, they insisted that the toxin was finally purified by cellulose TLC and assigned it as shikimic acid from its mass spectrum, which showed an ion peak at *m/z* 156, corresponding to a dehydrate ion of shikimic acid.

Reexamination according to the literature[33] gave an unexpected result. A fraction[33] after purification by cellulose TLC was proved to contain shikimic acid but not to contain 5-*O*-caffeoylshikimic acid. However, the fraction prior to the purification was proved to contain more than twice as much 5-*O*-caffeoylshikimic acid as shikimic acid when checked by TLC, and the EI mass spectrum of 5-*O*-caffeoylshikimic acid showed fragment ions at *m/z* 157.0506, 139.0398 and 138.0335, corresponding to the dehydrate ions of shikimic acids as already stated. Further, shikimic acid was stable to drying (sublimed at about 190°C), whereas the acute toxin of bracken fern was unstable to drying at room temperature. On the other hand, 5-*O*-caffeoylshikimic acid was proved to decompose on drying at room temperature. Nevertheless, Evans *et al.* reported that the toxin isolated from bracken fern and shikimic acid commercially obtained both exhibited the acute toxicity and carcinogenicity to mice.[33,34,36] In another report, on the contrary, shikimic acid was reported not to exhibit carcinogenicity to rats.[37]

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