(200 ml × 4). The combined AcOEt layers were washed with saturated aqueous NaCl solution and dried over Na_2SO_4. Removal of the solvent by evaporation yielded 2.40 g of *p*-aminophenyl acetate (96%). Recrystallization from petroleum ether gave white needles, mp 74—75° (lit.[9] 74°). MS *m/e*: 151 (M^+). IR ν_{max} cm^{-1}: 1748 (C=O). *Anal.* Calcd for $C_8H_9NO_2$: C, 63.57; H, 6.00; N, 9.27. Found: C, 63.59; H, 5.83; N, 9.18.

References and Notes

1) The work described in this paper was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April, 1978.
2) *a*) T. Satoh, S. Suzuki, Y. Miyaji, and Z. Imai, *Tetrahedron Lett.*, **1969**, 4555; *b*) T. Satoh, S. Suzuki, T. Kikuchi, and T. Okada, *Chem. Ind.* (London), **1970**, 1626; *c*) T. Satoh, Y. Suzuki, and S. Suzuki, *Yakugaku Zasshi*, **90**, 1553 (1970); *d*) T. Satoh, K. Namba, and S. Suzuki, *Chem. Pharm. Bull.*, **19**, 817 (1971); *e*) D. Satoh, and T. Hashimoto, *Chem. Pharm. Bull.*, **24**, 1950 (1976).
3) H.C. Brown and C.A. Brown, *J. Am. Chem. Soc.*, **84**, 2827 (1962).
4) T. Nelson, H.C.S. Wood, and A.G. Wylie, *J. Chem. Soc.*, **1962**, 371.
5) J. Jardine, *Chem. Commun.*, **1969**, 477.
6) H.H. Jaffe, *Chem. Rev.*, **53**, 191 (1953).
7) Y. Ogata and I. Sugiyama, *Science* (Japan), **19**, 232 (1949).
8) These compounds were identified by comparison of their melting points, IR spectra and mass spectra with those of authentic samples after isolation from the reaction mixture.
9) L. Galatis, *Chem. Ber.*, **59**, 849 (1926).

Chem. Pharm. Bull. 29(5)1445—1451(1981)

The Constituents of *Paris verticillata* M.*v.* Bieb.

Kimiko Nakano,[a] Kōtarō Murakami,[a] Toshihiro Nohara,*,[a] Toshiaki Tomimatsu[a] and Toshio Kawasaki[b]

Faculty of Pharmaceutical Sciences, Tokushima University,[a] Shomachi 1-78, Tokushima, 770, Japan, and Faculty of Pharmaceutical Sciences, Kyushu University,[b] Maedashi 3-1-1, Higashi-ku, Fukuoka, 812, Japan

(Received October 6, 1980)

Nine compounds (**1**—**9**) have been isolated from the whole plants of *Paris verticillata* M.*v.* Bieb. (Liliaceae) and their structures characterized. They can be divided into four groups; phytosteryl derivatives (**1**, phytosteryl (6′-palmitoyl)-β-D-glucopyranoside; **2**, the despalmitate of **1**), phytoecdysones (**3**, ecdysone; **4**, ajugasterone A; **5**, ecdysterone), pennogenin glycosides (**6**, pennogenin tetraglycoside (T-g); **7**, prototype glycoside of **6**), and kaempferol glycosides (**8**, kaempferol 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside; **9**, 7-O-β-D-glucopyranosyl kaempferol 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside).

Keywords——*Paris verticillata*; Liliaceae; phytosteryl (6′-palmitoyl)-glucoside; ecdysone; ajugasterone A; ecdysterone; pennogenin glycoside; furostanol glycoside; kaempferol glycoside

As a part of our studies on the constituents of *Paris* and *Trillium* plants (Liliaceae) with a view to obtaining the physiologically active components, we have surveyed whole plants of *Paris verticillata* M.*v.* Bieb. and have isolated nine compounds (**1**—**9**) including ecdysone.[1] It is noteworthy that the latter was obtained in a yield of 0.035% from this plant. This report deals with the isolation and the structure elucidation of the nine compounds by spectral and chemical means.

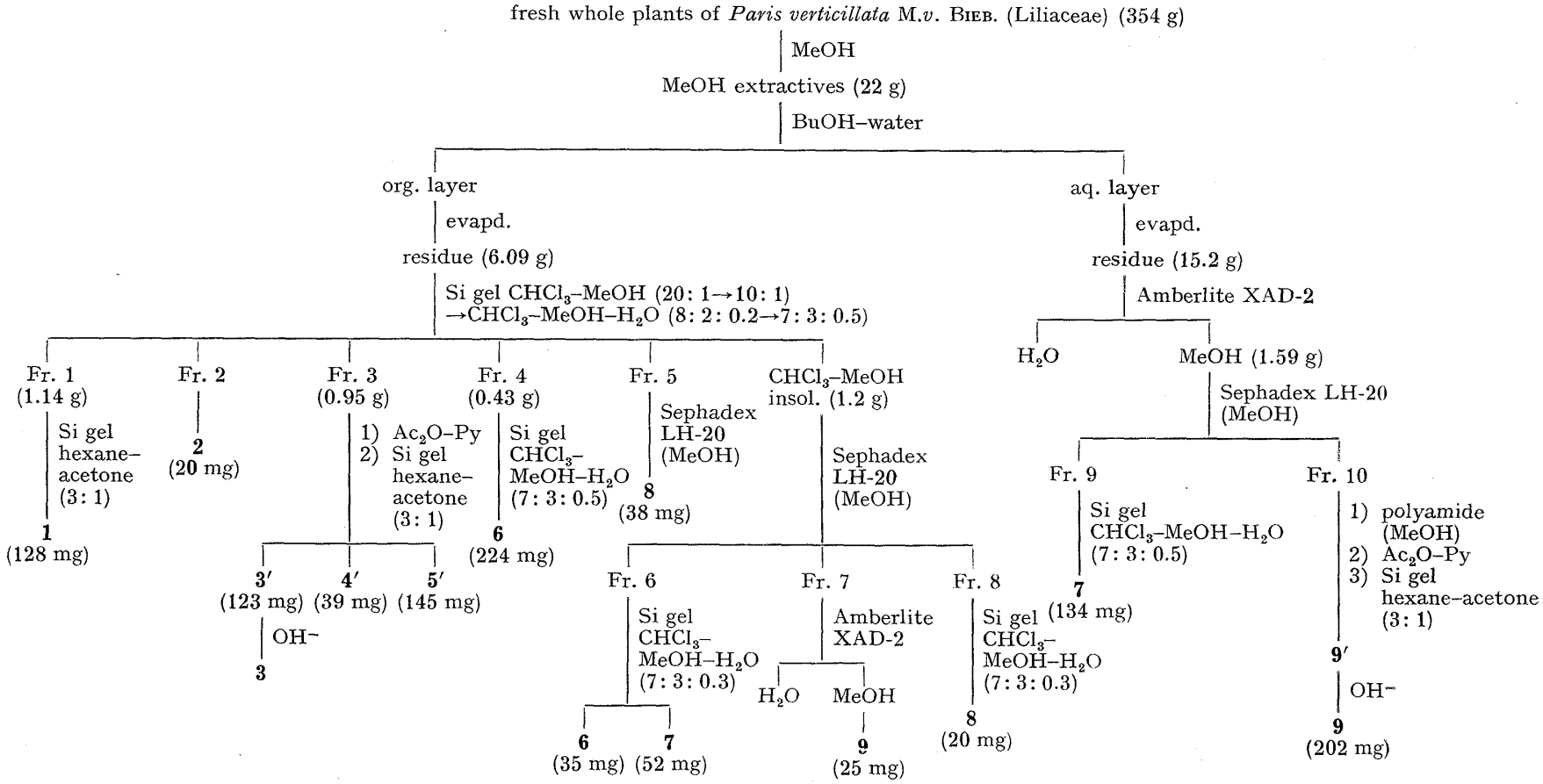


Chart 1

The methanolic extractive of the fresh whole plants was treated as shown in Chart 1 to give **1**, **2**, **3′**—**5′** and **6**—**9**.

1, an amorphous powder, $[\alpha]_D$ −73.3°, showed absorptions due to hydroxy (3500—3300 cm^{-1}) and carbonyl (1720 cm^{-1}) functions in its infrared (IR) spectrum. The proton nuclear magnetic resonance (PMR) spectrum of **1** showed signals due to the methyls (δ 0.65—1.00) of phytosterol, the methylene (δ 1.25) of fatty acid and the carbinol methines (δ 3.26—3.60) of the sugar component. Since **1** was assumed to be an ester of a phytosteryl glycoside and a fatty acid, it was saponified with a weak base to yield a phytosteryl glucoside, mp 292—295°, and palmitic acid. Gas–liquid chromatographic (GLC) analysis indicated that the steryl moiety is a mixture of β-sitosterol and stigmasterol in a ratio of 3:1. As regards the site of linkage of the palmitoyl residue to the sugar, it was deduced that fatty acid was bound at 6′-OH of the steryl glucosyl moiety, because the signals due to 2′-, 3′- and 4′-H appeared at 3.26—3.60 ppm, in the region of the methines adjacent to the free hydroxy groups of the glucosyl residue in the PMR spectrum. Therefore, **1** was considered to be a phytosteryl (consisting of a mixture of β-sitosterol and stigmasterol) (6′-palmitoyl)-β-D-glucopyranoside. **1** appears to be analogous to Glycoside P_1 isolated from rhizomes of *Panax japonicum* C. A. MEYER by Shoji *et al.*[2]

2 was identical with phytosteryl β-D-glucopyranoside obtained by alkaline treatment of **1** as described above.

3′, **4′** and **5′** were isolated after acetylation during the separation procedure. All the PMR spectra showed patterns characteristic of the insect moulting hormones.

3′, a white powder, $[\alpha]_D$ +75.0°, exhibited signals due to 13-CH_3 (singlet (s), δ 0.68), 20-CH_3 (doublet(d), J=6 Hz, δ 0.98), 10-CH_3 (s, δ 1.04) and 25-$(CH_3)_2$ (s, δ 1.24) together with three acetoxy groups (s each, δ 2.00—2.10), 9-H (multiplet (m), δ 3.10), 2-H, 22-H (m, δ 4.76—5.16), 3-H (broad(br) s, δ 5.30) and 7-H (br s, δ 5.84) in the PMR spectrum. From the PMR spectrum of **3′**, it was assumed to be ecdysone 2,3,22-triacetate.[3] **3′** on alkaline treatment gave the corresponding acetyl-free compound (**3**), mp 234—235°, which was identical with an authentic sample of ecdysone[4] as regards *Rf* value on thin-layer chromatography (TLC), mp, mass spectrum and PMR spectrum.

4′, colorless needles, mp 146—148°, $[\alpha]_D$ +76.5°, and **5′**, colorless needles, mp 152—155°, $[\alpha]_D$ +54.2°, were identified as ajugasterone A 2,3,22-triacetate[5] and ecdysterone 2,3,22-triacetate,[5] respectively, by comparison of their PMR spectra with those of authentic specimens.

Since **4′** and **5′** as well as **3′** on weak base treatment regenerated the acetyl-free compounds **4** and **5**, whose *Rf* values on TLC were coincident with those of compounds in the original methanolic extractive, it was considered that they probably exist in acetyl-free forms in the plant body.

Meanwhile, **6**, colorless needles, mp 251—253°, $[\alpha]_D$ −93.9° and **7**, a white powder, $[\alpha]_D$ −73.2°, were found to be identical respectively with pennogenin 3-O-α-L-rhamnopyranosyl-

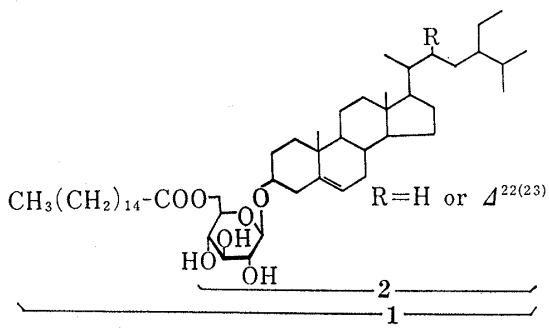
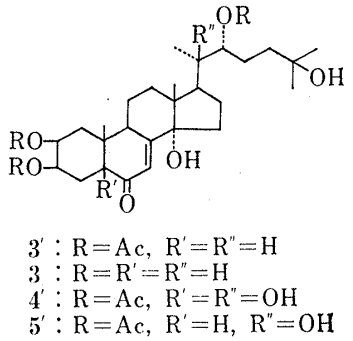


Fig. 1



3′ : R=Ac, R′=R″=H
3 : R=R′=R″=H
4′ : R=Ac, R′=R″=OH
5′ : R=Ac, R′=H, R″=OH

Fig. 2

(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (T-g) and the prototype compound of **6**, 26-O-β-D-glucopyranosyl-25 D-furost-5-ene-3β,17α,22,26-tetraol 3-O-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (T-h), which have been isolated from *Trillium kamtschaticum* PALL,[6] in regard of *Rf* values on TLC and the IR spectra.

8, pale yellow needles, mp 197—200°, $[\alpha]_D$ −58.8°, showed positive reactions with the $FeCl_3$ reagent and in the Mg-HCl test, indicating that **8** is a flavonoid derivative. **8** on acid hydrolysis gave kaempferol, mp 274—276°, D-glucose and L-rhamnose. The peracetate (**8'**) of **8**, a white powder, $[\alpha]_D$ −62.5°, showed peaks of *m/z* 561 and 273 due to the terminal peracetylated methylpentosyl hexosyl cation and the terminal peracetylated methylpentosyl cation,[7] respectively, in the mass spectrum. To determine the locations of the linkages of the rhamnosyl moiety to the glucosyl one and of the bisglycosyl moiety to the aglycone, ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy was undertaken. The signals were assigned as shown in the formula **8** by referring to a report by Markham *et al.*[8] It was concluded that the rhamnosyl residue was bound with C_2-OH of the glucosyl moiety and its bisglycosyl residue at C_3-OH of kaempferol. Consequently, the structure of **8** was deduced to be 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl kaempferol.[9]

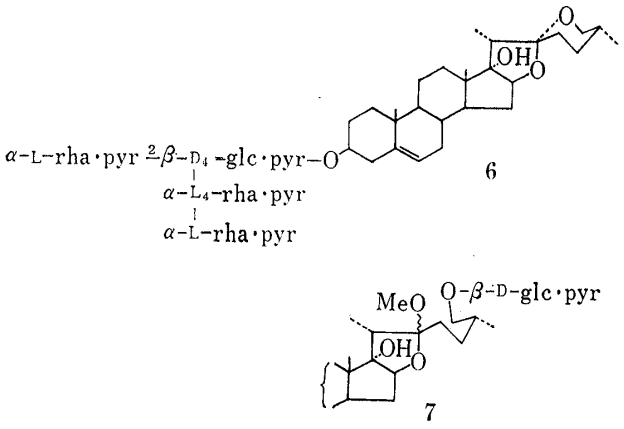


Fig. 3

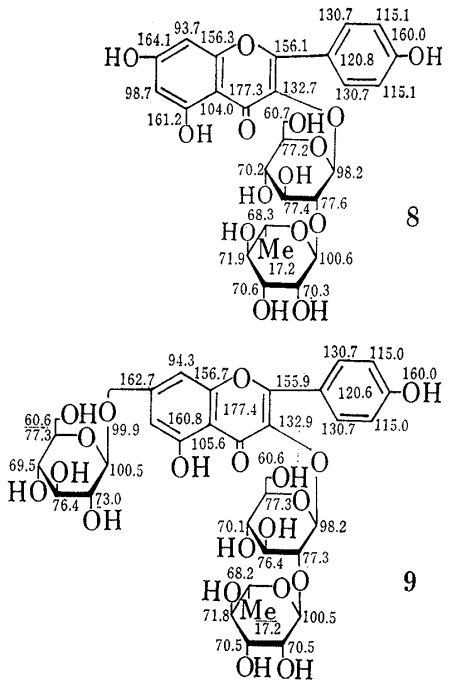


Fig. 4

Finally, **9**, pale yellow needles, mp 202—205°, $[\alpha]_D$ −103.6°, also exhibited colorization reactions ($FeCl_3$ and Mg-HCl reagents) characteristic of flavonoid derivatives. The mass spectrum of the peracetate (**9'**) of **9**, a white powder, $[\alpha]_D$ −58.8°, showed peaks of *m/z* 561,331 and 273, due to the terminal peracetylated methylpentosyl hexosyl, the peracetylated hexosyl and the peracetylated methylpentosyl residues, respectively. **9** on enzymatic hydrolysis with Taka-diastase liberated a flavonol glycoside identical with **8** and D-glucose. It was thus supposed that an additional glucosyl moiety is combined with **8** to form **9**. The location of this glucosyl bond on the aglycone was decided by ^{13}C-NMR spectroscopy to be as shown in the formula **9**. Furthermore, the permethylate (**9''**), mp 103—105°, $[\alpha]_D$ −94.6° (prepared by CH_2N_2-methylation followed by Kuhn's procedure) on methanolysis afforded methyl 2,3,4,6-tetra-O-methyl glucopyranoside, methyl 2,3,4-tri-O-methyl rhamnopyranoside and methyl 3,4,6-tri-O-methyl glucopyranoside, supporting the above ^{13}C-NMR evidence. There-

fore, the structure of **9** can be represented as 7-O-β-D-glucopyranosyl kaempferol 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.[9]

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured with a Union Giken PM-201 automatic digital polarimeter. IR spectra were obtained with a Hitachi EPI-G2 machine and ^{13}C-NMR (solv. d_6-dimethylsulfoxide) and PMR spectra were taken with JEOL JNM-FX-90Q (22.5 MHz) and JEOL-PS-100 (100 MHz) spectrometers. Chemical shifts are given on a δ (ppm) scale with tetramethylsilane as internal standard. Mass spectra were taken with a JEOL JMS D-300 machine. GLC was run on a Shimadzu GC-6A unit with a flame ionization detector. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel F_{254}, Merck) and detection was achieved by spraying 10% H_2SO_4 followed by heating or by UV irradiation (short wave length). Column chromatography was performed on Kieselgel 60 (Merck, 70—230 mesh).

Extraction and Separation——Fresh whole plants (354 g) of *Paris verticillata* M.*v.* Bieb., were collected at Mt. Tsurugi in Tokushima in July, 1979, were extracted with refluxing MeOH (4 l). The MeOH extractives (22 g) were treated as shown in Chart 1.

Phytosteryl (6′-Palmitoyl)-β-D-Glucopyranoside (**1**)——A white powder, $[\alpha]_D^{22}$ −73.3° (c=0.19, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500—3300 (OH), 2920, 2850, 1460 (methylene), 1720 (C=O). PMR ($CDCl_3$) δ: 0.65—1.00 (21H, m, 7×CH_3), 1.25 (−$(CH_2)_n$−), 3.26—3.60 (5H, m, H-3,2′,3′,4′ and 5′), 4.30 (3H, m, H-1′ and CH_2-6′), 5.32 (1H, m, H-6).

Alkaline Treatment of 1——A solution of **1** (15 mg) in 3% KOH–MeOH (4 ml) was left to stand for 15 min at room temperature then neutralized with 1 N HCl–MeOH. The solution was passed through Sephadex LH-20 with MeOH to remove the salts. The white crystals, mp 292—295°, deposited from the eluate were identical with **2**. The filtrate was subjected to GLC to detect methyl palmitate [column, 3% SE-30 on Chromosorb W 3 mm×2 m; column temp., 210°; carrier gas, N_2 33.6 ml/min; sample, t_R(min) 2.4 (=methyl palmitate)].

Acetylation of 1——**1** (10 mg) was acetylated with pyridine (1 ml) and Ac_2O (1 ml) in the usual way to give the acetate as a white powder, $[\alpha]_D^{23}$ +30.2° (c=0.32, $CHCl_3$). PMR ($CDCl_3$) δ: 0.68—1.00 (21H, m, CH_3×7), 1.25 (−$(CH_2)_n$−), 1.98, 1.99, 2.00, 2.02 (12H, all s, $OCOCH_3$×4), 3.70 (2H, m, H-3 and 5′), 4.12 (2H, m, CH_2-6′), 4.55 (1H, d, J=8 Hz, H-1′), 4.80—5.20 (3H, m, H-2′, 3′ and 4′), 5.30 (1H, m, H-6).

Acid Hydrolysis of 2——A solution of **2** (5 mg) in 2 N HCl–MeOH (2 ml) was refluxed for 2 hr on a hot bath, then neutralized and subjected to GLC. Peaks of β-sitosterol and stigmasterol were detected in a ratio of 3 to 1 [column, 1.5% SE-30 on Chromosorb W 3 mm×2 m; column temp., 270°; carrier gas, N_2 73.6 ml/min; sample, t_R(min) 8.0 (=stigmasterol), 9.1 (=β-sitosterol)].

Acetylation of 2——**2** (10 mg) was acetylated in the usual way to give the tetraacetate as white plates, mp 163—165.5°. MS (m/z): 396, 381, 331, 255, 169, 109.

Ecdysone 2,3,22-Triacetate (**3′**)——A white powder, $[\alpha]_D^{22}$ +75.0° (c=0.20, $CHCl_3$). MS (m/z): 554 (M^+-2H_2O), 494 ($M^+-2H_2O-AcOH$), 384, 172, 147, 135, 109. PMR ($CDCl_3$) δ: 0.68 (3H, s, CH_3-13), 0.98 (3H, d, J=6 Hz, CH_3-20), 1.04 (3H, s, CH_3-10), 1.24 (6H, s, $(CH_3)_2$-25), 2.00, 2.04, 2.11 (9H, all s, $OCOCH_3$×3), 3.10 (1H, m, H-9), 4.76—5.16 (2H, m, H-2 and 22), 5.30 (1H, m, H-3), 5.84 (1H, br s, H-7).

Saponification of 3′ giving Ecdysone (**3**)——**3′** (25 mg) in 3% KOH–MeOH (4 ml) was heated on a hot bath (70°) for 10 min, then neutralized with 1 N HCl–MeOH and evaporated to dryness under reduced pressure to give a residue, which was chromatographed on a silica gel column (solv. $CHCl_3$–MeOH=20:1) to give colorless needles, **3** (9 mg), mp 234—235°, which were identical with ecdysone. *Anal.* Calcd for $C_{27}H_{44}O_6$: C, 69.79; H, 9.55. Found: C, 69.61; H, 9.45. MS (m/z): 446 (M^+-H_2O), 428 (M^+-2H_2O), 410 (M^+-3H_2O), 300, 126, 109, 99, 81. PMR (d_5-pyridine) δ: 0.73 (3H, s, CH_3-13), 1.08 (3H, s, CH_3-10), 1.28 (3H, d, J=7 Hz, CH_3-20), 1.39 (6H, s, $(CH_3)_2$-25).

Ajugasterone A 2,3,22-Triacetate (**4′**)——Colorless needles, mp 146—148°, $[\alpha]_D^{21}$ +76.5° (c=0.20, $CHCl_3$). *Anal.* Calcd for $C_{33}H_{50}O_{11}$: C, 63.64; H, 8.09. Found: C, 63.33; H, 8.19. IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1725 (OAc), 1680—1650 (enone). MS (m/z): 568 (M^+-3H_2O), 546 ($M^+-3H_2O-AcOH$), 508, 445, 400, 325, 152, 126, 109, 99. PMR ($CDCl_3$) δ: 0.86 (3H, s, CH_3-13), 0.94 (3H, s, CH_3-10), 1.23, 1.28, 1.28 (9H, all s, CH_3-20 and $(CH_3)_2$-25), 2.02 ($OCOCH_3$×1), 2.10 ($OCOCH_3$×2), 3.20 (1H, m, H-9), 4.80 (1H, br d, H-22), 5.10—5.32 (2H, m, H-2 and 3), 5.94 (1H, br s, H-7).

Ecdysterone 2,3,22-Triacetate (**5′**)——Colorless needles, mp 152—155°, $[\alpha]_D^{22}$ +54.2° (c=0.25, $CHCl_3$). *Anal.* Calcd for $C_{33}H_{50}O_{10}\cdot H_2O$: C, 63.44; H, 8.39. Found: C, 63.26; H, 8.47. IR ν_{max}^{KBr} cm^{-1}: 3300 (OH), 1725 (OAc), 1650, 1625 (enone). MS (m/z): 570 (M^+-2H_2O), 552 (M^+-3H_2O), 492, 385, 327, 128, 109, 99. PMR ($CDCl_3$) δ: 0.86 (3H, s, CH_3-13), 1.04 (3H, s, CH_3-10), 1.21, 1.23, 1.26 (9H, all s, CH_3-20 and $(CH_3)_2$-25), 2.00 ($OCOCH_3$×1), 2.10 ($OCOCH_3$×2), 3.10 (1H, m, H-9), 4.79 (1H, br d, 22-H), 5.02 (1H, br d, H-2), 5.31 (1H, m, H-3), 5.82 (1H, m, H-7).

Pennogenin Tetraglycoside (**6**)——Colorless needles, mp 251—253°, $[\alpha]_D^{21}$ −93.9° (c=0.25, pyridine). *Anal.* Calcd for $C_{51}H_{82}O_{31}\cdot 3H_2O$: C, 56.45; H, 8.12. Found: C, 56.42; H, 8.09. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH),

980, 920, 898, 840 (spiroketal). **6** was identical with T-g.

Protype Glycoside (7) of Pennogenin Tetraglycoside——A white powder, *Rf* 0.14 (solv. $CHCl_3$–MeOH–H_2O=7:3:0.5), $[\alpha]_D^{23}$ −73.2° (*c*=1.32, pyridine). IR ν_{max}^{KBr} cm^{-1}: 3400—3200 (OH). **7** was identical with T-h. **7** was refluxed with aq.acetone to give the corresponding 22-OH compound, *Rf* 0.09. A mixture of **7** (16 mg) in dist.water (3 ml) and almond emulsin (Sigma Co.) was incubated at 37° overnight to liberate a spirostanol glycoside identical with T-g (5 mg) and glucose.

Flavonol Diglycoside (8)——Pale yellow needles, mp 197—200°, $[\alpha]_D^{22}$ −58.8° (*c*=1.36, MeOH), $FeCl_3$: dark green, Mg–HCl: reddish color. *Anal.* Calcd for $C_{27}H_{30}O_{15}$: C, 54.54; H, 5.09. Found: C, 54.28; H, 5.06. PMR (CD_3SOCD_3) δ: 0.76 (3H, d, *J*=6 Hz, rha CH_3-5), 5.07 (1H, s, rha H-1), 5.64 (1H, triplet(t)-like,[10] glc H-1), 6.20, 6.44 (each 1H, d, *J*=3 Hz, H-6 and 8), 6.88, 8.02 (each 2H, d, *J*=9 Hz, H-2′, 3′, 5′ and 6′).

Acetylation of 8——**8** (20 mg) was acetylated with Ac_2O (1 ml) and pyridine (1 ml) in the usual manner to yield the acetate as a white powder, $[\alpha]_D^{28}$ −62.5° (*c*=0.64, $CHCl_3$). MS (*m/z*): 561 (terminal peracetylated methylpentosyl hexosyl cation), 273 (terminal peracetylated methylpentosyl cation).

Acid Hydrolysis of 8——A solution of **8** (40 mg) in 2 N H_2SO_4 was refluxed for 2 hr and the product was separated by column chromatography over silica gel with $CHCl_3$–MeOH–H_2O=7:3:0.2 as the solvent to afford kaempferol, yellow needles, mp 276—278°, D-glucose, *Rf* 0.41 (solv. $CHCl_3$–MeOH–acetone–H_2O=3:3:3:1), $[\alpha]_D^{21}$ +56.4° (*c*=0.36, water), and L-rhamnose, *Rf* 0.68, $[\alpha]_D^{21}$ +10.2° (*c*=0.42, water).

Flavonol Triglycoside (9)——Pale yellow needles, mp 202—207°, $[\alpha]_D^{23}$ −103.6° (*c*=1.10, MeOH), $FeCl_3$: dark green, Mg–HCl: reddish color. *Anal.* Calcd for $C_{33}H_{40}O_{20}$: C, 52.38; H, 5.33. Found: C, 52.45; H, 5.32.

Acid Hydrolysis of 9——**9** (10 mg) was hydrolyzed with 2 N HCl–MeOH (2 ml) for 2 hr on a hot bath. After neutralization with 3% KOH–MeOH, the hydrolysate was examined by TLC. Kaempferol (*Rf* 0.76) and methylsides (*Rf* 0.61 and 0.34, solv. $CHCl_3$–MeOH–H_2O=7:3:0.5) of rhamnose and glucose were detected.

Enzymatic Hydrolysis of 9——A mixture of **9** (35 mg) and Taka-diastase (20 mg) in AcOH-AcONa buffer pH 5.1, 3 ml) was incubated at 38° for 1 hr then evaporated to dryness under reduced pressure to give a residue, MeOH was added, and the mixture was filtered. The filtrate was concentrated and applied to Sephadex LH-20 column. Elution with MeOH gave a flavonol glycoside, mp 198—201°, $[\alpha]_D^{22}$ −60.2° (*c*=0.42, MeOH), identical with **8** and D-glucose, $[\alpha]_D^{22}$ +48.2° (*c*=0.32, water).

Peracetate (9′) of 9——**9** (30 mg) was peracetylated with Ac_2O–pyridine (2 ml each) in the usual manner to give the peracetate (**9′**) as colorless needles, mp 227—230°, $[\alpha]_D^{19}$ −58.8° (*c*=1.02, $CHCl_3$). *Anal.* Calcd for $C_{57}H_{64}O_{32}$: C, 54.29; H, 5.08. Found: C, 54.06; H, 5.12. IR ν_{max}^{KBr} cm^{-1}: 1750 (OAc), 1630 (aromatic ring). MS (*m/z*): 561 (terminal peracetylated methylpentosyl hexosyl cation), 331 (terminal peracetylated hexosyl cation), 273 (terminal peracetylated methylpentosyl cation). PMR ($CDCl_3$) δ: 0.88 (3H, d, *J*=7 Hz, rha CH_3-5), 1.86—2.12 (OAc×10), 2.32, 2.46 (3H each, s, arom. OAc), 3.50—5.62 (19H, m, methylenes and methines of the sugar moieties), 6.68, 6.98 (each 1H, d, *J*=3 Hz, H-6 and 8), 7.28, 8.01 (each 2H, d, *J*=10 Hz, H-2′,3′,5′ and 6′).

Permethylate (9″) of 9——A solution of **9** (152 mg) in MeOH (10 ml) was treated dropwise with CH_2N_2–ether solution (6 ml) under cooling with ice-water. After standing for 4 days in a refrigerator, the solution was evaporated to dryness *in vacuo* to give a residue, to which dimethylformamide (4 ml), CH_3I (5 ml) and Ag_2O (1.5 g) were added (Kuhn's methylation), and the mixture was stirred at room temperature overnight. After usual work-up, the resulting syrup was re-methylated at 70° for 7 hr with the same quantities of the solvent and reagents. Purification of the product by silica gel column chromatography eluting with *n*-hexane–acetone=3:1 gave the permethylate **3″** (22 mg) as colorless needles, mp 103—105°, $[\alpha]_D^{19}$ −94.6° (*c*=1.11, $CHCl_3$). PMR ($CDCl_3$) δ: 0.98 (3H, d, *J*=6 Hz, rha CH_3-5), 4.90 (1H, *t*-like,[10] glc H-1), 5.23 (1H, br s, rha H-1), 5.60 (1H, d, *J*=8 Hz, glc H-1), 6.44, 6.64 (each 1H, d, *J*=3 Hz, H-6 and 8), 6.94, 8.04 (each 2H, d, *J*=10 Hz, H-2′,3′,5′ and 6′).

Methanolysis of the Permethylate (9″)——The permethylate (**9″**, 10 mg) was hydrolyzed with 3 N HCl–MeOH (3 ml) for 2 hr on a hot bath. After neutralization, the solution was examined by TLC (solv. *n*-hexane–AcOEt=2:3). Methylglycosides of 2,3,4,6-tetra-O-methyl glucopyranose (*Rf* 0.30), 3,4,6-tri-O-methyl glucopyranose (*Rf* 0.14) and 2,3,4-tri-O-methyl rhamnopyranose (*Rf* 0.59) were detected.

Acknowledgement We wish to express our thanks to Prof. T. Takemoto and Associate Prof. S. Arihara of Tokushima Bunri University for providing an authentic sample of ecdysone. Thanks are also due to Mr. H. Fujiwara and Mr. K. Goto of Otsuka Pharmaceutical Factory, Inc., for measurement of the FT-NMR and to the staff of the Analytical Laboratory of the Faculty of Pharmaceutical Sciences, Tokushima University, for elemental analysis and for the measurements of mass and PMR spectra.

References and Notes

1) Ecdysone has been obtained in very small amounts from several fern species in the plant kingdom (H. Hikino and Y. Hikino, "Fortschritte d. Chem. Org. Naturst," Vol. 28, ed. by W. Herz, H. Grisebach, and A.I. Scott, Springer-Verlag, Wien, 1970, p. 256; T. Takemoto, T. Okuyama, H. Jin, T. Arai, M.

Kawahara, C. Konno, S. Nabetani, S. Arihara, Y. Hikino, and H. Hikino, *Chem. Pharm. Bull.*, **21**, 2336 (1973)).
2) T.D. Lin, N. Kondo, and J. Shoji, *Chem. Pharm. Bull.*, **24**, 253 (1976).
3) T. Takemoto and H. Hikino, *Kagaku No Ryoiki*, **22**, 603 (1968).
4) P. Karlson, *Vitamins and Hormones*, **14**, 227 (1956); H. Hoffmeister, H.F. Grützmacher, and K. Dünnebell, *Z. Naturforsch*, **22b**, 66 (1967).
5) T. Matsuoka, S. Imai, M. Sakai, and M. Kamada, *Takeda Kenkyusho Nempo*, **28**, 221 (1969).
6) T. Nohara, K. Miyahara, and T. Kawasaki, *Chem. Pharm. Bull.*, **23**, 872 (1975).
7) H. Budzikiewicz, C. Djerassi, and D.H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol. II, Holden-Day. Inc., San Francisco, 1964, pp. 203—227; T. Komori, Y. Ida, Y. Mutou, K. Miyahara, T. Nohara, and T. Kawasaki, *Biomedical Mass Spectrometry*, **1975**, 65.
8) K.R. Markham, B. Ternal, R. Stanley, H. Geiger, and T.J. Mabry, *Tetrahedron*, **34**, 1389 (1978).
9) The linkage modes of sugar moieties were estimated on the basis of the molecular rotation differences, **9**—**8** (ΔM_D −434°) and **8**—kaempferol 3-O-β-D-glucopyranoside, mp 173—176°, $[\alpha]_D^{18}$ +16.9° (c=0.62, MeOH), obtained by partial hydrolysis of **8** (ΔM_D −425°).
10) This unusual splitting pattern is under investigation.

[Chem. Pharm. Bull. 29(5)1451—1455(1981)]

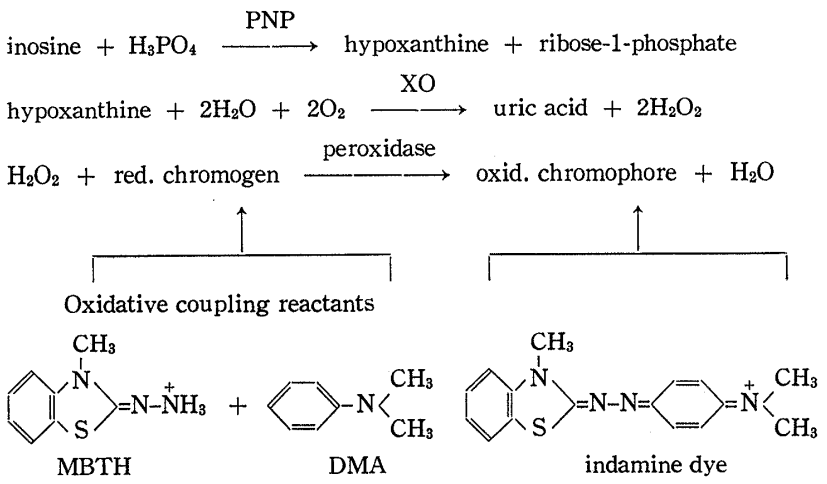
An Enzymic Method for the Determination of Inorganic Phosphate in Serum[1)]

MAMORU SUGIURA,*,[a] KENJI KATO,[a] TETSUO ADACHI,[a] YOSHIMASA ITO,[a] KAZUYUKI HIRANO,[a] and SHUNJI SAWAKI[b]

Gifu College of Pharmacy,[a] 6-1, Mitahora-higashi 5 chome, Gifu, 502, Japan and First Department of Internal Medicine, Aichi Medical University,[b] Nagakute-cho, Aichi-gun, Aichi, 480, Japan

(Received November 19, 1980)

We have established an enzymic method for the determination of inorganic phosphate in serum, based on the formation of hydrogen peroxide with purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO).



The method was evaluated for precision, accuracy, sensitivity and specificity, and was concluded to be useful as a clinical test for the determination of inorganic phosphate in serum.

Keywords——inorganic phosphate; purine nucleoside phosphorylase; hydrogen peroxide; enzymic determination; xanthine oxidase