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Soil Bacterial Hydrolysis leading to Genuine Aglycone. VII.[1] On Monoterpenoid Glucosides of *Scrophularia buergeriana* Miq. and *Paeonia albiflora* Pallas

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The soil bacterial hydrolysis method has been applied to two monoterpenoid glucosides, which were isolated from the roots of *Scrophularia buergeriana* Miq. and *Paeonia albiflora* Pallas. Although some chemical modification of the glucosides were needed beforehand, the microbiological method has been shown to be a useful procedure in the chemical investigation of the monoterpenoid glucosides.

With an intention to develop the further applicability of the soil bacterial hydrolysis method which has hitherto given rise to the successful result on the structure elucidation of triterpenoid,[3] diterpenoid,[1] and steroid,[4] glycosides, we have applied the microbiological method to two monoterpenoid glucosides which are obtainable from the roots of *Scrophularia buergeriana* Miq. and *Paeonia albiflora* Pallas. This paper deals with the detailed account of the result.[5]

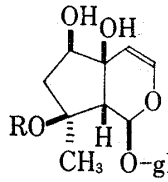
In 1967, we elucidated a mixture of two iridoid glucosides, harpagoside (Ia) and 8-O-(*p*-methoxycinnamoyl)-harpagide (Ib), from the roots of *S. buergeriana*.[6] On barium hydroxide treatment, the both glucosides afforded the common desacyl derivative harpagide (Ic).[7] However, it has never been accomplished to liberate the intact aglucone directly from harpagide either chemically or enzymatically as has usually been experienced on the hydrolysis of C_9-iridoid glucoside due to the instability of the aglucone. On the other hand, Lichti and Wartburg succeeded in the isolation of an aglucone enzymatically through the modified procedure.[7] They hydrolyzed dihydroharpagide (IIa) with emulsin or snail enzyme and obtained crystalline dihydroharpagenin (IIb) in a good yield. Therefore we initiated the application of our soil bacterial hydrolysis method with dihydroharpagide.

Dihydroharpagide (IIa) was prepared from a mixture of above mentioned two glucosides (Ia and Ib) by the barium hydroxide hydrolysis[6] followed by the catalytic hydrogenation over palladium-charcoal.[7] Since dihydroharpagide was noticed to decompose significantly during the sterilization procedure, the glucoside was added aseptically to the synthetic medium of inorganic salts which was sterilized beforehand. The aglucone (termed as G-1), mp 127—128°, obtained after 10 days cultivation, showed the proton magnetic resonance (PMR) spectrum resembled to that of dihydroharpagenin (IIb) which was previously prepared by Lichti and Wartburg through the enzymatic hydrolysis of IIa.[7] A direct comparison of G-1 with authentic IIb[8] established the identity of both substances.

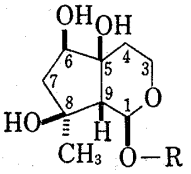
1) Part VI: I. Yosioka, S. Saijoh, J.A. Waters, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 2500 (1972).
2) Location: *Toneyama, Toyonaka, Osaka.*
3) *a*) I. Yosioka, M. Fujio, M. Osamura, and I. Kitagawa, *Tetrahedron Letters*, **1966**, 6303; *b*) I. Yosioka, K. Imai, and I. Kitagawa, *ibid.*, **1967**, 2577; *c*) I. Yosioka, T. Sugawara, A. Ohsuka, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **19**, 1700 (1971); *d*) I. Yosioka, S. Saijoh, and I. Kitagawa, *ibid.*, **20**, 564 (1972).
4) I. Yosioka, K. Imai, and I. Kitagawa, *Tetrahedron Letters*, **1971**, 1177.
5) Presented at the 21st Annual Meeting of Kinki Branch, Pharmaceutical Society of Japan, Osaka, November 1971, Abstract Papers, p. 22.
6) I. Kitagawa, T. Nishimura, M. Takei, and I. Yosioka, *Chem. Pharm. Bull.* (Tokyo), **15**, 1254 (1967).
7) H. Lichti and A. von Wartburg, *Helv. Chim. Acta*, **49**, 1552 (1966).
8) Kindly provided by Dr. Wartburg of Sandoz AG, Basel.

In regard to the glycoside constituents of the roots of *Paeonia albiflora*, Shibata and his co-workers clarified the structure of a major glucoside paeoniflorin (IIIa).[9] Due to the instability of the aglucone, the cleavage of the glucoside linkage was unaccomplished both chemically and enzymatically and they proceeded the structure elucidation without liberating the glucose moiety throughout the work.

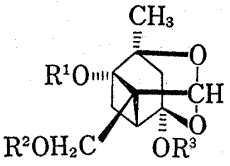
At first, we applied the soil bacterial hydrolysis method on paeoniflorin (IIIa) itself. Although an aglucone was detected by thin-layer chromatography (TLC) of the hydrolysate, its isolation was not attained presumably due to its instability. Therefore, the desbenzoylmethylether (product F) (IIIb),[10] which was considered to yield a stable aglucone, was taken as the starting glucoside.

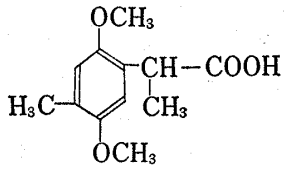


Ia: R = $C_6H_5CH{=}\overset{t}{C}HCO-$ harpagoside
Ib: R = p-CH_3O-$C_6H_4CH{=}\overset{t}{C}HCO-$
Ic: R = H harpagide



IIa: R = glucose dihydroharpagide
IIb: R = H dihydroharpagenin





IV

IIIa: R^1 = glucose, R^2 = C_6H_5CO-, R^3 = H paeoniflorin
IIIb: R^1 = glucose, R^2 = H, R^4 = CH_3 product F
IIIc: R^1 = R^2 = H, R^3 = CH_3 PF–S
IIId: R^1 = H, R^2 = CH_3CO, R^3 = CH_3
IIIe: R^1 = tetra-O-acetyl-glucose, R^2 = CH_3CO, R^3 = CH_3

Chart 1

TABLE I. τ Values in $CDCl_3$ at 60 MHz[a]

	PF–S(IIIc) (R=H)	PF–S monoacetate(IIId) (R=Ac)	Product F pentaacetate(IIIe)[b] (R=Ac)
-O-C(H)-O-	4.52 (s)	4.54 (s)	4.65 (s)
-C(H)CH_2OR	5.90 (s)	5.57 (br.s, $W_{h/2}$=4 Hz)	5.73 (d-like, J=3 Hz)
-OCH_3	6.57 (s)	6.55 (s)	6.58 (s)
-O-C(C)-CH_3	8.63 (s)	8.66 (s)	8.66 (s)

a) abbreviation: br.s=broad singlet, d-like=doublet like, s=singlet. All the spectra show the minor signals at 8.72 and 8.00τ probably due to the impurity, which are also observed in the spectrum of the starting product F(IIIb).
b) prepared from the product F according to Shibata, *et al.*[9]

Extraction of the culture broth followed by the chromatographic purification afforded the oily aglucone (termed as PF-S) (IIIc) in a good yield. Ordinary acetylation of PF-S with acetic anhydride and pyridine yielded a crystalline monoacetate (IIId), mp 57—58.5°, whose infrared (IR) spectrum shows the presence of unacetylated hydroxyl function at 3500

9) N. Aimi, M. Inaba, M. Watanabe, and S. Shibata, *Tetrahedron*, **25**, 1825 (1969).
10) Kindly provided by Prof. Shibata of the University of Tokyo.

cm^{-1}. The PMR spectrum of PF-S (IIIc) and the monoacetate (IIId) exhibits the presence of a methyl geminal to an oxygen function, a methoxyl, a primary carbinol, and a methine proton attached to a carbon bearing two oxygen function as given in Table I. The chemical shifts are comparable to those corresponding signals of product F pentaacetate (IIIe). It has been assumed therefore that PF-S (IIIc) retains the carbon framework of product F (IIIb) and is the genuine aglucone of the latter. Acid treatment of PF-S followed by methylation with dimethyl sulfate furnished a product, mp 110—112°, being identical to the leucodimethylether of aglycone H (IV)[11] which was prepared by Shibata and Nakahara from the product F through the same reaction sequence. Consequently, PF-S is assigned IIIc, which corresponds to the genuine aglucone of product F.

For comparison purpose, emulsin was also adopted for the hydrolysis of product F. As described in the experimental section, the same aglucone (IIIc) was obtained in a lesser yield than above and characterized by preparing the monoacetate (IIId).

It follows therefore that the soil bacterial hydrolysis method has been shown to be a versatile one. The present observation extends the usefulness of the procedure for the structure elucidation of the monoterpenoid glucoside, although some structural modification was needed to isolate the aglucone without difficulty. Furthermore, it is pointed out that, in case of the thermally unstable glucoside, the carbon source should be added aseptically after preparing the sterilized inorganic medium to avoid the decomposition of the glucoside during the sterilization procedure.

Experimental[12]

Soil Bacterial Hydrolysis of Dihydroharpagide (IIa)——After the sterilization (at 120°, 2 atm, 20 min) of the inorganic salts medium (330 ml of the following composition: KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.7 g, NaCl 1 g, $(NH_4)_2HPO_4$ 4 g, $FeSO_4 \cdot 7H_2O$ 0.03 g, and distilled water 1 liter), dihydroharpagide (IIa, 1.7 g) was added aseptically to the medium at room temperature as the sole carbon source. A soil bacterial strain (YSB-23, unidentified) selected on the synthetic medium as described previously[3a] was cultivated stationarily at 31° for 10 days. The residue obtained by complete evaporation of the total culture medium *in vacuo* was extracted with MeOH (100 ml) and then with *n*-BuOH. Evaporation of the former extract gave the brown resinous product (1.94 g), whereas from the latter was obtained the brown tarry product (0.2 g). The combined product (2.14 g) was chromatographed on silica gel (155 g). The substance (651 mg) obtained by 10% MeOH–$CHCl_3$ elution contained G-1 as the major which was chromatographed again using silica gel (45 g) to give G-1 (511 mg). The substance (110 mg) obtained by successive elution with 15% MeOH–$CHCl_3$ was revealed to be pure G-1 by TLC ($CHCl_3$: MeOH=4:1, detected by the Godin reagent[13]). The combined G-1 (621 mg) was then washed with MeOH, benzene, *n*-hexane, acetone, and ether successively and crystallized from acetone to give colorless plates (186 mg) of G-1, mp 127—128°, $[\alpha]_D$ −35.0° (*c*, 1.02 in H_2O). IR ν_{max}^{KBr} cm^{-1}: 3380 (br) (OH), 1066 (C-O-C). PMR (D_2O) τ: 8.69 (3H, s, $C_{(8)}$-C<u>H</u>$_3$), 8.1—8.5 (2H, $C_{(4)}$<u>H</u>$_2$), 8.00 (1H, d, J=5.4 Hz, $C_{(9)}$-<u>H</u>), *ca.* 8.02 (unclear due to the overlapping) and 7.68 (d.d, J=15 & 7.2 Hz) (2H, $C_{(7)}$<u>H</u>$_2$), 6.7—5.7 (2H, $C_{(3)}$<u>H</u>$_2$), 6.03 (1H, d.d, J=7.5 & 5.7 Hz, $C_{(6)}$-<u>H</u>), 5.07 (1H, d, J=5.4 Hz, $C_{(1)}$-<u>H</u>). G-1 thus obtained was identified with authentic dihydroharpagenin (IIb)[8] by mixed mp, IR (KBr), and TLC.

Soil Bacterial Hydrolysis of Product F (IIIb)——To the sterilized inorganic salts medium (500 ml of the composition as above) was added aseptically product F (IIIb, 1.5 g)[10] and a soil bacterial strain (YSB-24, unidentified) selected as described previously[3a] was cultivated stationarily at 31° for 9 days. Extraction of the culture broth with ether twice (500 ml each) followed by evaporation of the solvent afforded a residue (85 mg), which was treated again with ether to remove a trace amount of the insoluble substance. The

11) S. Shibata and M. Nakahara, *Chem. Pharm. Bull.* (Tokyo), **11**, 372 (1963).

12) The following instruments were used for the physical data. Melting points: Yanagimoto Micro-melting-point Apparatus (a hot-stage type); Specific rotations: Rex Photoelectric Polarimeter NEP-2; IR spectra: Hitachi EPI-S2 IR Spectrometer; PMR spectra: Hitachi R-20A NMR Spectrometer, TMS as the internal standard in $CDCl_3$ and DSS in D_2O. Silica gel Camag D-5 and silica gel Merck (70—325 mesh, ASTM) were used for TLC and column chromatography respectively.

13) *a*) P. Godin, *Nature*, **174** 134 (1954); *b*) A.P. MacLennan, H.M. Randall, and W.D. Smith, *Anal. Chem.*, **31**, 2020 (1959).

ether soluble substance (designated PF-S) showed a single spot on TLC ($CHCl_3$: MeOH=6:1). The culture broth was then extracted with *n*-BuOH saturated with water and the extract was evaporated *in vacuo* to give the brown residue (810 mg), which was purified by repeated preparative TLC ($CHCl_3$: MeOH=6:1, detected by I_2 vapor) to afford the additional amount of PF-S (IIIc, 502 mg) as an oily product (total yield: 58%). $[\alpha]_D$ +3° (*c*, 0.55 in $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450 (OH). PMR: As given in Table I.

Acetylation of PF-S (IIIc)——To a solution of PF-S (IIIc, 10 mg) in pyridine (2 ml) was added Ac_2O (2 ml) and the mixture was allowed to stand at room temperature for 24 hr. After the usual work-up followed by preparative TLC purification ($CHCl_3$: acetone=2:1, detected by I_2 vapor), the product (11 mg) was crystallized from $CHCl_3$–*n*-hexane to give colorless needles of IIId, mp 57—58.5°, $[\alpha]_D$ −26.3° (*c*, 0.3 in $CHCl_3$). *Anal.* Calcd. for $C_{13}H_{18}O_6$: C, 57.77; H, 6.71. Found: C, 56.95; H, 6.43. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3500 (w) (OH), 1742, 1235 (OAc), ν_{max}^{KBr} cm^{-1}: 1737, 1230 (OAc), 1030. PMR: As given in Table I.

Hydrolysis of Product F (IIIb) with Emulsin——i) To a solution of product F (IIIb, 200 mg) in AcOH–AcONa buffer (30 ml, pH 5.4) was added emulsin (100 mg)[14] and the total mixture was allowed to stand at 32° for 5 days. After addition of boiling EtOH (10 ml) to the total hydrolysate mixture, a small amount of Celite 535 (Wako Pure Chem.) was added and the mixture was stirred and filtered. The filtrate was extracted with ether and $CHCl_3$ successively and evaporation of the solvent of combined extracts afforded the residue (11 mg). The aqueous layer was then evaporated *in vacuo* to give a residue which was extracted with $CHCl_3$ and evaporation of the organic solvent gave the residue of 23 mg. Preparative TLC (as above) of the combined residue afforded PF-S (oily) in 30% yield. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3460 (OH), 1015. ii) Acetylation of the oily product (7 mg) with Ac_2O (2 ml) and pyridine (2 ml) at room temperature for 48 hr followed by usual work-up furnished an acetate (7 mg), which was purified by preparative TLC (as above) and crystallized from $CHCl_3$–*n*-hexane to give crystals of mp 57—58.5°. The acetate was identified with IIId obtained above by the soil bacterial hydrolysis method by mixed mp, IR (KBr), and TLC.

Acid Treatment of PF-S (IIIb) followed by Methylation——A mixture of PF-S (IIIb, 40 mg) of aq. 2N H_2SO_4 (1 ml) was heated in a boiling water-bath. Whenever the solution became turbid, it was extracted with ether. The procedure was repeated until the reaction mixture did not become cloudy again while heating. The combined ether extracts were evaporated to dryness and the residue was methylated with $(CH_3)_2SO_4$ (2 ml), MeOH (40 ml) and KOH (2 g) at reflux for one hour. After cooling, MeOH was evaporated and the reaction mixture was acidified with aq. 2N H_2SO_4 and extracted with ether. Usual work-up of the ether extract gave a methylated product (21 mg), which was purified by preparative TLC ($CHCl_3$: MeOH=10:1, detected by I_2 vapor) and crystallized from *n*-hexane to give the methylether (2.5 mg), mp 110—112°. IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 1702 (COOH), 1040 (C-O-C). A direct comparison (mixed mp, IR (KBr), and TLC) of the methylether with the authentic leucodimethylether of algycone H (IV) established the identity of both substances.

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14) Kindly provided by Prof. Inouye of Kyoto University.