Summary

Penicillum brunneum UDAGAWA was fed with malonate [2-^{14}C], and rugulosin-^{14}C isolated from the mycelia was degraded to prove that the terminal C-CH_3 group was not labelled with ^{14}C.

Using acetate [1-^{14}C] with or without competition of inactive malonic acid, a predominant incorporation of acetate unit into the terminal C-CH_3 unit was revealed.

It has been established that the fungal anthraquinone series compounds are biosynthesized by the malonate-acetate condensation.

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67. Shoji Shibata*1 and Motoko Nakahara*2 : Studies on the Constituents of Japanese and Chinese Crude Drugs. VIII.*3 Paeoniflorin, A Glucoside of Chinese Paeony Root. (1).

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The Chinese Paeony root (the root of *Paeonia albiflora* PALLAS (: *P. lactiflora* PALLAS)) is well-known as one of the important medicaments in the traditional old Chinese medicine.

However, none of the especial principle, except benzoic acid,[1] has been isolated from the Paeony root. Recently, Ohta, *et al.*[2] suggested that benzoic acid must exist combined with some principle in the Paeony root, on which, however, they gave no further evidence.

From the methanolic extracts of the Paeony root a colorless hygroscopic amorphous substance has now been isolated, which, so far, has not been obtained in a crystalline form in spite of many efforts in purification using chromatography and counter current distribution.

The uniformity of this principle has almost been established as it gave a single spot on paper chromatogram and single peak of fractionation in the counter current distribution.

The principle has now been named paeoniflorin, with which the present paper chiefly concerns.

Accompanying with paeoniflorin, a high content of sucrose in Paeony root is noted, the yield of which is variable depending on the sources of material.

Paeoniflorin is a neutral glucoside in which benzoic acid exists as a benzoyl grouping. It gives no coloration with ferric chloride and does not reduce Fehling's reagent.

The glucoside was affected neither by emulsin nor snail enzyme, whereas it was hydrolyzed readily by dilute mineral acid to give D-glucose and benzoic acid quantitatively, but the aglycone was failed to be obtained in a pure state due to its instability

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1) Y. Asahina, S. Okuno : Yakugaku Zasshi, **27**, 1237 (1907).
2) T. Ohta, T. Miyazaki : Tokyo Yakka-Daigaku Nenpo (Ann. Rep. Tokyo Coll. of Pharmacy), **1954**, 266.

giving only a dark resinous substance. Paeoniflorin is unstable in aqueous alkaline solution to separate benzoic acid quantitatively, and the remaining part is turned into reddish brown resinous substance.

On stepwise acetylation, paeoniflorin afforded firstly a partially acetylated crystalline product with a free hydroxyl, m.p. 196° (tentatively named acetate I), which was then completely acetylated to give acetate II, m.p. 158°.

The analytical results showed that the acetate I, $C_{31}H_{36}O_{15}$, is tetraacetate,*4 and the acetate II, $C_{33}H_{38}O_{16}$, is pentaacetate*4 of paeoniflorin. The molecular formula of paeoniflorin would be represented as $C_{23}H_{28}O_{11}$ which is deduced from the analytical figures of its crystalline tetra- and pentaacetates.

The ultraviolet absorption curves of the acetates of paeoniflorin are almost superimposable with those of alkyl benzoates[3] showing the absence of any particular chromophoric structure except benzoyl grouping in the molecule of paeoniflorin.

Although the infrared spectra of acetates I and II, ozonization, catalytic hydrogenation and perbenzoic acid titration gave no positive result to prove the unsaturated bond, the presence of very hindered double bond could not be ruled out.

On deacetylation and simultaneous debenzoylation of acetates I and II by the action of lithium aluminium hydride, crystalline product A, $C_{16}H_{24}O_{10}$, m.p. 186° (decomp.) was formed, while under some different condition, only amorphous product A′ was obtained.

On methylation with methyl iodide and silver oxide, the acetate I afforded a methyl ether, $C_{32}H_{38}O_{15}$, m.p. 123°, tentatively named product E acetate. The product E acetate liberated its benzoyl and acetyl groupings by the action of lithium aluminium hydride to form product F, $C_{17}H_{26}O_{10}$, m.p. 194°, which gave pentaacetate, $C_{27}H_{36}O_{15}$, m.p. 131.5°.

Based on the analytical results and molecular weight determinations of acetates and other derivatives of paeoniflorin, it has been concluded that paeoniflorin is a glucoside whose debenzoylated aglycone part, which has not been obtained in a pure state, would be corresponded to $C_{10}H_{14}O_5$.

The product F gives no absorption in the ultraviolet region and no infrared absorption band of carbonyl group and double bond. It showed resistance against enzymatic hydrolysis, while it was hydrolyzed by mineral acid to form an amorphous aglycone, aglycone F, which showed strong reducing activity turning into a dark colored resinous substance on standing. The ultraviolet absorption spectrum of aglycone F suggested the presence of aromatic ring.

As it was difficult to isolate pure aglycone F, the hydrolyzed product was treated instantly with chromic acid in 1*N* sulfuric aicd at room temperature to yield a yellow crystalline compound, named aglycone H, m.p. 133° (decomp.). The aglycone H, $C_{10}H_{10}O_4$, is a sublimable yellow pigment with quinonic nature, whose infrared spectrum shows the presence of C=O and COOH groupings.

The ultraviolet spectral curve also gave an evidence that the aglycone H is a benzoquinone derivative.[4] As has been shown in several benzoquinone derivatives,[5,6] the aglycone H is photosensitive to be decolorized at the crystalline state and much more rapidly in the solution. The variation of ultraviolet absorption spectral curve of the aglycone H is shown in Fig. 2.

*4 The acetyl estimation of acetates I and II gave the acetyl values of 1 acetyl excess. This might be caused by 1 mole of HCOOH liberated from the molecule of acetates I and II during the process of acetyl estimation, as the product A yielded HCOOH on boiling with aq. alkali.

3) H.E. Ungnade, R.W. Lamb : J. Am. Chem. Soc., **74**, 3789 (1952).
4) W. Flaig, J.C. Salfeld, E. Baume : Ann., **618**, 117 (1958).
5) R.C. Cookson, J. Hudec : Proc. Chem. Soc., **1959**, 11.
6) L.I. Smith, R.W.H. Tess : J. Am. Chem. Soc., **66**, 1323 (1944).

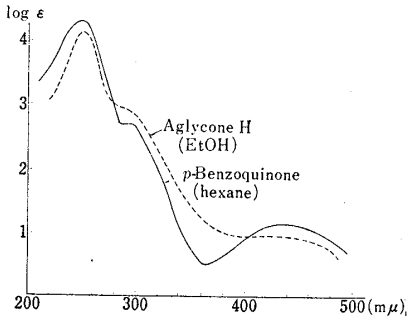


Fig. 1.

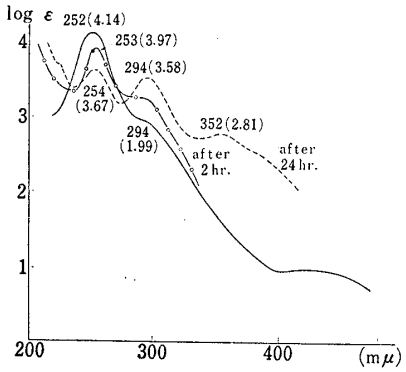
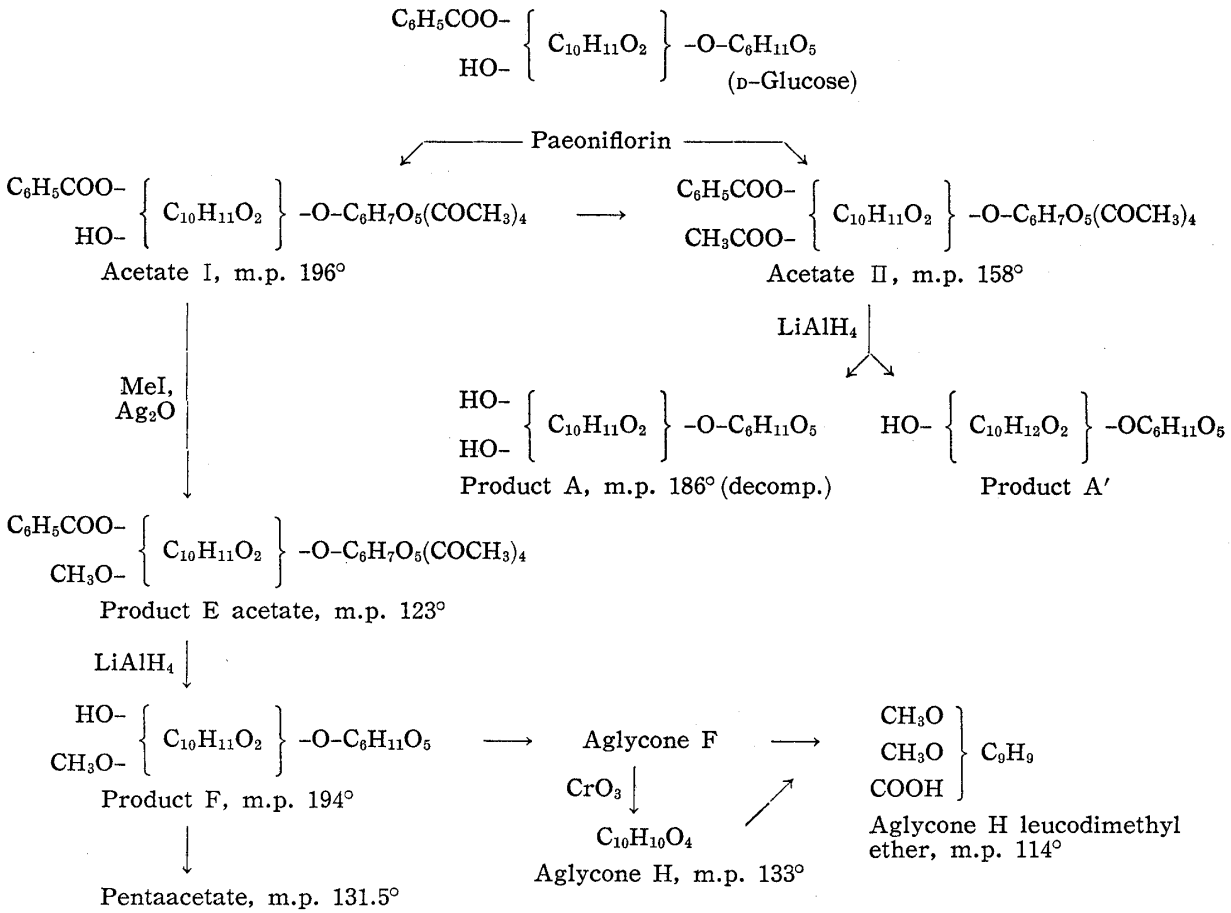


Fig. 2.

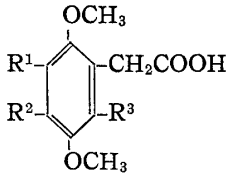
It has been established that leucodimethyl ether of aglycone H is identical with the methyl ether derived from the amorphous aglycone F.

The series of reactions and derivation of paeoniflorin mentioned above are formulated as follows :

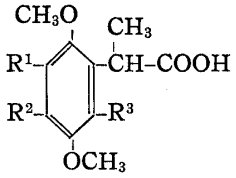


The analytical figures, the molecular weight determination by iodometric titration and the C-methyl group determination by Kuhn-Roth method (2 C-CH_3) suggested that the aglycone H would be a dimethyl-*p*-benzoquinone-acetic acid.

Thus the leucodimethyl ether of aglycone H should be represented by one of the following structures :



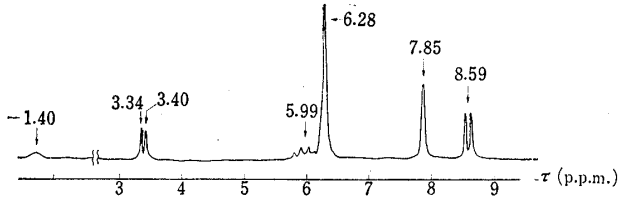
I : R^1, $R^2=CH_3$, $R^3=H$
II : R^1, $R^3=CH_3$, $R^2=H$
III : R^2, $R^3=CH_3$, $R^1=H$



IV : $R^3=CH_3$, R^1, $R^2=H$
V : $R^2=CH_3$, R^1, $R^3=H$
VI : $R^1=CH_3$, R^2, $R^3=H$

Of these structures, the formula (I) is of the known compound, m.p. 121°[7] which must be excluded, and the compound (II), m.p. 138° has been synthesized[8] to prove as being not identical with the leucodimethyl ether of aglycone H.

The nuclear magnetic resonance spectrum of leucodimethyl ether of aglycone H in carbon tetrachloride was measured at 60 Mc. using Varian Associates PD-60 apparatus and cyclohexane as the internal reference (Fig. 3).



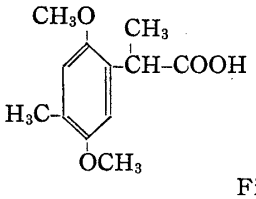


Fig. 3.

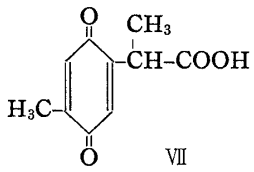
The nuclear magnetic resonance spectrum of leucodimethyl ether of aglycone H resembles that of the lactone A, an ozonolytic product of *o*-diacetylusnic acid.[9]

The doublet signal at τ : 8.62 and 8.56 (J : 7.04 c.p.s.) would couple with the quartet centered at 5.99, corresponding apparently with the doublet at τ : 8.43 and 8.59, and the quartet at τ : 6.07, 6.22, 6.35, and 6.49 in the nuclear magnetic resonance spectrum of the lactone A, which showed the presence of a system-CH-CH_3. Thus one of the methyl groups of aglycone H leucodimethyl ether (and also that of aglycone H) would exist at the side chain to form -CH-COOH system.
CH_3

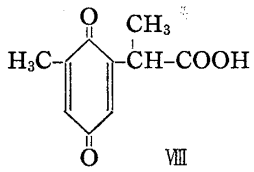
The nuclear magnetic resonance signal at τ : 7.85 shows the existence of an aromatic methyl group (τ : 8.04 in the lactone A, and τ : 8.11 in the lactone B), whose areal intensity indicates 3 protons in comparison with 6 protons of 2 methoxyl groups at τ : 6.28.

Consequently, the result of nuclear magnetic resonance spectral analysis indicated that the formulas (IV), (V), and (VI) should be considered for leucodimethyl ether of aglycone H. Furthermore, the signal of aromatic protons appeared as a doublet at τ : 3.34 and 3.40, which much more favored the formula (V) or (VI). The formula (IV) could be ruled out, as it should give a typical AB type nuclear magnetic resonance spectrum accompanying outer pair of lines, and it is not the case of the nuclear magnetic resonance spectrum of leucodimethyl ether of aglycone H.

The formulas (V) and (VI) of aglycone H leucodimethyl ether would be initiated to the formulas of benzoquinones (VII) and (VIII), one of which should represent the aglycone H.



VII



VIII

7) L.I. Smith, F.L. Austin : J. Am. Chem. Soc., **64**, 528 (1942).
8) S. Shibata, N. Aimi, M. Nakahara : This Bulletin, **11**, 379 (1963).
9) S. Shibata, J. Shoji, N. Tokutake, Y. Kaneko, H. Shimizu, H.C. Chiang : *Ibid.*, **10**, 477 (1962).

It should be noted that the aglycone H possesses all the number of carbon atoms (C_{10}) of the original aglycone of paeoniflorin.

The synthetical approach for the establishment of the structure of aglycone H or aglycone H leucodimethyl ether is being pursued.[8]

Experimental*5

Isolation of Paeoniflorin——The hot methanolic extract of Paeony root was treated with $(AcO)_2Pb$, and the precipitate was removed. After introducing H_2S to remove excess of Pb^{++}, the clear orange filtrate was concentrated under diminished pressure to give syrup, which was washed with Et_2O, and exhaustively extracted with hot AcOEt. The AcOEt solution dried over Na_2SO_4 was evaporated to dryness *in vacuo* to give amorphous hygroscopic white powder, the crude paeoniflorin, whose yield from commercial Chinese Paeony root was 3.1%.

The extraction must be carried out cautiously to keep the solution neutral during the process, otherwise the yield decreased greatly. This may be attributed to the extremely high sensitivity of the glucoside to acid.

In the case of dealing with Japanese Paeony root, a large amount of sucrose was obtained when some portion of EtOH was added to the hot-AcOEt-insoluble syrupy residue. Purification of the crude paeoniflorin was attempted by the counter current distribution using the solvent system, 5% Na_2SO_4–AcOEt–BuOH (5:5:1). Paeoniflorin thus obtained is hygroscopic amorphous powder, $[\alpha]_D^{16}$ −12.8° (c= 4.6, MeOH), which showed a single spot on paper chromatogram developed by BuOH–AcOH–H_2O (4:1:5) and detected as Fe hydroxamate. Paeoniflorin did not consume perbenzoic acid, while it reacted with $NaIO_4$ to give 0.82 mole of HCOOH. It did not reduce Fehling's solution even on heating.

Hydrolysis of Paeoniflorin——1) Acid Hydrolysis: Paeoniflorin was warmed in 2*N* H_2SO_4 on a boiling water bath. Within a few minutes, the solution became cloudy then gradually brownish red. From the ethereal extract of the hydrolysate benzoic acid was obtained and from the aqueous layer glucose was isolated, which was identified by paper chromatography and the formation of phenylglucosazone. A red resinous substance formed during the hydrolysis dissolved neither in Et_2O nor in H_2O and showed strong reducing activity.

2) Methanolysis: A solution of paeoniflorin (0.5 g.) dissolved in 15 cc. of 1*N* MeOH–HCl was refluxed for 1 hr. The solvent was removed by distillation, and the residue was washed with Et_2O and extracted with H_2O. On evaporation α-methyl glucoside was obtained as colorless needles, m.p. 168°.

3) Alkaline Hydrolysis: A solution of paeoniflorin (0.7 g.) in dil. $Ba(OH)_2$ (30 cc.) was warmed on a boiling water-bath under N_2 stream for 1 hr. The neutralized solution was extracted with Et_2O. The ethereal extract yielded on evaporation benzoic acid (170 mg. 85% of theor. amount).

4) Enzymatic Hydrolysis: The hydrolysis with emulsin and snail enzyme was attempted to give a negative result.

Paeoniflorin used for the above experiments was the material purified by counter current distribution.

Paeoniflorin Tetraacetate (Acetate I)——To a cold pyridine solution of paeoniflorin (1 g. in 1.5 cc.) Ac_2O (1.5 cc.) was added under ice cooling, and the solution was left to stand overnight in a refrigerator. The resulting crystalline mass was poured into ice H_2O. The precipitate formed was collected and recrystallized from $CHCl_3$–hexane or from 80% EtOH to obtain colorless needles, m.p. 196°. Yield, 0.9~1.0 g. *Anal.* Calcd. for $C_{31}H_{36}O_{15}$: C, 57.41; H, 5.50; acid residue (eq.), 5; mol. wt., 648. Found: C, 57.20, 57.24; H, 5.45, 5.48; acid residue (eq.), 5.79, 5.47; mol. wt. 651, 639, 631. IR ν_{max}^{Nujol}: 3350 cm^{-1} (OH).

Acid Residue Estimation——About 10 mg. of a sample was weighed into 0.1*N* NaOH (2 cc.) and the solution was refluxed under N_2 stream for 1 hr. After cooling the excess of alkali was titrated with 0.05*N* HCl using phenolphthalein as an indicator.

Paeoniflorin Pentaacetate (Acetate II)——On heating the foregoing acetate I (1 g.) in Ac_2O (1 cc.) and pyridine (1 cc.), or in Ac_2O (1 cc.) containing AcONa (0.5 g.) in a boiling water-bath for 30 min. A fully acetylated product was obtained which was recrystallized from 80% EtOH to give fine colorless needles, m.p. 158°, $[\alpha]_D^{20}$ +13.5°(c=4.13, MeOH). *Anal.* Calcd. for $C_{33}H_{38}O_{16}$: C, 57.39; H, 5.51; O, 37.10; acid residue (eq.), 6; mol. wt., 690. Found: C, 57.44, 57.67, 57.66, 57.53, 57.35; H, 5.53, 5.53, 5.54 5.54, 5.59; O, 36.89; acid residue (eq.), 6.33, 6.89, 6.45; mol. wt. 706, 707, 690.

*5 All melting points were not corrected. The molecular weights were determined by Rast's method. The volatile acid evolved by the Kuhn-Roth C-methyl determination and acetyl estimation was identified by paper chromatography.[10]

10) B. Lindquist, T. Storgärds: Acta Chem. Scand., 7, 87 (1953); C. F. Garbers, H. Schmid, P. Karrer: Helv. Chim. Acta, 37, 1336 (1954).

Product E Acetate——A mixture of acetate I (4 g.), MeI (3 cc.) and Ag_2O (0.5 g.) in Me_2CO (50 cc.) was refluxed for 3 hr. The reaction mixture was filtered and the filtrate was concentrated to dryness, and the residue was recrystallized from 50% EtOH to needles, m.p. 123°. *Anal.* Calcd. for $C_{32}H_{38}O_{15}$: C, 58.01; H, 5.74; MeO–, 5.00; acid residue (eq.), 5; mol. wt., 662. Found: C, 57.80, 57.75, 57.77, 57.56; H, 5.70, 5.70, 5.63, 5.56; MeO–, 4.88, 4.68; acid residue (eq.), 4.89, 5.00, 4.95; mol. wt. 660, 666, 670.

Product F——The etherial solution of product E acetate (5 g.. in 500 cc.) was added with the ethereal suspension of $LiAlH_4$ and refluxed for 3 hr. After decomposing the excess of $LiAlH_4$ by a careful addition of a few drops of H_2O, 500 cc. of H_2O was added to the ethereal solution. The upper layer was separated and the remaining aqueous layer was filtered to remove the precipitate of $Al(OH)_3$. After deionization through a column of ion exchange resin, IR-120, the solution was evaporated to dryness *in vacuo*. The residue was recrystallized from MeOH to prisms, m.p. 193~194°. The yield was almost quantitative. *Anal.* Calcd. for $C_{17}H_{26}O_{10}$: C, 52.31; H, 6.67; C-Me, 3.38. Found: C, 52.36, 52.42, 52.61, 52.52; H, 6.64, 6.64, 7.74, 6.73; C-Me, 2.99 (NMR. one C-Me).

Product F Acetate——Crystalline product F was acetylated in a usual method using pyridine and Ac_2O. On recrystallization from 60% EtOH, it afforded product F pentaacetate as needles, m.p. 131°. *Anal.* Calcd. for $C_{27}H_{36}O_{15}$: C, 54.00; H, 6.00; MeO–, 5.17; acid residue (eq.), 5; mol. wt. 600. Found: C, 54.61, 54.47; H, 5.58, 5.92; MeO–, 4.93, 5.22; acid residue (eq.), 5.14, 5.02; mol. wt., 593, 605, 595.

Product A——The ethereal solution of acetate I (or acetate II) was treated with $LiAlH_4$ in the same way as in the case of preparing product F from product E acetate. Recrystallization from MeOH gave fine needles, m.p. 186°. Yield, 80~90%. *Anal.* Calcd. for $C_{16}H_{24}O_{10}$: C, 51.07; H, 6.38. Fond: C, 51.47, 51.63, 51.17, 51.39; H, 6.48, 6.48, 6.49, 6.75.

$NaIO_4$ Oxidation: $NaIO_4$ consumed: 1.3 moles for 1 hr., 1.5 moles for 2 hr., 1.85 moles for 7 hr., 1.85 moles for 12 hr.

Alkaline Decomposition: About 10 mg. of product A was weighed into 0.5*N* NaOH (2 cc.) and was refluxed for 1 hr. under N_2 stream. After adding dil. H_2SO_4 the evolved acid was distilled and the titration with 0.1*N* NaOH showed that the evolved volatile acid in the distillate was equivalent to 0.75 mole, and 0.80 mole. The volatile acid was identified as HCOOH by the reducing spot on the paper chromatogram developed with the same solvent system as that given in the ref. 10.

Aglycone F Methyl Ether (Leucodimethyl Ether of Aglycone H)——A solution of product F (0.5 g.) dissolved in 3 cc. of 2*N* H_2SO_4 was heated on a boiling water-bath. On 3 or 4 min. heating, the clear solution became cloudy, from which on cooling formed white precipitate. The precipitate was extracted with Et_2O and the aqueous layer was heated on the water-bath for a subsequent few minutes again, and the cooled mixture was extracted again with Et_2O. This process was repeated several times until no precipitate was produced on cooling. The combined ethereal extract, which reduced $AgNO_3$-NH_4OH solution immediately at room temperature and gave acidic character and blue fluorescence, was evaporated to dryness under diminished pressure. To the pale yellowish residue, a mixture of MeOH (20 cc.) and Me_2SO_4 (2 cc.) was added, and then, to the boiling mixture KOH (2 g.) was added at a time. After refluxing for 1 hr., the solution was concentrated, and extracted with Et_2O after acidification. The residue obtained on evaporation of Et_2O was recrystallized from hexane to give colorless needles, m.p. 113~114°, $[\alpha]_D = 0°$. Yield, 190 mg. *Anal.* Calcd. for $C_{12}H_{16}O_4$: C, 64.29; H, 7.14. Found: C, 64.01, 64.32; H, 7.16, 7.04.

UV λ_{max}^{EtOH} of the ethereal solution of aglycone F: 297 mμ (log ε 3.3 ca.).

IR ($CHCl_3$) cm^{-1} of aglycone F: 1790, 1710, suggesting that aglycone F was the mixture of a lactone and the corresponding carboxylic acid.

Aglycone H——The ethereal solution of aglycone F (about 50 cc., prepared from 0.5 g. product F) was diluted with the equal volume of benzene, and Et_2O was evaporated on a water-bath below 50°. The resulting benzene solution (30 cc.) of aglycone F was added with 2*N* H_2SO_4 (10 cc.) containing CrO_3 (100 mg.), and the mixture was stirred for 2 or 3 hr. at room temperature, when the upper layer turned orange within a few minutes. The benzene layer was concentrated and extracted with Na_2CO_3. The extract was acidified and extracted again with Et_2O. The ethereal extract afforded, after recrystallization from Me_2CO-hexane, orange needles, m.p. 133° (decomp.). It was sublimable at 120° under reduced pressure (20 mm.Hg) without decomposition. Yield, 150 mg. *Anal.* Calcd. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.16; 2 C-Me, 15.46; mol. wt., 194. Found: C, 61.42, 62.22; H 5.51, 5.25; C-Me, 14.84, 14.61; mol. wt.,[11] 195.6, 195.9.

Aglycone H Leucodimethyl Ether (Aglycone F Methyl Ether) from Aglycone H——1) Aglycone H (70 mg.) was catalytically reduced in methanolic solution using 5% Pd-C as a catalyst. After the solution was decolorized, the catalyst was removed and the methanolic solution of the product was treated with Me_2SO_4 and KOH as previously described in the case of methylation of aglycone F.

11) Mol. wt. of the compound was determined by iodometry. T. Posternak: Helv. Chim. Acta, **21**, 1326(1938).

The methyl ether of reduced aglycone H was obtained as colorless needles, m.p. 113° by recrystallization from hexane. (Yield, 30 mg.).

2) The ethereal solution of aglycone H (70 mg. in 50 cc.) was shaken with a small portion of freshly prepared saturated aqueous solution of $Na_2S_2O_4$. The decolorized ethereal solution was evaporated under reduced pressure. The residue was dissolved in MeOH and treated with Me_2SO_4 and KOH as mentioned above. On recrystallization from MeOH, aglycone H leucondimethyl ether formed colorless needles, m.p. 113° (Yield, 15 mg.).

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Summary

An amorphous glucoside, named paeoniflorin, which was isolated from the roots of *Paeonia albiflora* PALLAS yielded crystalline tetraacetate, $C_{31}H_{36}O_{15}$, and pentaacetate, $C_{33}H_{38}O_{16}$. Alkaline hydrolysis showed that paeoniflorin possesses a benzoyl group. On treatment with lithium aluminium hydride, the acetates afforded product A, $C_{16}H_{24}O_{10}$.

Methylation of paeoniflorin tetraacetate yielded a methyl ether, $C_{32}H_{38}O_{15}$ (product E acetate). By the action of lithium aluminium hydride, the product E acetate was converted into product F, $C_{17}H_{26}O_{10}$. On treatment with sulfuric acid, the product F was hydrolyzed to yield an amorphous aglycone (aglycone F) liberating D-glucose. Oxidation of the aglycone F with cromium trioxide afforded a yellow crystalline quinonic compound (aglycone H), $C_{10}H_{10}O_4$. The nuclear magnetic resonance spectral analysis of aglycone H leucodimethyl ether showed that it should be represented as 2-(4-methyl-2,5-dimethoxyphenyl) or 2-(3-methyl-2,5-dimethoxyphenyl) propionic acid.

Paeoniflorin was shown to be D-glucoside of benzoylated C_{10}-compound ($C_{10}H_{14}O_5$).

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