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Studies on the Components of *Clerodendron trichotomum* Thunb. III.[1] A New Glycoside, Acacetin-7-glucurono-(1→2)-glucuronide from the Leaves[2]

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A new glycoside (I) was isolated from the leaves of *Clerodendron trichotomum* Thunb. and confirmed to be acacetin-7-β-D-glucurono-(1→2)-β-D-glucuronide, the sugar part of which was the same as that of glycyrrhizin in licorice.

Chemical reports[4-7)] on the constituents of an Indian medicinal plant, *Clerodendron infortunatum* Gortn. (Verbenaceae) stimulated us to find bitter principles[8)] and some root components[1)] from a Japanese plant, *C. trichotomum* Thunb. (Japanese name, kusagi). The present paper deals with the isolation and characterization of a new glycoside, acacetin-7-β-D-glucurono-(1→2)-β-D-glucuronide (I) from the leaves of this plant.

The glycoside (I) was obtained as its salts (mainly as a calcium salt) when extracted with boiling water and concentrated. The yield of the glycoside from dried leaves varied with season as shown in Fig. 1. This findings suggest that flowering would depress the content of the glycoside. On treating with dilute hydrochloric acid the salts changed to yellow precipitates which on purification from a large amount of acetone gave a pale yellow compound, $C_{28}H_{28}O_{17}$ (I), mp 191—205° (decomp.). The color tests with ferric ion and magnesium–hydrochloric acid, and the ultraviolet (UV) spectrum, $\lambda_{max}^{ethanol}$: 270.2 mμ (29800) and 326 mμ

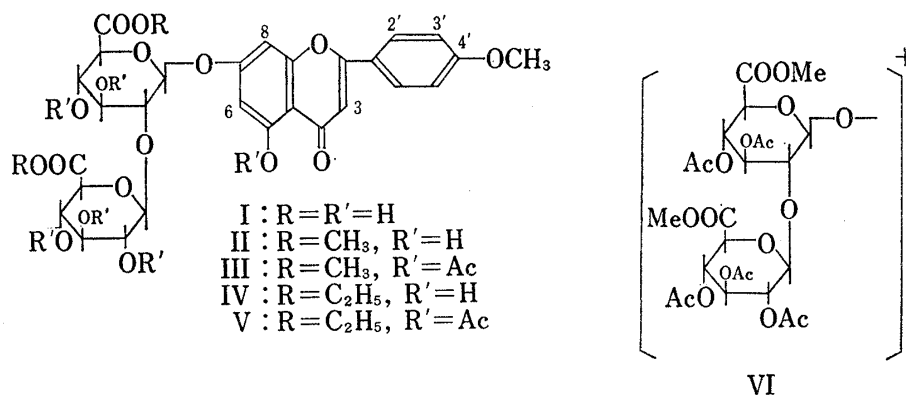

Chart 1

1) Part II: N. Kawano, H. Miura and Y. Kamo, *Yakugaku Zasshi*, **87**, 1146 (1967).
2) Preliminary communication of this work, *Tetrahedron Letters*, **1970**, 2935.
3) Location: *a*) *1-14 Bunkyo-machi, Nagasaki*; *b*) A guest researcher from Chugai Pharmaceutical Co. Ltd., Tokyo; *c*) *3-1 Hongo 7-chome, Bunkyo-ku, Tokyo.*
4) D.H.R. Barton, H.T. Chung, A.D. Cross, L.M. Jackmann and M. Martin-Smith, *J. Chem. Soc.*, **1961,** 5061.
5) I.C. Paul, G.A. Sim, T.A. Hamor and J.M. Robertson, *J. Chem. Soc.*, **1962,** 4133.
6) M. Manzoor-i-Khuda and S. Sarela, *Tetrahedron*, **21**, 797 (1965).
7) M. Manzoor-i-Khuda, *Tetrahedron*, **22**, 2377 (1966).
8) N. Kawano, H. Miura and T. Yamada, *Yakugaku Zasshi*, **84**, 472 (1964).

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(3200) of this compound suggested an apigenin derivative. The infrared (IR) spectrum indicated bands associated with ketone ($1735\ cm^{-1}$) and carboxylic acid ($1700\ cm^{-1}$). I was soluble in a sodium bicarbonate solution showing to have an acid character. The nuclear magnetic resonance (NMR) spectrum (in pyridine solution) gave a signal (3.75 ppm) corresponding to a methoxy group attached to aromatic ring.

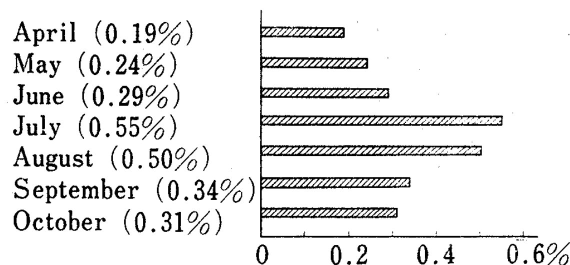

Fig. 1. Seasonal Change of the Yield from Dried Leaves

A dimethyl ester, $C_{30}H_{32}O_{17}$ (II), mp 256° (decomp.) was formed by refluxing a methanol solution of I with a small amount of hydrogen chloride. II gave a hexaacetate, $C_{42}H_{44}O_{23}$ (III), mp 234—235°, the NMR spectrum ($CDCl_3$) of which showed two methyl ester groups (3.60 and 3.63 ppm), one aromatic methoxy group (3.82 ppm) and six acetoxy groups (1.98 (6H), 2.01 (6H), 2.08 (3H) and 2.39 ppm (3H, aromatic)). Similarly, a diethyl ester of I, $C_{32}H_{36}O_{17}$ (IV), mp 240—242° and its hexaacetate, $C_{44}H_{48}O_{23}$ (V), mp 222—223° were formed. The mass spectrum of III showed a parent peak, *m/e* 916,[9] a diglucuronide fragment, 607.153 ($C_{24}H_{31}O_{18}^+$ (VI) requires 607.151) and an acacetin monoacetate fragment, 326.080 ($C_{18}H_{14}O_6^+$ requires 326.079).

When hydrolyzed with β-D-glucuronidase, I gave acacetin and D-glucuronic acid detected by circular paper chromatography (Table I), whereas the dimethyl ester (II) remained unchanged by the same treatment. The UV absorption spectrum of the glycoside (I) was unchangable on addition of sodium acetate[10] showing that a sugar part should be attached to 7-position of acacetin. Repeated methylation of I with diazomethane followed by hydrolysis with hydrochloric acid afforded apigenin-4′,5-dimethyl ether, mp 262—263°.

Table I. Circular Paper Chromatography

Uronic acids	*Rf* value
Glucuronic acid	0.21 (0.44)
Galacturonic acid	0.31
Hydrolyzed solution	0.20 (0.44)

Number in parenthesis indicates week spot caused by D-glucuronolactone.
Flowing solvent: *n*-BuOH:AcOH:H_2O (4:1:5) Toyo Roshi No. 51, UH type was used.

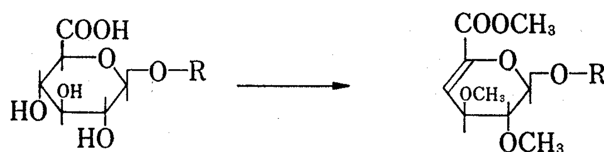

Chart 2

Sum up these findings it was concluded that the glycoside (I) should be acacetin 7-β-D-glucurono-β-D-glucuronide but the linkage between the two glucuronic acids was still unknown. The usual method for determination of sugar linkage is methylation with methyl iodide followed by hydrolysis to detect partially methylated monosaccharides. However, in the case of glucuronide, β-elimination is apt to occur to give 4-deoxy-4,5-dehydrosugar[11] (Chart 2) and hydrolysis will be accompanied with considerable decomposition of glucuronic acid.

9) Milimass could not be read because PFK did not reach to this region.
10) L. Jurd and R.M. Horowitz, *J. Org. Chem.*, **22**, 1618 (1957).
11) T. Imanari and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **15**, 1677 (1967).

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Accordingly, the dimethyl ester (II) was reduced with sodium borohydride to give a neutral sugar[12] and then methylated by Hakomori's procedure[13] which is better[14] than the other methylation methods.[15–17] Hydrolysis was performed with 2.5N trifluoroacetic acid in a sealed tube and the methylated sugars were detected by gas chromatography (GLC) after reduction with sodium borohydride in order to avoid peaks due to α and β anomers[18,19] (Chart 3). Since reduction of glucuronide with sodium borohydride into glucoside proceeded incompletely (for example, 89.5% was estimated by GLC analysis in case of phenylglucuronide), unreduced glucuronide or glucuronic acid was removed from hydrolyzed solution by passing through Amberlite CG-400 (Ac).

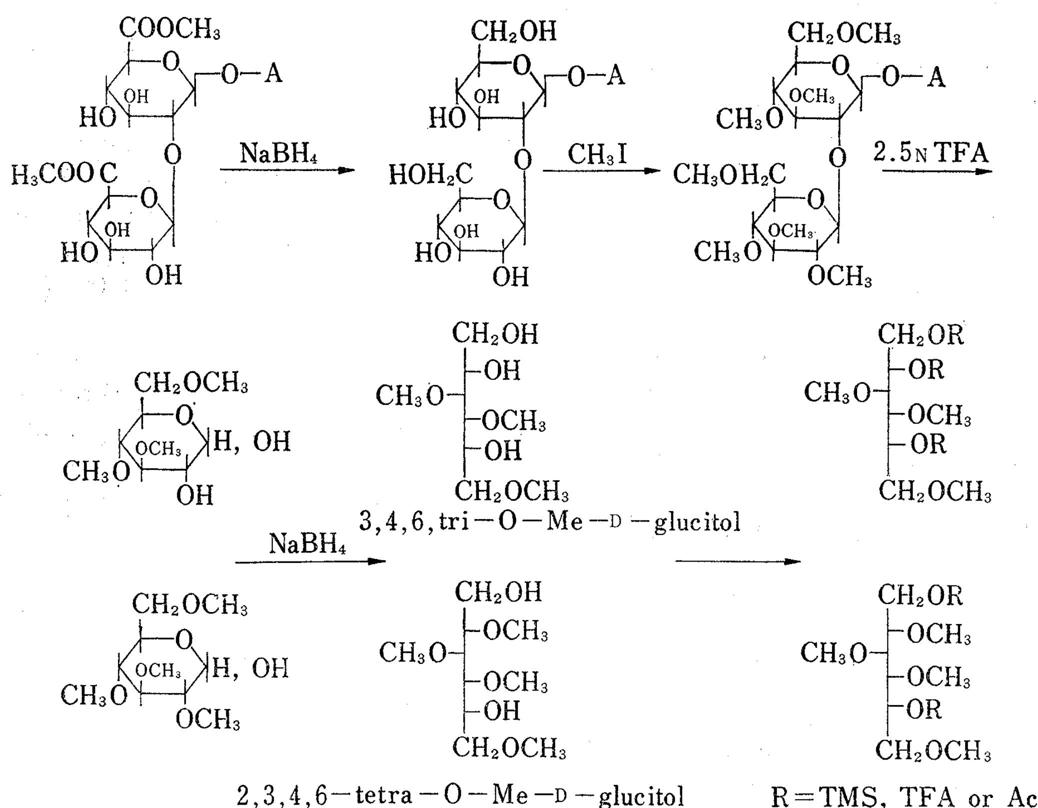

Chart 3

GLC detection was achieved in three derivatives, trimethylsilyl ether (TMS), trifluoroacetate (TFA) and acetate (Ac), and two peaks corresponding to 2,3,4,6-tetra-O-methyl-D-glucitol and 3,4,6-tri-O-methyl-D-glucitol were detected in all derivatives as shown in Table II. Standard compounds of 2,3,4,6-tetra-O-methyl-D-glucitol, 2,3,6-tri-O-methyl-D-glucitol, 2,4,6-tri-O-methyl-D-glucitol and 3,4,6-tri-O-methyl-D-glucitol were prepared from 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-glucose, 3-O-benzyl-D-glucose (mp 128—129.5°, derived from diacetoneglucose) and glycyrrhizin (1→2 diglucuronide)[20] respectively.

12) M.C. Wolfrom and K. Anno, *J. Amer. Chem. Soc.*, **74**, 5583 (1952).
13) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
14) Y.S. Ovodov and E.V. Evtushenko, *J. Chromatog.*, **31**, 527 (1967).
15) T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1031 (1903); *idem, ibid.*, **85**, 1049 (1904).
16) W.H. Haworth, *J. Chem. Soc.*, **107**, 8 (1915).
17) R. Kuhn, H. Trischmann and I. Löw, *Angew. Chem.*, **67**, 32 (1955); *idem, ibid.*, **75**, 1014 (1963)
18) H. Björndal, B. Lindberg and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967).
19) H.G. Jones and J.K.N. Jones, *Can. J. Chem.*, **47**, 3269 (1969).
20) B. Lythgoe and S. Trippett, *J. Chem. Soc.*, **1950**, 1983.

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Table II. GLC Relative Retention Time

Derivatives	TMS		TFA	Ac	
Column	A	B	B	A	B
Column temperature	160°	140°	110°	180°	160°
2,3,4,6-Tetra-O-methyl-D-glucitol	1.00 (6.80 min)	1.00 (13.30)	1.00 (13.80)	1.00 (5.30)	1.00 (7.00)
3,4,6-Tri-O-Me-D-glucitol	1.265	1.401	0.673	1.523	1.457
2,4,6-Tri-O-Me-D-glucitol	1.280	1.419	0.699	1.512	1.464
2,3,6-Tri-O-Me-D-glucitol	1.290	1.422	0.687	1.695	1.562
O-Methyl-D-glucitols obtained from I	1.00 1.266	1.00 1.404	1.00 0.673	1.00 1.522	1.00 1.455

A: 2% Silicon GE XF-1105(2.0 m×4 mm *i.d.*), B: 2% Silicon OV-1 (1.5 m×4 mm *i.d.*). carrier gas: N_2 60 ml/min. temperature: injection port 230°, detector 230°.

Therefore, the sugar linkage between the two glucuronic acids in the glycoside (I) should be 1→2 linkage similarly as that of glycyrrhizin in licorice, and I was confirmed to be acacetin-7-β-D-glucurono-(1→2)-β-D-glucuronide, the second example of naturally occurring glucurono-glucuronide.

Experimental[21]

Isolation of Glycoside (I)——Air-dried leaves (1 kg) of *Clerodendron trichotomum* collected in Nagasaki were extracted with boiling water (6 liters) two times for 3 hr each. Combined filtrates separated while hot were concentrated on a steam bath to *ca.* 600 ml. After cooling, grayish deposits were collected, washed with water and treated with 10% hydrochloric acid to give yellow precipitates, which were washed with water and recrystallized from acetone (10 liters) three times to give pale yellow crystals (3 g, 0.3%), mp 191—205° (decomp.), $[\alpha]_D^{22}$ −48° (c=1.3%, pyridine), Mg-HCl (orange), $FeCl_3$ (brown), soluble in $NaHCO_3$ aq. under bubbling. TLC (solvent A): one spot *Rf*=0.19. UV λ_{max}^{EtOH} mμ (ε): 270.2 (29800), 326 (3200). IR (KBr) cm^{-1}: 1735 (ketone), 1700 (carboxylic acid). NMR (pyridine): 3.75 ppm (s, OMe). *Anal.* Calcd. for $C_{28}H_{28}O_{17}\cdot 2H_2O$: C, 51.32; H, 4.92. Found: C, 51.01; H, 4.90. Seasonal change of yield is shown in Fig. 1.

Dimethyl Ester (II)——A solution of the glycoside (I, 100 mg), anhydrous methanol (100 ml) and concentrated hydrochloric acid (0.2 ml) was refluxed on a steam bath for 2 hr and concentrated *in vacuo* to about 30 ml to give precipitates under cooling, which were collected and recrystallized from dioxane to afford pale yellow needles (77 mg), mp 256° (decomp.). TLC (solvent A): *Rf*=0.58. NMR (pyridine): 3.75 (6H, s), 3.62 ppm (3H, s). *Anal.* Calcd. for $C_{30}H_{32}O_{17}\cdot H_2O$: C, 52.79; H, 5.02. Found: C, 52.41; H, 5.02. The dimethyl ester (II, 100 mg) was acetylated with pyridine (2.5 ml) and acetic anhydride (2 ml) to give a hexa-acetate (III), colorless needles (96 mg) from 50% methanol, mp 234—235°. NMR ($CDCl_3$): 3.60, 3.63 (3H each, s, ester methyl), 3.82 (3H, s, aromatic methoxy), 1.98 (6H, s), 2.01 (6H, s), 2.08 (3H, s, five acetoxy groups) and 2.39 (3H, s, aromatic acetoxy group), 4.0—5.5 (10H, m, suger protons), 6.48 (1H, s, position 3 in flavone nucleus), 6.51 (1H, d, J=3 cps, position 6), 6.67 (1H, d, J=3, 8), 6.96 (2H, d, J=9, 3′, 5′), 7.80 (2H, d, J=9, 2′, 6′). *Anal.* Calcd. for $C_{42}H_{44}O_{23}$: C, 52.74; H, 4.64. Found: C, 52.52; H, 4.60.

Diethyl Ester (IV)——Similar procedure to above. Pale yellow needles from ethanol, mp 240—242°. TLC (solvent A): *Rf*=0.60. NMR (pyridine): 1.18 (6H, t, J=6 cps), 4.05 (2H, q, J=6), 4.11 (2H, q, J=6), 3.76 (3H, s). *Anal.* Calcd. for $C_{32}H_{36}O_{17}\cdot H_2O$: C, 54.10; H, 5.55. Found: C, 54.23; H, 5.68. Hexaacetate (V), colorless needles from 50% ethanol, mp 222—223°. NMR ($CDCl_3$): 1.26 (6H, t, J=7.5), 2.05 (6H, s), 2.08 (6H, s), 2.17 (3H, s), 2.45 (3H, s, 5-acetoxy), 3.90 (3H, s, 4′-methoxy), 4.17 (4H, q, J=7.5, two ethyl methylenes), 4.0—5.4 (10H, m, sugar protons), 6.54 (1H, s, position 3), 6.76 (1H, d, J=3, 6), 7.01 (2H, d, J=9, 3′, 5′), 7.11 (1H, d, J=3, 8) and 7.83 (2H, d, J=9, 2′, 6′). *Anal.* Calcd. for $C_{44}H_{48}O_{23}$: C, 55.93; H, 5.12. Found: C, 55.78; H, 4.95.

Acid Hydrolysis of I——I (50 mg) was treated with 20% hydrochloric acid (5 ml) on a steam bath for 2 hr to yield yellow precipitates, which were washed with water and recrystallized from acetone (30 ml) to give yellow crystals (10 mg), mp 256—257°, identified with an authentic sample of acacetin by mixed mp and IR spectrum.

21) All melting points are uncorrected. NMR spectra were recorded on a Hitachi H-60 instrument using TMS as internal standard. Mass spectra were determined on a JEOL JMS-01 SG double forcus high resolution spectrometer. TLC analysis were performed on silica gel G acc. to Stahl (Merck). Solvent A, ethyl acetate: methyl ethyl ketone: formic acid: water (5:3:1:1); solvent B, toluene: ethyl formate: formic acid (5:4:1).

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Hydrolysis of I by β-D-Glucuronidase——I (10 mg) suspended in an acetate buffer solution (20 ml) of pH 5.0 was mixed with β-D-glucuronidase (Sigma Chemical Co., U.S.A., 100 mg) and kept for 24 hr at 37°. Filtrates separated from pale yellow precipitates showing the same spot with acacetin by TLC (solvent B) were concentrated *in vacuo* and subjected to circular paper chromatography to give the results shown in the Table I.

Methylation of I with Diazomethane followed by Hydrolysis——I (100 mg) was dissolved in methanol (100 ml) and treated repeatedly with diazomethane in ether solution until ferric reaction became negative. The resulted compound was hydrolyzed with 20% hydrochloric acid (5 ml) on a steam bath for 2 hr to give insoluble yellow compound, which was washed with water and recrystallized from ethanol (20 ml) to give yellow prisms (21 mg), mp 262—263° (apigenin-4′,5-dimethyl ether, lit.[22] 264°). Acetylation with acetic anhydride and anhydrous sodium acetate gave colorless needles from ethanol, mp 203—204° (lit.[22] 204°). NMR (pyridine): 3.83 (3H, s), 3.74 (3H, s), 2.30 (3H, s).

Preparation of Standard Methylated D-Glucitols——A) 2,3,4,6-Tetra-O-methy-D-glucitol: A solution of 2,3,4,6-tetra-O-methyl-D-glucose (1 mg) in 50% methanol (3 ml) was mixed with 3% $NaBH_4$ solution (1 ml) and kept for 1 hr at room temperature. Excess $NaBH_4$ was decomposed with Amberlite CG–120 (H^+) and the reaction mixture was passed through a column of Amberlite CG–120 (H^+) to remove salts and concentrated under reduced pressure. In order to eliminate boric acid as methyl borate methanol (4 ml) was added and evaporated *in vacuo* twice.

B) 2,3,6-Tri-O-methyl-D-glucitol: 2,3,6-Tri-O-methyl-D-glucitol was prepared from 2,3,6-tri-O-methyl-D-glucose (1 mg) by similar treatment to above.

C) 3,4,6-Tri-O-methyl-D-glucitol: Glycyrrhizin (3 mg) dissolved in methanol was methylated with diazomethane and reduced with 3% $NaBH_4$ solution (1 ml) in 50% methanol as described above. Passing through Amberlite CG–120 (H^+) column followed by methanol treatment the reduced material was dissolved in dimethyl sulfoxide (0.5 ml) and kept for 10 min at room temperature after adding (under ice-cooling) 0.5 ml of a reaction mixture of sodium hydride (200 mg, washed with *n*-hexane several times) and dimethyl sulfoxide (5 ml) at 50° for 45 min. The reaction mixture was mixed with methyl iodide (0.5 ml) and left for 20 min at room temperature under occasional stirring. Chloroform was added under ice-cooling and washed with water. Chloroform layer was separated, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure to give methylated compounds, which were dissolved in minimum amount of methanol, mixed with 2.5N trifluoroacetic aicd (2 ml) and hydrolyzed in a sealed tube at 100° for 4 hr. The mixture was evaporated to dryness, dissolved in water, passed through a column of Amberlite CG–400 (AcO^-) and again evaporated to dryness to give a mixture of methylated glucose, which was reduced as described above in A) to a mixture of 3,4,6-tri-O-methyl-D-glucitol and 2,3,4,6-tetra-O-methyl-D-glucitol (almost equal amount).

D) 2,4,6-Tri-O-methyl-D-glucitol: 3-O-Benzyl-D-glucose (mp 128—129.5°, 3 mg) was methylated by Hakomori's method, hydrolyzed and reduced as described above. The reduced material was dissolved in methanol, hydrogenated for 10 hr in the presence of 5% Pd-C (30 mg), filtered, passed through a column of QAE-sephadex (borate form) and evaporated to dryness to give 2,4,6-tri-O-methyl-D-glucitol.

Methylated D-Glucitols from II——The dimethyl ester (II) dissolved in 50% methanol was reduced with 3% $NaBH_4$ solution (1 ml) for 1 hr at room temperature, treated with Amberlite CG–120 (H^+) and methanol (twice). Methylation, hydrolysis and reduction were performed as described above in C).

Detection by Gas Chromatography——Shimadzu GC–4APF equipped with hydrogen flame ionization detector was used. 1 μl of a reaction mixture was supplied for detection.

1) TMS Derivatives: Sample dissolved in pyridine (4 drops) was mixed with hexamethyldisilazane (2 drops) and trimethylchlorosilane (2 drops) and kept for 10 min at 60°.

2) TFA Derivatives: Sample dissolved in ethyl acetate (4 drops) was mixed with trifluoroacetic anhydride (2 drops) and kept for 10 min at room temperature.

3) Acetyl Derivatives: Sample dissolved in pyridine (4 drops) was mixed with acetic anhydride (2 drops) and kept for 20 min at 100°.

22) E. Vongerichten, *Ber*, 33, 2909 (1900).

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