

difficult to be separated from it, was detected and it was assumed to be glucogitofucoside, separated as the acetate from the leaves of *Digitalis lanata*, by paper partition chromatography of the sugar portion of the acid hydrolyzate.

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27. Kazuo Miyatake, Atsuji Okano, Kazuhiko Hoji, and Tōsaku Miki :

Studies on the Constituents of *Digitalis purpurea* L. III. Gitostin, a New Cardiotonic Glycoside from Digitalis Seeds.

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It was shown in the preceding paper¹⁾ that a large number of unknown cardioglycoside-like substances were present, besides the known glycosides such as digitalinum verum, in the seeds of *Digitalis purpurea*. First, the fraction of a strongly polar glycoside, thought to be contained in a comparatively large amount, was taken up. The presence of a similar glycoside in the leaves was detected by paper partition chromatography and Sasakawa²⁾ also detected an unknown glycoside of gitoxigenin series by paper partition chromatography.

Reichstein and others employed the technique of hydrolysis with various enzymes for strongly polar glycosides and used the so-called secondary glycosides so formed for the isolation. Since the method of isolation described in the preceding paper had been established, the primary glycoside was used, *per se*, for the separation.

The fraction containing a substance which gives a spot with smaller R_f value than that of digitalinum verum and which is more soluble in water, the fraction Nos. 10~16 (see Part II, Table I on p. 161 of this issue) obtained on alumina chromatography, was treated by the method of purification used for digitalinum verum.¹⁾ A new cardiotonic glycoside was successfully separated.

This fraction Nos. 10~16 contains a small amount of various substances, besides several kinds of cardioglycosides. It was therefore submitted to alumina chromatography using water-saturated butanol to remove the occluded substances. The cardioglycosides were developed rapidly and each fraction was submitted to paper partition chromatography as described in the preceding paper. It was thereby assumed that the substance A-VIII would be present in the largest amount. Therefore, fraction No. 11, which contained the largest amount of A-VIII, was recrystallized but only a small amount crystallized out, the majority remaining in the solution. This fraction was then submitted to partition chromatography with Celite 535 (Johns-Manville product) as a carrier and water-saturated methyl ethyl ketone as the developing solvent, by which the substance A-VIII was isolated in a pure state and easily crystallized.

Repeated recrystallization of this substance from water-saturated butanol and hydrated ethanol-ether or methanol-ether afforded colorless needle crystals (Fig. 1), m.p. 252~254°; $[\alpha]_D^{25} -3.4 \pm 2^\circ (c=2.14, \text{MeOH})$. It is extremely bitter to the taste, easily soluble in methanol and hydrated ethanol, soluble in dehydrated ethanol and water, and insoluble in chloroform, ether, and benzene. It gives positive Legal and Raymond reactions and its ultraviolet spectrum exhibits maximum absorption at 218 m μ (EtOH),

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1) Part II : This Bulletin, 5, 157(1957).

2) Y. Sasakawa : J. Pharm. Soc. Japan, 75, 947(1955).

TABLE I. Paper Partition Chromatography of Fraction Nos. 10~16

| Fraction No. | Eluted Product | Keller-Kiliani reaction (H ₂ SO ₄) | Raymond reaction |
|--------------|---------------------------|---|------------------|
| 1~6 | A-VI, -VII, B-II, C-I | Orange red | + |
| 7~8 | A-VII, -VIII, B-III, C-II | Red | + |
| 9~10 | A-VII, -VIII, C-II | " | + |
| 11 | A-VIII, -IX | " | + |
| 12~16 | A-VIII, -IX, B-IV | " | + |
| 17~23 | A-IX, B-IV | " | + |

Toyo Roshi No. 50, ascending method.

Stationary phase: Water. Moving Phase: Methyl ethyl ketone

indicating the presence of an unsaturated lactone of cardioglycosides. Keller-Kiliani reaction gives colorless glacial acetic acid layer and carmine red sulfuric acid layer, same as gitoxigenin, and the Gregg-Gisvold reaction is negative, indicating the absence of 2,6-desoxysugar.

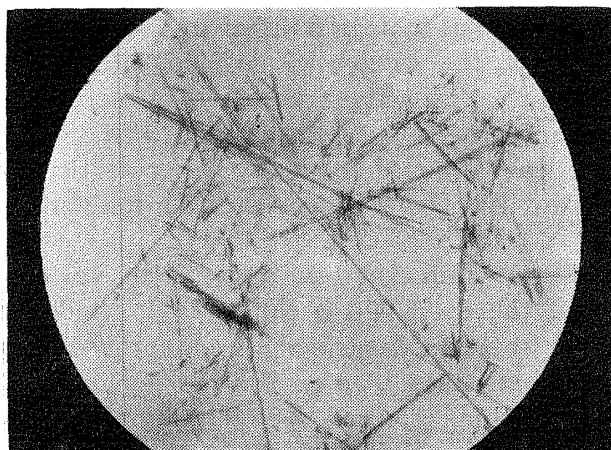


Fig. 1. Gitostin

×80

Hydrolysis of this substance by heating with 3.5% hydrochloric acid affords needles, m.p. 212~214°, as the aglycone, and this was identified as dianhydrogitoxigenin, obtained by the hydrolysis of digitalinum verum under the same conditions, through melting point determination, paper partition chromatography, and ultraviolet absorption spectrum. The sugar portion gave two spots respectively identical with digitalose and glucose, same as digitalinum verum, in paper chromatogram, but the coloration of the glucose spot was much more intense than that from digitalinum verum. Therefore, this substance has the same composition as that of digitalinum verum, with gitoxigenin as the aglycone and digitalose and glucose as the sugars. However, it was assumed from molecular absorption coefficient and the yield of aglycone from hydrolysis that there are three moles of sugar present and the analytical values agree well with C₄₂H₈₆O₁₉·2H₂O, with one mole of hexose added to digitalinum verum.

Acetylation of this substance by the usual method gives an acetate as plate crystals of m.p. 163~166°, whose analytical values were quite close to those of a nonacetate, C₈₀H₈₄O₂₈, derivable from the foregoing formula.

These experimental evidences make it almost certain that the sugar portion is composed of three moles of sugars but the amount of glucose formed by hydrolysis was determined by the Borel-Hostettler-Deuel method.³⁾ Tsukamoto and Kawasaki utilized this method for the determination of the sugar portion of diostin⁴⁾ and conditions of their hydrolysis were referred to in the present case. Digitalinum verum was hydrolyzed under various conditions and the glucose formed was determined.

3) E. Borel, F. Hostettler, H. Deuel: *Helv. Chim. Acta*, **35**, 115(1952).

4) T. Tsukamoto, T. Kawasaki, T. Yamauchi: *This Bulletin*, **4**, 35(1956).

It was learned that the glucose could be determined without any loss by carrying out the hydrolysis in carbon dioxide atmosphere by refluxing in a solution of 1:1 mixture of dioxane and water containing 3.5% of hydrochloric acid for six hours on a boiling water bath. These conditions were adopted for the hydrolysis of this substance and the presence of two moles of glucose was determined (Table II).

TABLE II. Determination of Glucose

| Substance | Sample mg. | Glucose found | | Glucose calcd. | |
|-------------------|---------------|---------------|------|----------------|-------------|
| | | mg. | % | 1 mole (%) | 2 moles (%) |
| Digitalinum verum | 41.00 | 9.4 | 22.9 | 24.05 | — |
| Gitostin (A-VIII) | 28.95 | 11.2 | 38.7 | 19.78 | 39.55 |
| Gitostin (A-VIII) | 28.95 | 13.2 | 45.6 | 19.78 | 39.55 |

It is known that substances possessing an acetyl group in 16-position of gitoxigenin, such as oleandrin and digitalinum verum hexaacetate, easily undergo deacetylation by treatment with adsorption chromatography with alumina and form substances with anhydrogitoxigenin, such as nerium-E⁵⁾ (anhydroöleandrin)⁶⁾ and anhydrodigitalinum verum pentaacetate,⁷⁾ exhibiting maximum absorption at 270 m μ in the ultraviolet spectra.

Alumina chromatography of the acetate of the present substance gave a fraction whose ultraviolet spectrum exhibited a new absorption band at 270 m μ . It follows, therefore, that the said acetate has an acetyl group in the 16-position, which excludes the bonding of extra glucose in this position, and since the hydroxyl in 14-position cannot be considered from the established idea on glycosides, it must be assumed that the extra glucose is also in the 3-position, as in digitalinum verum, composing a triglycoside. Bonding of the sugar is still unaccountable. As a glycoside of gitoxigenin series not possessing a 2,6-desoxysugar, separation of triglycoside has not been effected. The said glycoside (substance A-VIII) is a new glycoside and it was named gitostin.

As a glycoside possessing one mole of digitalose and two moles of glucose as the sugar portion, there is odorotriptide-G isolated from *Nerium odorum* SOL. by Reichstein and others,⁸⁾ but this substance has not been obtained in crystalline state. These workers derived odorotriptide to octaacetate which was purified, saponified, and the crystalline odorotriptide-G monoacetate so obtained was put to enzymatic decomposition to be separated into odorobioside-G monoacetate and odoroside-H. They reported that odorotriptide-G monoacetate has a structure of digitoxigenin- β -*d*-glucosido- β -*d*-glucosido- β -*d*-acetyldigitaloside.

Gitostin obtained in the present series of experiments may also possess one mole of glucose bonded to the terminal end of the glucose in digitalinum verum and structural studies by enzymatic decomposition will be described later. It is interesting that a series of mono-, di-, and triglycosides are present in *Digitalis purpurea* and *Nerium odorum*, strosposide, digitalinum verum, and gitostin in the former and odoroside-H, odoroside-F,⁹⁾ and odorotriptide-G in the latter.

Strosposide = gitoxigenin + digitalose

Digitalinum verum = gitoxigenin + digitalose + glucose

Gitostin = gitoxigenin + digitalose + glucose + glucose

Odoside-H = digitoxigenin + digitalose

Odoside-F = digitoxigenin + digitalose + glucose

Odosotriptide-G = digitoxigenin + digitalose + glucose + glucose

5) M. Okada : J. Pharm. Soc. Japan, **73**, 86(1953).

6) A. Hunger : Helv. Chim. Acta, **34**, 898(1951).

7) A. Hunger, T. Reichstein : *Ibid.*, **33**, 76(1950).

8) A. Rheiner, A. Hunger, T. Reichstein : *Ibid.*, **35**, 687(1952).

9) Odoroside F = gracilioside (J.P. Rosselet, T. Reichstein : Helv. Chim. Acta, **36**, 787(1953)).

Gitostin shows a fair cardiotonic action but detailed data on its physiological action will be reported later. Gitostin is more easily soluble in water than any of the known digitalis glycosides and is stable due to the absence of 2,6-desoxysugar, and it is presumed that the solution of gitostin in physiological saline would be of valuable asset in clinical application.

Deep gratitude is expressed for the kind guidance and encouragement from Dr. Junzo Shinoda, President of this Company, and Mr. Isamu Nakano, Director of the Yanagishima Factory. The writers are indebted to Messrs. Negishi and Abe of this Factory for elemental analyses.

Experimental

Alumina Adsorption Chromatography—The orange yellow residue (ca. 12 g.) from fraction Nos. 10~16, obtained by alumina chromatography as reported in Part II of this series,¹⁾ was dissolved in MeOH, mixed with silica gel (50~100 mesh), and dried in vacuum. A chromatographic column was prepared with 120 g. of alumina washed in with water-saturated BuOH into a glass tube of 3 cm. diameter. Water-saturated BuOH was added to the column, the silica gel with the sample was added in small portions, taking care not to allow air bubbles to remain in the silica gel layer, and the column was developed with water-saturated BuOH when the original BuOH layer receded to the top of alumina. Effluent was collected in 25-cc. fractions and paper partition chromatographic examination of each fraction gave results presented in Table I.

Fraction No. 11 was found to contain a small amount of substance A-VII, besides A-VIII, but it was recrystallized from water-saturated BuOH and 100 mg. of aggregated needle crystals, m.p. 248~250°, was obtained. Repeated recrystallizations afforded 60 mg. of the substance A-VIII as crystals melting at 250~252°.

Partition Chromatography—The fraction Nos. 7~16 (ca. 5 g.) containing substance A-VIII from the foregoing adsorption chromatography was adsorbed on Celite 535, as described in the preceding paper,¹⁾ and developed on a column (4.8×65 cm.) of 300 g. of Celite 535 with water-saturated MeCOEt, and the effluent was collected in 200-cc. fractions. Each fraction was examined by paper partition chromatography.

Substance A-VIII was found to be eluted in fraction Nos. 13~22 and the portion was recrystallized from water-saturated BuOH and from hydrated EtOH-ether mixture, affording 1 g. (0.05% from the seeds) of needle crystals were obtained.

Crystalline Substance A-VIII (Gitostin)¹⁰⁾—Recrystallization from water-saturated BuOH gives colorless, aggregated needles and that from hydrated EtOH-ether, microneedles, m.p. 252~254°. U. V. $\lambda_{\max}^{\text{EtOH}}$ 218 m μ (log ϵ 4.21. Calcd. as C₄₂H₆₆O₁₉·2H₂O=910.98). Other properties were described in the text. No loss in weight was observed on drying over P₂O₅ for 10 hrs. at 100° in vacuum (0.01 mm. Hg). Anal. Calcd. for C₄₂H₆₆O₁₉: C, 57.65; H, 7.60. Calcd. for C₄₂H₆₆O₁₉·2H₂O: C, 55.37; H, 7.75. Found: C, 54.77; H, 7.75.

Acetylation of Crystalline Substance A-VIII—Acetylation of 50 mg. of the crystalline substance A-VIII (m.p. 252~254°) by the usual method by standing with 2 cc. of pyridine and 1.5 cc. of Ac₂O for 48 hrs. and recrystallization from acetone-ether-petr. ether afforded colorless plates, m.p. 163~166°; $[\alpha]_D^{25}$ -16.5°±3°(c=1.57, CHCl₃). Anal. Calcd. for C₆₀H₈₄O₂₈ (Gitostin nonaacetate); C, 57.50; H, 6.76. Found: C, 57.03; H, 7.07.

Hydrolysis of Crystalline Substance A-VIII—a) A mixture of 20 mg. of the crystals in 10 cc. of 3.5% HCl-MeOH was refluxed in CO₂ atmosphere, on a water bath, for 6 hrs., MeOH was distilled off under a reduced pressure, and the colorless needles that precipitated out were recrystallized from dil. MeOH to needle crystals, m.p. 212~214°, undepressed on admixture with dianhydrogitoxigenin obtained by the hydrolysis of digitalinum verum. Its ultraviolet absorption maximum of 338 m μ (log ϵ 4.30) (EtOH), positive yellow coloration with tetranitromethane, and Rf 0.56 in paper partition chromatogram on Toyo Roshi No. 50, developed with the upper layer of cyclohexane: AcOH: CHCl₃: H₂O (100: 30: 20: 1), all agreed with those of dianhydrogitoxigenin.

The aqueous solution obtained after removal of the foregoing dianhydrogitoxigenin was treated with Amberlite IR-4B and the residue from its effluent was submitted to partition chromatography, using Toyo Roshi No. 50, developed by the ascending method at 20° with upper layer of BuOH: AcOH: H₂O (4: 1: 5). Coloration with aniline hydrogen phthalate revealed two spots, at Rf 0.11 and 0.40, which agreed respectively with the Rf values of glucose and digitalose, the sugars obtained on the hydrolysis of digitalinum verum. Identical spots were also obtained in multiple chromatography.

10) All m.p.s were measured on a Kofler block and uncorrected, ultraviolet spectra with Hitachi Model EPU-2 photoelectric spectrophotometer.

Determination of Glucose—A mixture of 28.95 mg. of the crystalline substance A-VIII (m.p. 251~254°) in a solution of 1:1 mixture of dioxane and water, containing 3.5% of HCl, was refluxed in CO₂ atmosphere for 6 hrs. on a water bath of 90~95°, the solution was concentrated to one-half the original volume under a reduced pressure and below 50°, and extracted with CHCl₃ to remove the aglycone. The CHCl₃ extract was washed with water, washing was combined with the aqueous layer, and treated with Amberlite IR-4B. The effluent was concentrated under a reduced pressure and the syrupy residue was dissolved in water in a 2-cc. measuring flask and diluted to the mark. This solution was submitted to paper chromatography by spotting 0.1 cc. of this solution on Toyo Roshi No. 50 and developed by the ascending method with the same solvent as that used for the development of sugars. The spot of glucose was cut out, extracted, and the extracted solution was concentrated. This concentrated extract was treated by the method of Borel and others and its optical density was measured at 520 m μ by a spectrophotometer. The same measurement was carried out with digitalinum verum and the results obtained are listed in Table II.

Summary

Of the unknown cardioglycosides contained in the seeds of *Digitalis purpurea* reported in the preceding paper, substance A-VIII was isolated in crystalline state. This substance occurs as a very bitter needle crystals, m.p. 252~254° (Kofler, uncorr.); $[\alpha]_D^{25}$ -3.1°, C₄₂H₆₆O₁₉·2H₂O; negative to the Keller-Kiliani reaction, sulfuric acid layer in carmine red, and positive to Legal and Raymond reactions; U.V. $\lambda_{\max}^{\text{EtOH}}$ 218 m μ (log ϵ 4.21). Its hydrolysis with 3.5% hydrochloric acid afforded dianhydrogitoxigenin and the sugar portion was found to be digitalose and glucose by paper partition chromatography. Determination of glucose by the Borel-Hostetteler-Devel method showed the presence of two moles. This substance was found to be a new glycoside, a triglycoside, of gitoxigenin series, and was named gitostin. A structure of gitoxigenin-glucosido-glucosido-digitaloside for gitostin as the gitoxigenin-series glycoside corresponding to odoroside-G was forwarded.

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28. Atsuji Okano, Kazuhiko Hoji, Tōsaku Miki, and Kazuo Miyatake :

Studies on the Constituents of *Digitalis purpurea* L. IV.¹⁾

Enzymatic Decomposition of Gitostin.

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It was shown in the preceding paper¹⁾ that a new cardioglycoside, gitostin, had been isolated from the leaves of *Digitalis purpurea* L., and that this glycoside is a triglycoside possessing gitoxigenin as the aglycone and one mole of digitalose and two moles of glucose as the sugars. It was assumed from the bonding order of the sugars in odorotrioxide-G²⁾ that the structure of gitostin would be gitoxigenin-glucosido-glucosido-digitaloside, with one more mole of glucose bonded to the terminal glucose in digitalinum verum.

Reichstein and others utilized enzymatic hydrolysis in their structural studies on cardioglycosides and revealed the structure of the sugar portion. Stoll also used the autoenzyme³⁾ taken out of fresh digitalis leaves for the enzymatic decomposition of true glycosides such as digilanid and purpurea glycoside and also examined the manner

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1) Part III: This Bulletin, 5, 163(1957).

2) A. Rheiner, A. Hunger, T. Reichstein: Helv. Chim. Acta, 35, 687(1952).

3) A. Stoll, W. Kreis: Ibid., 16, 1390(1933).