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30. Atsuji Okano : Studies on the Constituents of *Digitalis purpurea* L. IX[1]. The Structures of Gitostin and Neogitostin.

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The new cardiotonic glycoside, neogitostin,[1] isolated from the seeds of *Digitalis purpurea*, is a triglycoside, an isomer of gitostin, that possesses gitoxigenin as the aglycone and one mole of digitalose and two moles of glucose as the sugars. In Part IV[2] and VII[3] of this series, enzymatic decomposition of glycoside with an enzyme obtained from a snail[2] (*Euhadra quaesita* DESHAYES) was described and the same enzymatic method was adopted in the examination of the structure of neogitostin.

Aqueous solution of amorphous neogitostin was mixed with an enzyme solution and stood for 5 days. The enzyme powder was extracted with acetate buffer (pH 5.4) and neogitostin was found to be hydrolysed to strospeside and digitalinum verum. This result proves that neogitostin is gitoxigenin-glucosido-glucosido-digitaloside, which is the same as gitostin.

It was shown in Part VIII[1] that neogitostin monoacetate was clearly distinguished from gitostin monoacetate. The acetyl group of these was supposed to be in the digitalose portion, but it had not been proved. Therfore, an examination was made to see if the positions of these acetyl groups could be proved by hydrolysis with snail enzyme.

It had been reported in a previous paper that application of an enzyme solution obtained from a snail and treated with distilled water effected cleavage of only one mole of glucose from gitostin[2] and glucodigifucoside[3] during a short period, and digitalinum verum and digiproside were obtained in a good yield. Decomposition under the same conditions was first attempted with gitostin monoacetate. The state of hydrolysis of gitostin monoacetate was traced at definite intervals of time by paper chromatography and it was found that gitostin monoacetate was first transformed quantitatively into digitalinum verum monoacetate by elimination of only one mole of

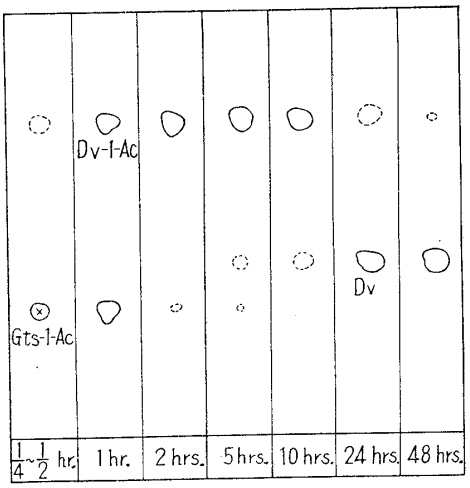

Fig. 1.
Paper Chromatography of Enzymatic Decomposition from Gitostin Monoacetate to Digitalinum verum

Toyo Roshi, No. 50. Ascending method, at 18~25°C.
Moving phase : Water-saturated MeCOEt
Stationary phase : Impregnated with H_2O•acetone (1:4)
Coloring agent : 20% $SbCl_3$-$CHCl_3$ solution.
Dv : Digitalinum verum
Dv-1-Ac : Digitalinum verum monoacetate
Gts-1-Ac : Gitostin monoacetate

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1) Part VIII. A. Okano : This Bulletin, **6**, 173(1958).
2) Part IV. K. Miyatake, *et al.* : *Ibid.*, **5**, 167(1957)
3) Phrt VII. A)(1957).

glucose, during only 2 hours, and then deacetylation followed, as shown in Fig. 1. In neogitostin monoacetate, the same treatment gave the same result as gitostin monoacetate, evidenced by paper chromatography.

Therefore, both gitostin monoacetate and neogitostin monoacetate were treated for 2 hours under the foregoing hydrolytic conditions, the reaction mixture was extracted, and purified by partition chromatography. Digitalinum verum monoacetate was obtained in a good yield and was identified with specimens prepared from digitalinum verum.[4]

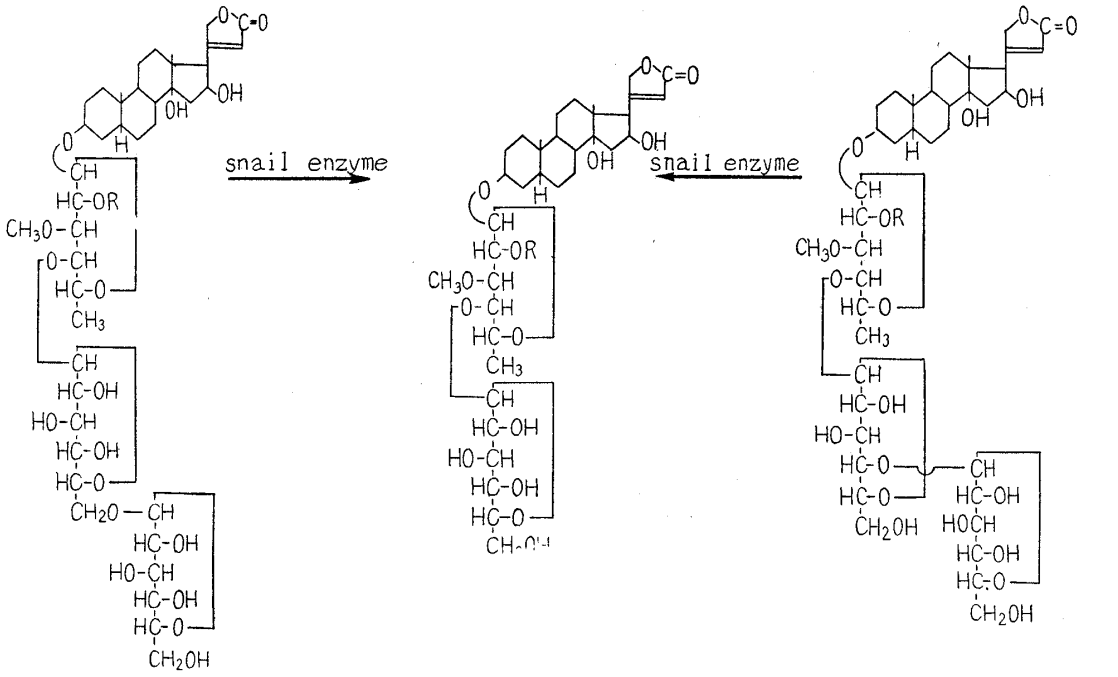
In the fermentation of gitostin and neogitostin monoacetate, the aqueous layer left after extraction of digitalinum verum and digitalinum verum monoacetate with chloroform–ethanol (2:1, v/v) mixture contained glucose liberated from glycosides and this glucose was determined by the Sumner's reagent.

From these results it was found that gitostin and neogitostin monoacetate were quantitatively transformed into digitalinum verum and digitalinum verum monoacetate. In other words, the enzymatic decomposition of these glycosides proceeded step by step.

It had been shown in a previous paper[2] that the snail enzyme first effected deacetylation and then cleavage of glucose from digitalinum verum monoacetate. It is interesting to note that in the triglycoside monoacetates, this enzyme first hydrolyzed one mole of glucose very rapidly and then the acetyl group.

The foregoing results have shown that the acetyl group in both is in digitalose portion, and that terminal glucose in gitostin monoacetate and neogitostin monoacetate is linked to the terminal glucose in digitalinum verum monoacetate. Consequently the structural difference between gitostin and neogitostin is only in the glucosidic linkage of the terminal glucose unit.

The linkage of terminal disaccharide in all the known cardiotonic triglycosides,



(I) Neogitostin R=H
(III) Neogitostin monoacetate R=CH_3CO–
(V) Digitalinum Verum R=H
(VI) Digitalinum verum monoacetate R=CH_3CO–
(II) Gitostin R=H
(IV) Gitostin monoacetate R=CH_3CO–

Fig. 2. Enzymatic Decomposition of Neogitostin Monoacetate and Gitostin Monoacetate

4) Part II. K. Miyatake, *et al.*: This Bulletin, **5**, 157(1957).

namely odoroside G,[5] k-strophanthoside,[6] echujin,[7] thevetin,[8] tanghinoside,[9] and odoroside K[10] are described as the 1,6-β linkage, indicated by the production of α-octaacetyl gentiobiose on acetolysis of their glycoside acetates.

The acetolysis of gitostin acetate and neogitostin acetate respectively gave α-octaacetyl-cellobiose and α-octaacetyl-gentiobiose, each respectively identified with authentic sample.

As shown in Fig. 2, gitostin (I) is gitoxigenin-β-cellobiosido-β-D-digitaloside and neogitostin (II) is gitoxigenin-β-gentiobiosido-β-D-digitaloside.

The known cardiotonic glycosides, which consist of the same aglycone and same sugars, but differing from each other in the sugar linkage, are found in only one pair of glycosides, odorobioside G and graciloside[11] (odoroside F).[12] However, these two glycosides are not isolated from the same plant in the strict sense, because odorobioside G is not contained in *Nerium odorum* but is formed by the partial enzymatic decomposition of odorotrioside G. It is very interesting that two isomeric triglycosides, gitostin and neogitostin, are present in the seeds of *Digitalis purpurea*.

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Experimental[13]

Enzymatic Decomposition of Neogitostin—Enzyme solution prepared from 300 mg. of snail enzyme powder and 50 cc. of acetate buffer (pH 5.2) was added to a solution of 80 mg. of amorphous neogitostin dissolved in 200 cc. of distilled water, 4 cc. of toluene added, and the mixture was allowed to stand at 32° for 5 days. The white turbid liquid was concentrated to 20 cc. under a reduced pressure at a bath temp. of 45° and 100 cc. of EtOH was added to the concentrated solution. The enzyme that precipitated out on standing was removed by the use of Hyflo Super Cel (Johns Manville product), the filtrate was concentrated to 15 cc. under a reduced pressure, and the concentrate was shaken 3 times with 9:1 mixture and 3 times with 2:1 mixture of $CHCl_3$-EtOH. $CHCl_3$-EtOH(9:1) portion was evaporated under a reduced pressure and the residue was chromatographed on 2 g. of alumina (Merck product). The residue from $CHCl_3$-EtOH(19:1) effluent was recrystallized from acetone-ether and 10 mg. of prismatic crystals, m.p. 241~248°, were obtained, which showed no depression of m.p. on admixture with strospeside, m.p. 242~246°. They were identified by paper partition chromatography.[14]

$CHCl_3$-EtOH(2:1) extracted fraction was submitted to partition chromatography with a column prepared from 30 g. of Celite 535 with water-saturated MeCOEt. The effluent was fractionated into 25 cc. each. Fraction Nos. 4~10 (40 mg.) revealed a spot of digitalinum verum in paper chromatography and its residue was recrystallized to 10 mg. of digitalinum verum, m.p. 235~239°.

This recrystallization mother liquor was acetylated by the usual method and 8 mg. of digitalinum verum hexaacetate was obtained as needle crystals, showing double melting point of 171~174°/218~225°. These crystals were proved by mixed m.p. and paper partition chromatography.

Examination of the State of Enzymatic Decomposition—2~5 mg. of gitostin, gitostin monoacetate, neogitostin, or neogitostin monoacetate was dissolved in 5~10 cc. of distilled water. A supernatant solution from 1~2 mg. of enzyme powder treated with 5 cc. of distilled water and centrifuged, was added to this solution together with 2 drops of toluene, and the mixture was allowed to stand in a thermostat

5) A. Rheiner, A. Hunger, T. Reichstein: Helv. Chim. Acta, **35**, 687(1952).
6) A. Stoll, J. Renz, W. Kreis: *Ibid.*, **20**, 1484(1937).
7) J.C. Hess, A. Hunger, T. Reichstein: *Ibid.*, **35**, 2202(1952).
8) H. Helfenberger, T. Reichstein *Ibid.*, **31**, 1470(1948).
9) M. Frerejacque, V. Hasenfratz: Compt. rend., **220**, 268(1948).
10) W. Rittel, T. Reichstein: Helv. Chim. Acta, **37**, 1361(1954).
11) A. Aebi, T. Reichstein: *Ibid.*, **34**, 1277(1951).
12) W. Rittel, T. Reichstein: *Ibid.*, **36**, 554(1953).
13) All m.p.s. were measured on a Kofler block and are uncorrected.
14) Toyo Roshi No. 50 impregnated with formamide and developed with a mixed solvent of xylene: MeCOEt(1:1) and toluene:BuOH(3:1), both saturated with formamide.

of 32°. About 1 cc. of every reaction mixture was taken out at definite intervals of time, 15 min., 30 min., and 1, 2, 5, 10, 24, and 48 hrs., and 5 cc. EtOH was added to each of them. They were filtered on Hyflo Super Cel and the filtrate was evaporated under a reduced pressure. Each residue was submitted to paper chromatography using a water-saturated MeCOEt on water-impregnated paper.

It was found that gitostin and neogitostin were similarly transformed into digitalinum verum very rapidly in only 2 hrs. and this digitalinum verum was not changed for 48 hrs. The other two monoacetates showed identical Rf values with that of digitalinum verum monoacetate and after 48 hrs. these spots were converted to the spot of smaller Rf value which was identical with that of digitalinum verum. All decompostion therefore proceeds stepwise.

Enzymatic Decomposition of Gitostin Monoacetate—Enzyme solution prepared from 20 mg. of enzyme powder and 10 cc. of distilled water was added to a solution of 40 mg. of gitostin monoacetate, m.p. 256~259°, dissolved in 150 cc. of distilled water, 2 cc. of toluene was added, and the mixture was allowed to stand at 32° for 2 hrs. This was treated as in the case of gitostin, chromatographed through a column prepared from 25 g. of Celite 535 and developed with water-saturated MeCOEt. The effluent fractions, whose residue showed identical Rf values with digitalinum verum monoacetate, were combined and recrystallized from acetone-ether and dil. MeOH to 24 mg. of needles, m.p. 242~245°, which showed no depression of m.p. on admixture with digitalinum verum monoacetate.

Enzymatic Decomposition of Neogitostin Monoacetate—a) Formation of Digitalinum verum Monoacetate from Neogitostin Monoacetate : Enzyme solution prepared from 5 mg. of enzyme powder and 2 cc. of distilled water was added to a solution of 14.8 mg. of neogitostin monoacetate, m.p. 234~241°, dissolved in 30 cc. of distilled water, 0.5 cc. toluene was added, and the mixture was allowed to stand at 32° for 2 hrs. The liquid was concentrated to ca. 7 cc. under a reduced pressure at a bath temperature of 45° and 35 cc. of EtOH was added to the concentrated solution. The enzyme that precipitated out on standing was removed by filtration through Hyflo Super Cel, the filtrate was concentrated to 5 cc. under a reduced pressure, and the concentrate was shaken with $CHCl_3$-EtOH(2:1) mixture. $CHCl_3$ layer was washed with water and evaporated under a reduced pressure. This extracted product was submitted to partition chromatography with a column prepared from 5 g. of Celite 535 and the eluted substance was recrystallized from dil. MeOH as in the case of gitostin monoacetate, to needles, m.p. 239~242°, which showed no depression of m.p. on admixture with digitalinum verum monoacetate.

b) Determination of Glucose : Foregoing aqueous layer and washing were combined and evaporated under a reduced pressure. The syrupy residue was dissolved in water in a 10-cc. measuring flask and diluted to the mark. One cc. of this solution was heated with 2 cc. of Sumner's reagent for 5 mins. on a boiling water bath and the mixture was cooled for 3 min. at room temperature. Then the mixture was diluted to 25 cc. in a measuring flask and its optical density was measured at 520 mμ by a spectrophotometer. The same measurement was carried out with gitosin and the results obtained are shown in Table I.

Table I. Determination of Glucose in Enzymatic Decomposition

Substance	Sample (mg.)	Glucose		
		Found		Calcd.
		mg.	%	1 mole (%)
Gitostin ($C_{42}H_{66}O_{19}\cdot 2H_2O$)	19.2	3.5	18.23	19.78
Neogitostin monoacetate ($C_{44}H_{68}O_{20}\cdot H_2O$)	14.8	2.7	18.24	19.28

Acetolysis of Gitostin Nonaacetate—530 mg. of gitostin nonaacetate, m.p. 165~168°, was added to a mixture of 9 cc. Ac_2O and $ZnCl_2$ (125 mg.), and the mixture was heated in a boiling water bath for 0.5 hr. The reddish brown reaction mixture was allowed to stand at room temperature for 15 mins. and poured into ice water. After 15 hrs. the precipitate was collected by filtration, washed successively with water, 10% NaOH, 10% Na_2CO_3, 5% H_2SO_4, and water, and the precipitate was dissolved in $CHCl_3$. This solution was dried over anhyd. Na_2SO_4, $CHCl_3$ was evaporated under a reduced pressure, and the residue was treated with petr. ether (b.p. 40~60°). The insoluble substance was submitted to chromatography through a column prepared from a mixture of 8.5 g. of Florisil (Floridin Co., U. S. A.) and 1.5 g. of Celite 535. The first 150-cc. benzene eluate was very small. The following 180-cc. benzene eluate containing 1% EtOH easily crystallized from EtOH, and repeated recrystallisation from EtOH and MeOH-$CHCl_3$ gave 140 mg. of needle crystals, m.p. 224~227°, $[\alpha]_D^{24}$ +38.4°±3°(c=1.537 in $CHCl_3$). These crystals showed no depression of m.p. on admixture with α-octaacetyl-cellobiose, m.p. 227~229°, $[\alpha]_D^{24}$ +41.4°±2°(c=0.942 in $CHCl_3$), prepared from cellulose.[15]

Acetolysis of Neogitostin Nonaacetate—To a mixture of 10 cc. Ac_2O and 140 mg. $ZnCl_2$, 600 mg.

15) D. H. Brauns : Org. Syntheses, XVII, 36.

of neogitosin nonaacetate, m.p. 194~197°, was added and the mixture was heated in a boiling water bath for 30 mins. It was treated as in the case of gitosin nonaacetate and chromatographed on a mixture of 10 g. Florisil and 2 g. Celite 535, developing with benzene and benzene-EtOH. From the eluate of benzene containing 1% EtOH, 120 mg. of needles, m.p. 187~190°, $[\alpha]_D^{24}$ +50.3°±2°(c=1.315 in $CHCl_3$), was obtained. This substance gave no depression of m.p. on admixture with authentic sample of α-octaacetyl-gentiobiose, m.p. 188~190°, $[\alpha]_D^{24}$ +52.7°±2°(c=1.023 in $CHCl_3$), prepared from the root of *Gentiana lutea* L.[16]

Summary

The structure of neogitostin, newly isolated from digitalis seeds by the writer and described in the preceding paper,[1] was examined by enzymatic decomposition using the same enzyme isolated from a snail (*Euhadra quaesita* DESHAYES) as described in Part IV of this series.[3] It was found that hydrolysis of amorphous neogitostin afforded strospeside and digitalinum verum. However, hydrolysis of monoacetates of gitostin and neogitostin, under the same conditions as those used in the partial decomposition of gitostin to form digitalinum verum but by shortening the duration of hydrolysis, glucose alone was found to be hydrolyzed from these monoacetates, affording digitalinum verum monoacetate. From these results, it was found that gitostin and neogitostin are isomeric triglycosides whose terminal glucose is linked to the terminal glucose in digitalinum verum. By respective acetolysis of gitostin nonaacetate and neogitostin nonaacetate, it was established that gitostin is gitoxigenin-β-cellobiosido-β-D-digitaloside and neogitostin is gitoxigenin-β-gentiobiosido-β-D-digitaloside.

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16) C. S. Hudson, J. M. Johnson: J. Am. Chem. Soc., **39**, 1272(1917).