N-Methylation of the Ketal-lactam (XLI)—A mixture of the ketal-lactam (XLI) (100 mg.) and NaH (80 mg.) (50% in mineral oil) in dry benzene (30 ml.) was heated under reflux for 2 hr. After cooling, methyl iodide (1 ml.) was added to the benzene solution which was refluxed for 1 hr. The reaction mixture was washed with H₂O, dried and evaporated to dryness to give the N-methyl-ketal-lactam (XLI) (85 mg.) which was crystallized from acetone–ether as prisms, m.p. 179–180°C. IR ν̇cm⁻¹: 1640 (amide C=O). Anal. Calcd. for C₁₃H₂₀O₅N: C, 66.07; H, 6.71; N, 4.06. Found: C, 65.79; H, 6.80; N, 4.07.

Tetrahydrooxocinine Methine (XLIII)—A solution of the N-methyl-ketal-lactam (XLI) (70 mg.) and LiAlH₄ (80 mg.) in tetrahydrofuran (30 ml.) was heated under reflux for 5 hr. The excess reagent was decomposed by addition of a small amount of H₂O and the precipitate which formed was removed by filtration. The filtrate was concentrated to dryness to give a solid which was taken up in CHCl₃. The CHCl₃ solution was extracted with diluted HCl and the aqueous layer was basified with aqueous NH₄OH and extracted with ether. The ether extract was washed with H₂O, dried and evaporated to dryness to afford tetrahydrooxocinine methine (XLIII) (50 mg.) which crystallized from EtOH–ether as prisms, m.p. 147–148°C, identical in all respects with an authentic sample. UV λₚₑₘₚoₚₖ mµ (log ε): 242 (3.62), 290 (3.58). IR ν̇cm⁻¹: 1705 (C=O). Anal. Calcd. for C₁₇H₂₁O₅N: C, 71.05; H, 7.37; N, 4.87. Found: C, 71.33; H, 7.45; N, 4.93.

**Summary**

Tetrahydrooxocinine methine (XLIII) has been synthesized by a sequence of reactions including ring enlargement of a tetralone (XXXVI) to a seven-membered nitrogenous ring compound (XXXVIII) by the use of Schmidt reaction.

(Received November 20, 1964)

---

56. Toshio Kawasaki, Itsuo Nishioka, Tatsuo Yamauchi, Kazumoto Miyahara, and Miyoko Enbutsu: Digitalis Saponins, III.*¹ Enzymatic Hydrolysis of Leaf Saponins of Digitalis purpurea L. (Faculty of Pharmaceutical Sciences, Kyushu University*²)

In the preceding paper*¹ of this series it was reported that the crude saponin of *Digitalis purpurea* leaves precipitated by cholesterol from the butanol extracts of the remainings from the manufacture of cardiac glycosides contained two saponins (I and II). I was composed of tigogenin- and gitogenin-tetraglycosides (desgalactotigogenin and F-gitonin) and II was a mixture of three pairs of more polar oligosides each of which seemed also to consist of tigogenin- and gitogenin-glycosides. Since I and its component saponins had*⁰ the greater capacity than II and commercial "digitonin" in forming molecular compound of great insolubility with cholesterol, it was desired to obtain enough amount of I in order to investigate the structures and properties of its component saponins in detail. This paper concerns a convenient method to prepare I, and subsequently the component saponins, from the crude saponin and more efficiently from the original butanol extract by a specific partial hydrolysis of II with commercially available enzyme preparations.

---

*² Katakasu, Fukuoka (川崎敏男, 西岡五夫, 山内辰郎, 宮原一元, 円仏美代子).
The content of I in the crude saponin*1 was less than that of II, the isolation of I by alumina chromatography as reported*1 is not always practical for a large scale experiment, and I has remained relatively inaccessible. However, co-existence of I and II suggests that the former might be the prosapogenin of the latter and II could be converted enzymatically*3 into I. As a preliminary experiment, when II obtained by alumina chromatography was treated in aqueous alcohol with emulsin, snail enzyme, hemicellulase and amylase and the products were examined by paper chromatography, II was found to be cleaved to give a saponin (tentatively represented as FSE) corresponding to I and glucose. The experiment was then conducted in a larger scale and a considerable amount of amorphous precipitates*3 was obtained as a product. The product was extracted with chloroform–methanol (1:1) mixture, the solution was concentrated and left to stand to give a crystalline solid which revealed one spot of FSE on paper chromatograms and was shown on thin-layer of silica gel G to be composed of two saponins (FSE-1 and -2) which correspond very likely to desgalactotigogenin and F–gitonin (Fig. 1). The mixture was separated on cellulose powder impregnated with formamide*1 or preferably on silica gel column*4 into FSE-1, fine needles, m.p. 284–286° (decomp.), [ã]D +68° (c=0.50, pyridine), and FSE-2, fine needles, m.p. 251–255° (decomp.), [ã]D −66° (c=0.50, pyridine).

By acid hydrolysis followed by qualitative and quantitative determinations of the products they were found to be tigogenin– and gitogenin–glycosides both of which had the same kind and number of monosaccharide units (2 glucose+galactose+xylene) and were identified with desgalactotigogenin and F–gitonin, respectively, by direct comparisons with the authentic samples.**1 According to it was proved that FSE is nothing but I and that desgalactotigogenin and F–gitonin are the prosapogenins of II and selectively yielded by enzymatic cleavage from the corresponding parent saponins.

In the above experiment it was noted that the tetracyclisides formed were not likely to undergo further degradation*4,5 and the method seemed applicable to a mix-

---

*1 M. M. Krider, J. R. Branaman, M. E. Wall (J. Am. Chem. Soc., 77, 1238 (1955)) have reported that the partial hydrolysis of steroid saponin of Yuca schidigera (water–soluble) with native enzymes or dil. HCl or H2SO4 produced water–insoluble prosapogenins, and that the products of enzymatic cleavage (consisting primarily of sarsasapogenin linked to glucose–galactose oligosides) were obtained in the crystalline form. An attempted partial hydrolysis of II with acid resulted in producing less polar compounds together with I and II. Kikuh–saponin (diosgenin + 3 glucose + rhamnose) of Dioscorea septemloba, was cleaved with emulsin to give gracillin (diosgenin + 2 glucose + rhamnose) as precipitates from the reaction mixture (T. Kawasaki, T. Yamauchi, R. Yamauchi: This Bulletin, 10, 698 (1962)).

*2 Chromatography on silica gel was advantageous over that on formamide–impregnated cellulose powder in easiness of the procedure and in resolving power of the component saponins. According to the private communication (March 6, 1964) from Prof. Tscheshche, some saponin mixtures were successfully separated by silica gel chromatography also in his laboratory.

*3 In part, at least, it may be due to the insolubility of the product. The BuOH extract and the crude saponin from the leaves harvested in September contained originally a trace amount of less polar compound (substance x) besides I and II (cf. Fig. 2a, b, c).

---

Fig. 1. Thin-layer Chromatograms on Silica Gel G
Solvent: CHCl₃-MeOH-H₂O (65:35:10)
a: Saponin II*1 c: Desgalactotigogenin*1
b: FSE
f: FSE-2
c: Saponin I*1 g: F–Gitonin*1
d: FSE-1
ture of I and II. Hence the hydrolysis of the crude saponin was carried out in the same way and I was successfully obtained in 27～53% yield.*6

Meanwhile, a treatment of the original butanol extract with decolorizing carbon in methanol provided a highly hygroscopic (giving a clear aqueous solution) yellowish-brown powder (Ex) which showed a thin-layer chromatogram (Fig. 2c) quite similar*8 to that (Fig. 2b) of the crude saponin. Therefore the direct use of the extract was thought to offer a more efficient and economical method for the preparation of I. When an aqueous solution of Ex was allowed to stand with a commercial amylase or cellulase preparation, the reaction mixture yielded precipitates quite analogously to the above experiment for the crude saponin. They were extracted with chloroform-methanol (extract: TLC, Fig. 2d; yield, 24～40%) and recrystallized to give I revealing only two spots of the expected tetracyclosides on thin-layer (Fig. 2e).

I thus obtained from the crude saponin or from the butanol extract was fractionated into desgalactotigogenin and F-gitonin by silica gel chromatography as mentioned afore, and when the latter is contained in I about 85%,*8 pure F-gitonin was provided only by repeated recrystallization of I from dilute ethanol and from butanol saturated with water.

Thereby saponin (I) in the Digitalis purpurea leaves, accordingly also the two component tetracyclosides, particularly F-gitonin, became available in large amounts.

Recently Tschesche and Wulff have reported*7 that the hydrolyses of “digitonin” (Merck) (seed saponins of Digitalis purpurea) in water with four kinds of enzyme preparations resulted in a slight splitting (by β-glucosidase and “Luizyme”) of xylene, glucose and galactose. The difference in behaviors of seed- and leaf-saponins against enzyme is worth of note.

---

*6 In one case when a sample was cleaved in H₂O, I was obtained in up to 60% yield (cf. experimental part).
*7 Several unknown substances*9 detected on paper chromatograms of the original BuOH extract were almost removed by the treatment with carbon in MeOH. The relative amounts of the tetracyclosides (vs. the polar oligosides) in Ex was apparently less than those in the crude saponin (cf. preceding paper*9).
*8 The content (purity) was calculated from the yields of gitogenin and tigogenin obtained by hydrolysis of a saponin mixture followed by alumina chromatographic separation of the sapogenin fraction. The yield of gitogenin from the BuOH extract or Ex was almost same as those from crude saponin*9 precipitated by cholesterol and from enzymatically prepared I (=FSE), while it varied to a considerable extent depending on the harvest time of the leaves: leaves harvested in September, gitogenin ca. 60%; those harvested in October- November, ca. 85% (cf. Fig. 2a, b). F. Weiss and O. Manns (Pharm. Zentralhalle, 98, 437 (1959)) have reported that “tigogenin” was the major component of a leaf saponin (yield of sapogenin : tigogenin, 40～70%; gitogenin, 20～30%).
Experimental

Enzymatic Hydrolysis of Saponin (II)—As a preliminary experiment, II (50 mg.) was incubated with an enzyme, the reaction mixture was diluted with H₂O, extracted with BuOH, and BuOH and H₂O layers were respectively evaporated in vacuo and examined by the conventional paper chromatography (PC). The results are summarized in Table I.

<table>
<thead>
<tr>
<th>Medium (ml.)</th>
<th>Enzyme (mg.)</th>
<th>Time (days)</th>
<th>Temp. (°C)</th>
<th>BuOH layer (saponin)</th>
<th>H₂O layer (sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% EtOH 100</td>
<td>emulsins⁵)</td>
<td>5</td>
<td>25</td>
<td>FSE⁶)</td>
<td>glucose⁷)</td>
</tr>
<tr>
<td>AcOH</td>
<td>0.2</td>
<td>500</td>
<td></td>
<td>II (trace)</td>
<td></td>
</tr>
<tr>
<td>30% EtOH 100</td>
<td>snail enzyme⁸</td>
<td>5</td>
<td>25</td>
<td>FSE</td>
<td>glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% EtOH 50</td>
<td>hemicellulase⁹</td>
<td>7</td>
<td>25</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>AcOH</td>
<td>0.1</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% EtOH 50</td>
<td>&quot;takadiastase-A&quot; (¹⁰)</td>
<td>7</td>
<td>30</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>AcOH</td>
<td>0.1</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b) Kindly furnished by Dr. M. Shimizu of Daiichi Seiyaku Co., Ltd., to whom the authors are grateful.
c) Commercial preparation (Tokyo Kasei Co., Ltd.).
d) Kindly furnished by Dr. M. Iwal of Sankyo Co., Ltd., to whom the authors are grateful.
e) Stand for the product of Rf 0.45 on paper chromatograms⁴) (solv. BuOH-AcOH-H₂O 4:1:5; Rf values of reference compounds, saponin (1) 0.45, saponin (II) 0.20).
f) Examined by paper chromatography.⁴¹

Subsequently II (gigogenin glycoside content: ca. 85%)⁸) (2.0 g.) was dissolved in 20% EtOH (500 ml.), and AcOH (0.2 ml.), toluene (0.3 ml.) and hemicellulase or "takadiastase-A" (4.0 g.) were added. The mixture was allowed to stand at 30°C for 7 days. The amorphous precipitates formed were collected by filtration, dried, extracted with CHCl₃-MeOH (1:1) and the solution was concentrated and left stand to give a crystalline solid (FSE) (gigogenin glycoside: ca. 85%) (yield, 930 mg.; PC Rf 0.45; TLC, Fig. 1). From another sample of II (gigogenin glycoside: ca. 60%)⁸) FSE (gigogenin glycoside: ca. 60%) was similarly obtained.

Fractionation of the Product (FSE) into the Component Saponins (FSE-1 and -2)—Cellulose powder chromatography: FSE (150 mg.) above obtained (gigogenin glycoside: ca. 60%) was separated into four fractions by chromatography on cellulose powder impregnated with formamide in the same way as reported before using CHCl₃-tetrahydrofuran-pyridine (10:10:2)/formamide 4) as the solvent. Fr. 2 (TLC: FSE-1 +, FSE-2 −) was recrystallized from CHCl₃-MeOH to give a crystalline powder (12 mg.). Further recrystallization from CHCl₃ or CHCl₃-MeOH afforded thin-layer chromatographically pure FSE-1 as a crystalline powder, m.p. 284~268° (decomp.). Fr. 4 (TLC: FSE-1 −, FSE-2 +) was recrystallized from CHCl₃-MeOH and from BuOH saturated with H₂O (BuOH-H₂O) to give pure (TLC) FSE-2 as fine needles (18 mg.), m.p. 252~255° (decomp.).

Silica gel chromatography: FSE (500 mg.) in CHCl₃-MeOH-H₂O (7:3:1) (bottom layer) (3 ml.) was placed on a silica gel (Kantō Kagaku Co., Ltd., 100~200 mesh, 250 g) column (diameter, 5 cm.), eluted with the same solvent, fractionated into 416 portions and each portion (ca. 5 ml.) was examined by TLC: Fr. 1~240, none; Fr. 241~260, 3 mg., unknown substance (x); Fr. 261~265, 25 mg., x ±, FSE-1 +; Fr. 266~300, 116 mg., FSE-1; Fr. 301~310, 29 mg., FSE-1 +, FSE-2 ±; Fr. 311~325, 27 mg., FSE-1 +, FSE-2 ±; Fr. 326~337, 62 mg., FSE-1 ±, FSE-2 ±; Fr. 338~380, 178 mg., FSE-2; Fr. 391~416, 47 mg., FSE-2 ±, polar substance ±. Fr. 417~300 was recrystallized from dill. MeOH to give thin-layer chromatographically pure FSE-1 as fine needles, m.p. 284~268° (decomp.), [α]²⁰ −68° (c=0.5, pyridine). Fr. 388~390 was recrystallized from dill. MeOH or BuOH-H₂O to afford pure (TLC) FSE-2 as fine needles, m.p. 251~255° (decomp.), [α]²⁰ −68° (c=0.5, pyridine).

¹⁰ Paper chromatography (PC) of saponins, sopogenins and sugars, thin-layer chromatography (TLC) of saponins, hydrolysis of saponins and qualitative and quantitative determinations of the products were all carried out in the same ways as described before.¹¹ All melting points were taken on a Kofler block and are uncorrected.
FSE-1—Acid hydrolysis of FSE-1 (6.6 mg.) yielded tigogenin (2.6 mg.), and xylose, glucose and galactose in a ratio of 1:1.7:1 (calcld. for desgalactotigotin dihydrate: tigogenin, 2.64 mg.; sugar ratio, xylose-glucose-galactose=1:2:1). Indentified with desgalactotigotin*1 by mixed melting point, comparisons of \([\alpha]_D^25\) and IR spectra and co-chromatography on thin-layer.

FSE-2—Acid hydrolysis of FSE-2 (10.0 mg.) yielded tigogenin (4.3 mg.), and xylose, glucose and galactose in a ratio of 1:1.8:0.9 (calcld. for F-gitonin dihydrate: tigogenin, 3.98 mg.; sugar ratio, xylose-glucose-galactose=1:2:1). Identical with F-gitonin*1 in every respect.

Enzymatic Hydrolysis of the Crude Sapoin (a Mixture of Sapoinos (I and II))—Crude sapoim (tigogenin glycoside: ca. 85%) (TLC, Fig. 2a) (10 g.) obtained via molecular compound with cholesterol was dissolved in 20% EtOH (3 L) containing AcOH (3 ml.) and toluene (2 ml.) and the solution was treated with "takadiastase-A" or hemicellulase (20 g.) at 37° for 8 days. The reaction mixture (amorphous substances being deposited) was diluted with H₂O (1 L.) and the precipitates were collected by filtration, extracted with CHCl₃-MeOH and the solution was concentrated and left stand to give FSE (1 l.) as a crystalline powder (3.9 g.), PC, Rf 0.45; TLC, Fig. 2c. In several runs the yields were in the range of 27~58%. Another sample of crude sapoim (tigogenin glycoside: ca. 60%) (TLC, Fig. 2b) provided FSE in 45% yield in the same way as above, and the same sample in H₂O (1%) with "takadiastase-A" (half amount as much as that of the sapoin) gave FSE in about 60% yield.

Enzymatic Hydrolysis of the Butanol Extract of the Remains of the Manufacture of Cardiac Glycosides—BuOH extract (25 g.) in 90% MeOH (250 ml.) was refluxed with decolorizing carbon (5 g.) for 1 hr., carbon was filtered off and the filtrate was evaporated in vacuo to provide an yellowish-brown, highly hygroscopic powder (Ex) (21 g.). It gave a clear aqueous solution and the thin-layer chromatogram as shown in Fig. 2c. An eq. solution of Ex was subjected to enzymatic hydrolyses at 37° in various conditions: concentration of Ex, 0.5~10%; kind and amount of enzyme preparation, "takadiastase-A", "B",**10 hemicellulse, "sanastase"**11 and "'mecelase"**12 twice~one twentieth weight as much as that of Ex; incubation time, 1~4 days. The precipitates separated out from the reaction mixture were collected by centrifugation, washed with H₂O (again centrifuged), dried in vacuo, and extracted with CHCl₃-MeOH (1:1). The solution was evaporated to dryness in vacuo, weighed and examined by TLC. Satisfactory results were obtained by incubation of 0.5~2% Ex solution with twice~one-fourth amount of any enzyme used (except for hemicellulase) as much as that of Ex for 4 days. Some examples are as follows: Ex (tigogenin glycoside: ca. 60%) (TLC, Fig. 2c), 2 g.; H₂O, 200 ml.; "takadiastase-B", 0.5 g., 1 g., 2 g., 4 g.; at 37° for 4 days; yield of CHCl₃-MeOH extract (TLC, Fig. 2d), 0.54 g., 0.52 g., 0.5 g., 0.48 g., respectively. In larger scale experiments, above Ex (40 g.) and "takadiastase-B" (10 g.) in H₂O (4 L.) at 37° for 4 days gave FSE (10.6 g.) as a crystalline solid (recrystallized from CH₂Cl₂-MeOH) (TLC, Fig. 2e), while another Ex (tigogenin glycoside: ca. 85%) (30 g.) and "takadiastase-B" (60 g.) afforded FSE (12 g.) as needles (recrystallized from BuOH-H₂O).

Preparation of F-Gitonin and Desgalactotigotin—FSE thus obtained from the crude sapoin or from the BuOH extract was separated into pure F-gitonin and desgalactotigotin in a larger scale by silica gel chromatography as described for that from I.

In the case when FSE contained ca. 85% F-gitonin, the sample (9.2 g.) was recrystallized from 70% EtOH and then from BuOH-H₂O to give F-gitonin (6.2 g.) of ca. 94% purity**8 as fine needles, m.p. 246~252° (decomp.), and further recrystallization (8 times) from BuOH-H₂O afforded that of almost 100% purity as fine needles, m.p. 243~252° (decomp.). However, in another sample of FSE (F-gitonin: ca. 60%) of which solubility in BuOH-H₂O was relatively high, it was hard to obtain pure F-gitonin only by recrystallization. Thus, the sample (500 mg.) gave a crystalline powder (200 mg.) (F-gitonin: ca. 60%), m.p. 256~258° (decomp.) (from dil. MeOH and MeOH or CH₂Cl₂-MeOH), or needles (120 mg.) (F-gitonin: ca. 70%), m.p. 256~260° (decomp.) (from BuOH-H₂O (twice)).

The authors thank Shionogi & Co., Ltd. for the butanol extracts of the digitalis leaves, Prof. T. Momose and Dr. Mukai for the kind advices and helps in the micro determination of sugars, and Miss N. Nasu for technical assistance.

*10 Commercial preparation (Sankyo Co., Ltd.).
*11 Commercial preparation (Meiji Seika Co., Ltd.). An enzyme mixture (α- and β-amylases (main) and protease, lipase, cellulase, etc.) from Aspergillus niger. Kindly furnished by Dr. S. Kawaji of Meiji Seika Co., Ltd., to whom the authors are grateful.
*12 Commercial preparation (Meiji Seika Co., Ltd.). An enzyme mixture (cellulase (main) and cellobiase, xylanase, amyrase, etc.) from Trichoderma koningi. Kindly furnished by Dr. S. Kawaji of Meiji Seika Co., Ltd., to whom the authors are grateful.
Summary

Saponin (II) (a mixture of polar oligosides), the major component of the leaf saponins of Digitalis purpurea L., was found to be enzymatically cleaved to yield solely the minor component, saponin (I) (a mixture of two tetracylglycosides, desgalactotigotin and F-gitonin).

Saponin (I), subsequently the component two tetracylglycosides, were conveniently prepared from the crude leaf saponins precipitated by cholesterol and more efficiently from the butanol extract of the leaves by the specific partial hydrolysis of saponin (II) with some commercial enzyme preparations.

(Received December 19, 1964)


(Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.*1)

Recently, Vitamin B₁ has been improved to provide maintainable efficiency, higher absorbability and inertness to Thiaminase. Vitamin B₁ analogues can be classified into two groups as regards the thiazole ring cyclizing or not, and the latter case can be divided into the symmetric disulfide type and other sulfide derivatives.

In the present paper, the chemical shifts and the temperature dependence of the proton magnetic resonance spectra were studied to obtain the conformational relation between pyrimidine ring and thiazole ring or N-formyl group.

Experimental

For the following various vitamin B₁, either recrystallized from the commercial reagents or synthesized by the usual method, the proton magnetic resonance (NMR) spectra were measured on Japan Electron Co. J.N.M-60 spectrometer at 60 Mc. About 15% solutions of the samples, in N/2 HCl acidic deuterium oxide added a few drops of dioxane as internal standard, or in CDC₃ with TMS, were prepared. The temperature of these sample was changed over the range from −2° to 80° by Japan Electron Co. JES-VT-2 Temperature Controller.

Samples:
- Thiamine [T]
- Thiothiamine [TT]
- S-Methylthiamine [MT]
- Thiamine diphosphate [TDP]
- O-Benzoylthiamine [OBT]
- S-Benzoylthiamine monophosphate [BTMP]
- Thiamine disulfide [TDS]
- Thiamine disulfide monophosphate [TDMP]
- O-Benzoylthiamine disulfide [BTDS]

Results and Discussion

The proton chemical shifts of various vitamin B₁ are shown in Chart 1. Further the influence of substituted oxyethyl group and cyclization of thiazole ring are described in Table 1.

*1 Kashima-cho, Higashiyodogawa-ku, Osaka (小寺啓司).