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92. Masashi Okada,*1 Atsushi Yamada,*1 and Morizo Ishidate*2 : Bioconversion of Cardiac Aglycones by *Gibberella saubinettii* (MONT.) SACC.

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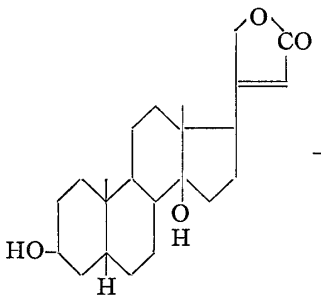
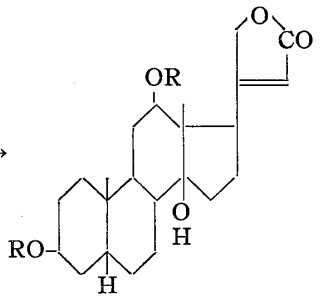
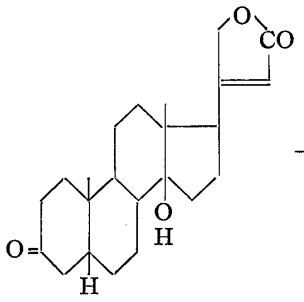
Quite recently a series of papers concerning microbiological transformations of cardiac aglycones, glycosides, and bufogenins by *Fusarium lini* (BOLLEY) have been presented by Tamm and Gubler.[1~4] The present authors had also been working independently on the same subject and the results obtained by the use of a strain of *Gibberella*



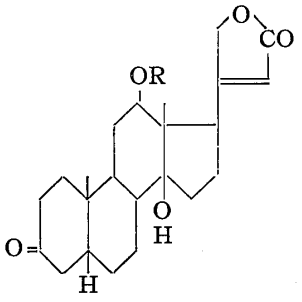
(I)



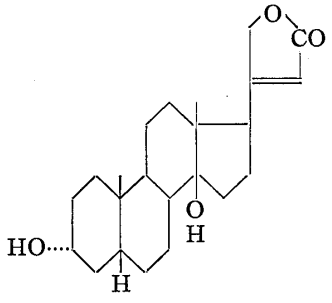
(IIa) R=H
(IIb) R=$COCH_3$



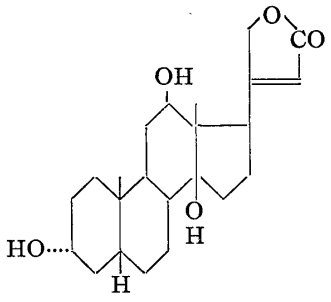
(III)



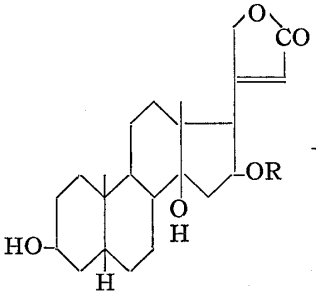
(IVa) R=H
(IVb) R=$COCH_3$



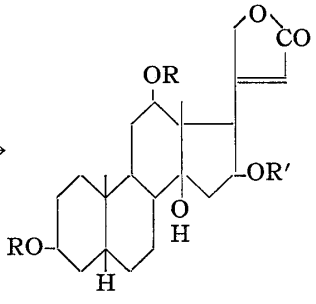
(V)



(VI)



(VII) R=H
(VIII) R=$COCH_3$



(IX) R=R'=H
(X) R=H, R'=$COCH_3$
(XI) R=R'=$COCH_3$

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1) A. Gubler, Ch. Tamm : Helv. Chim. Acta, **41**, 297(1958).
2) *Idem* : *Ibid.*, **41**, 1762(1958).
3) *Idem* : *Ibid.*, **42**, 239(1959).
4) *Idem* : *Ibid.*, **42**, 473(1959),

saubinettii (MONT.) SACC.*3 will be described in this paper. This work was initiated as a preliminary experiment to investigate the catabolism of cardiac aglycones and glycosides of digitalis in organisms, on which some observations have been reported recently.[6,7]

After screening various fungus strains using digitoxigenin (I) and gitoxigenin (VII) as a substrate, it was found by paper chromatographic method that a strain of *G. saubinettii* dissimilates these compounds considerably. Actually, digoxigenin (II) was obtained in a good yield (over 70%*4) as a sole transformation product when the strain was incubated with (I) in preparative quantities. Residual starting material could not be detected in this bioconversion. Identification of the transformation product with digoxigenin was made by comparison with an authentic sample and preparation of the acetate (IIb).

Digitoxigenone (III) was converted into three substances,*5 3-dehydrodigoxigenin (IVa), 3-epidigitoxigenin (V), and 3-epidigoxigenin (VI). The structure of the principal transformation product (IVa) (53% yield) was definitely established by converting it into the acetate which was shown to be identical with 3-dehydro-12-acetyldigoxigenin (IVb) prepared by another unequivocal way reported elsewhere.[8] The second transformation product, 3-epidigitoxigenin (V) (3.5% yield), was identical with an authentic sample prepared by sodium borohydride reduction of (III).[8,9] The third one, the formation of which was indicated by paper-chromatographic method alone, was reasonably inferred as 3-epidigoxigenin (IV) by paper-chromatographic comparison with an authentic sample prepared from (IVa) by sodium borohydride reduction[3,8] and the isolation of (V) in this bioconversion. It has been observed that microbiological reduction of 3-oxo group in cardiac aglycones with A/B-*cis* ring juncture yields equatorial 3α-hydroxyl group exclusively.[2,3]

Conversion of gitoxigenin (VII) to diginatigenin (IX)[10,11] by the present strain was achieved as expected in much higher yield (ca. 6%) than in the case of *F. lini* (reported

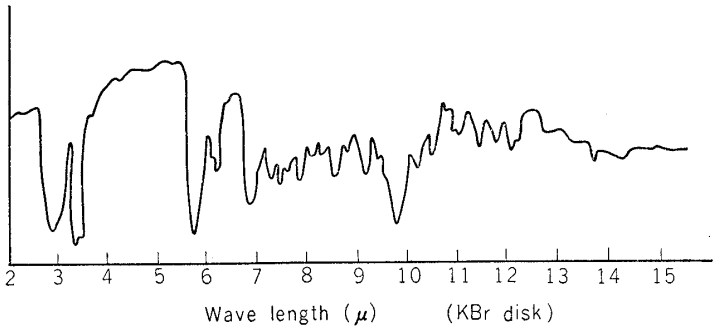

Fig. 1. Infrared Absorption Spectrum of Diginatigenin

*3 According to the footnote cited in the paper of Tamm, *et al.*,[3] strains of *Fusarium avenaceum* and *Trichothecium roseum* were found to convert digitoxigenin into digoxigenin. A research group of the Research Laboratories, Takeda Pharmaceutical Industries, Ltd., has also observed recently the bioconversion of digitoxigenin and gitoxigenin by strains of *Helicostylum piriforme*, *Cunninghamella blakesleeana*, and *Gibberella fujikuroi*.[5]

*4 Reported yield of this conversion by *F. lini* is ca. 40%.[3]

*5 Reported yield of this conversion by *F. lini*[3]; (IVa) ca. 5%, (V) 12.5%, (VI) ca. 1%.

5) H. Nawa, *et al.*: Nature, **184**, 469(1959).
6) J. J. Ashley, B. T. Brown, G. T. Okita, S. E. Wright: J. Biol. Chem., **232**, 315(1958).
7) K. Repke: Naturwiss., **45**, 366(1958); **46**, 173(1959). 8) A. Yamada: This Bulletin, 8, 18(1960).
9) H. P. Sigg, Ch. Tamm, T. Reichstein: Helv. Chim. Acta, **36**, 985(1953).
10) J. E. Murphy: J. Am. Pharm. Assoc., **44**, 719(1955).
11) The structure of diginatigenin, in the mean time, has been definitively established by the authors as formulated here. cf. The following paper (M. Okada, A. Yamada, M. Ishidate: This Bulletin, 8, 535(1960)).

yield,[2] 0.5%). The identification of this product with diginatigenin was made by comparison with an authentic sample kindly supplied by Dr. Murphy. Furthermore, the infrared spectrum (Fig. 1) of this diginatigenin was identical in every respect with that reported by Murphy and Tamm.[2] Acetylation of (IX) gave a triacetate (XI), which is presented in this paper for the first time. More than 70% of the starting material was found to be recovered in this bioconversion.

Incubation of oleandrigenin (VIII) with the strain of *G. saubinettii* provided the most interesting and stimulating findings. The principal dissimilation product isolated was entirely a new compound, 16-monoacetyldiginatigenin (X), which was obtained in approximately 15% yield. It gave the same acetate (XI) as that prepared from diginatigenin (IX) and underwent mild hydrolysis with potassium hydrogencarbonate in the usual way to yield (IX). Its infrared spectrum exhibited a band (8.12 μ) showing the presence of acetoxyl group besides the bands due to α,β-unsaturated lactone grouping of the cardenolide. Moreover, the observed maximum (270 mμ) in its absorption spectrum, when (X) was adsorbed on alumina, indicated the formation of $\Delta^{16,17}$-cardenolide, which is demonstrated in various cardiac aglycones and glycosides possessing 16β-acetoxyl group when they are treated with alumina.[12] From these facts, therefore, the assigned structure of (X) has been established beyond doubt.

Diginatigenin (IX), the second dissimilation product in this bioconversion, was isolated in ca. 2% yield. Nearly 40% of oleandrigenin (VIII) was recovered, while the expected formation of gitoxigenin (VII) from (VIII) by enzymatic hydrolysis during incubation was found to be quite small. Indeed, although the presence of (VII) was indicated by paper chromatography in various fractions of chromatographic separation procedures employed, it was not possible to isolate (VII) in pure crystalline state even after repeated chromatography. Therefore, the amount of gitoxigenin produced from oleandrigenin during incubation would be extremely small, if any. On the other hand, the strain of *G. saubinettii* proved to produce considerably effective esterase as well as 12β-hydroxylase by converting acetyldigitoxigenin into digoxigenin and digitoxigenin, and 3-acetylgitoxigenin into gitoxigenin and diginatigenin.*6

It has been repeatedly demonstrated that esterase activities of various molds are very high and hence acetylated steroidal substrates undergo enzymatic hydrolysis completely during incubation when they are hydroxylated.[13]

The present findings described in this paper concerning bioconversion of oleandrigenin, coupled with the recent observations that honghelοside-A acetate[3] and 16-acetyl-digitalinum verum monoacetate[14] underwent enzymatic hydrolysis to give honghelοside-A and 16-acetyl-digitalinum verum as the principal product, demonstrate conclusively that the 16β-acetoxyl group stands against enzymatic hydrolysis, so far as cardiac aglycones and glycosides of the cardenolide-type are concerned. It is well-known, on the other hand, that this acetoxyl group is hydrolyzed off by mild alkali without difficulty. Furthermore, the bioconversion of gitoxigenin and oleandrigenin by this strain of *G. saubinettii* reported here has provided the first instance, so far as is known, in which the presence of an ester group did promote microbiological introduction of a hydroxyl group into the steroid nucleus.

*6 Unpublished data.

12) Ch. Tamm: Fortschr. Chem. org. Naturstoffe, **13**, 142(1956).

13) S. H. Eppstein, P. O. Meister, H. C. Murray, D. H. Peterson: Vitamins and Hormones, **14**, 359 (1956).

14) K. Miyatake, A. Okano, K. Hoji, T. Miki, A. Sakashita: This Bulletin, **7**, 634(1959).

Experimental*7

Conversion of Digitoxigenin (I) to Digoxigenin (IIa)—A medium was prepared from potato extract (200 g. of peeled potato was extracted with distilled water at 40~60° for about 1 hr. and then at 100° for 20 min.), 10 g. of glucose, and 3 g. of sodium glutamate, made up to 1 L. with distilled water. 100 cc. of this medium was placed in a 500-cc. shake flask, which was sterilized and inoculated with a loopful of *Gibberella saubinettii* culture which had been maintained on nutrient agar. The culture was then incubated at 27°, shaken at 120 r.p.m. for 48 hr., 20 mg. of (I) in 2 cc. of MeOH was added, and incubation with shaking was continued for 72 hr. The reaction mixture was homogenized and extracted thoroughly with AcOEt, the extract was washed with 2% $NaHCO_3$ and H_2O, dried, and the solvent evaporated. The residue (24 mg.), which showed only one spot corresponding to (IIa) on paper chromatogram, was chromatographed on 2 g. of acid-washed alumina by successive elution with benzene, $CHCl_3$, and MeOH. The fraction eluted with $CHCl_3$ and $CHCl_3$ containing MeOH (1~3%) gave 9 mg. of (IIa), m.p. 206~209°, after recrystallization from MeOH and H_2O. The m.p. of the mixture with an authentic sample showed no depression.

Acetylation of the mother liquor of (IIa) in the usual way with Ac_2O and pyridine yielded 4.5 mg. of (IIb), m.p. 217~220°. This showed no depression on admixture with an authentic sample of digoxigenin diacetate.

Bioconversion of Digitoxigenone (III) by *G. saubinettii*—20 mg. of (III) in 2 cc. of MeOH was added to a 48-hr. growth of *G. saubinettii* in 100 cc. of the afore-mentioned medium (total amount of (III) used, 200 mg.). After incubation for 5 days with shaking, the reaction mixture was processed as described above. Upon evaporation of the solvent 425 mg. of a residue was obtained, which was chromatographed on 10 g. of acid-washed alumina. The fraction eluted with benzene containing $CHCl_3$ (10~20%) gave 53 mg. of (III), m.p. 192~202°, after crystallization from Me_2CO-petr. ether. Recrystallization of the fraction eluted with benzene-$CHCl_3$ mixture (4:1 and 2:1) from MeOH afforded 7 mg. of (V), m.p. 280~284°, which showed no depression on admixture with an authentic sample prepared by reduction of (III)[8] with $NaBH_4$. The fraction eluted with benzene-$CHCl_3$ mixture (2:1, 1:9) and $CHCl_3$ yielded 105 mg. of (IVa), m.p. 251~254°, after recrystallization from Me_2CO-Et_2O. $[\alpha]_D^{21}$ +32° (MeOH-$CHCl_3$). UV λ_{max}^{EtOH} mμ : 219 (log ε 4.24); 280 (shoulder). IR λ_{max}^{KBr} μ : 5.63, 5.74, 5.94, 6.14. Acetylation of (IV) in the usual way with Ac_2O and pyridine yielded (IVb), m.p. 193~197°, which showed no depression on admixture with a sample of (IVb) prepared in a different way.[8] The presence of (VI)[8] (Rf 0.11) in the fraction eluted with $CHCl_3$ containing MeOH (1~5%) was indicated by paper chromatography, but attempts to obtain (VI) in crystalline form failed.

Conversion of Gitoxigenin (VII) to Diginatigenin*9 (IX)—800 mg. of (VII) in MeOH was added to a 72-hr. growth of *G. saubinettii* in Czapek-Dox medium (20 mg. of (IV) in 50 cc. of the medium). After incubation at 28° for 5 days with shaking the mycelium was collected by filtration, the filtrate and mycelium were each extracted with AcOEt, the extracts were washed with H_2O, dried, and the solvent evaporated. The combined residue (1.9 g.) was chromatographed on 15 g. of Florisil. The fraction eluted with $CHCl_3$ and $CHCl_3$ containing 1% MeOH gave 580 mg. of (VII), m.p. 219~223°. The fraction eluted with $CHCl_3$ containing 5% MeOH afforded 47 mg. of (IX). After repeated recrystallization from H_2O it melted at 154~156°. $[\alpha]_D^{20}$ +34° (MeOH). UV : λ_{max}^{EtOH} 218 mμ (log ε 4.18).

Its chromogenic properties and mobility (Rf 0.01) on paper were identical with those of an authentic sample of diginatigenin,*8 and its infrared spectrum (Fig. 1.) was identical in all respects with that of diginatigenin reported by Tamm, *et al.*[2]

10 mg. of (IX) was acetylated in the usual way with Ac_2O and pyridine to give (XI), m.p. 217~220°, after recrystallization from Me_2CO-Et_2O-petr. ether.

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*7 All m.p.s are not corrected. Paper chromatograms were run in the solvent system of formamide and $CHCl_3$,[15] and examined after reaction with Kedde reagents[16] (3,5-dinitrobenzoic acid and KOH) or trichloroacetic acid in $CHCl_3$.[17] Infrared spectra were measured with Perkin-Elmer Model 21 Double-beam Spectrophotometer and ultraviolet spectra were measured with Beckman Model DU Spectrophotometer.

*8 The authors are grateful to Dr. J. E. Murphy, Burroughs Wellcome & Co., (U. S. A.) Inc., for the supply of diginatigenin.

*9 Fermentation and extraction processes in these experiments were performed at the Institute for Fermentation, Osaka, and the Research Laboratories, Takeda Pharmaceutical Industries, Ltd., for which the authors express their appreciation.

15) O. Schindler, T. Reichstein : Helv. Chim. Acta, **34**, 108(1951).
16) I. E. Bush, D. A. H. Taylor : Biochem. J., **52**, 643(1952).
17) A. B. Svendsen, K. B. Jensen : Pharm. Acta Helv., **25**, 241(1950).

Bioconversion of Oleandrigenin (VIII) by *G. saubinettii*[*9]—By incubating *G. saubinettii* with 750 mg. of (VIII) in the same way as described above, 1.47 g. of AcOEt extract was obtained, which was chromatographed on 15 g. of Florisil. The fraction eluted with $CHCl_3$ gave 283 mg. of (VIII), m.p. 218~223°. From the fraction eluted with $CHCl_3$ and $CHCl_3$ containing 1% MeOH, 108 mg. of (X) was obtained after crystallization from Me_2CO-Et_2O. The analytical sample was recrystallized from MeOH and melted at 233~238°. $[\alpha]_D^{22}$ +6°(MeOH). UV : λ_{max}^{EtOH} 218 mμ (log ε 4.22). IR λ_{max}^{KBr} μ : 5.55, 5.75, 5.84, 6.13, 8.12. *Anal.* Calcd. for $C_{25}H_{36}O_7$: C, 66.94; H, 8.09. Found : C, 66.69; H, 8.47.

To a solution of 2 mg. of (X) in 0.2 cc. of MeOH, a solution of 2 mg. of $KHCO_3$ in 0.05 cc. of H_2O was added and the solution was stored at room temperature for 10 days. The solution was extracted with AcOEt. The presence of (IX)(Rf 0.01) besides (X)(Rf 0.11) in the AcOEt extract was shown by paper chromatography. When (VIII)(Rf 0.85) was hydrolyzed with $KHCO_3$ in the same way, its conversion to (VII)(Rf 0.30) was also indicated.

A solution of 2 mg. of (X) in 0.7 cc. of a mixture of $CHCl_3$ and MeOH was adsorbed on 1 g. of alumina (activated alumina of E. Merk, Darmstadt) and this was allowed to stand at room temperature for 7 days. The alumina was extracted with $CHCl_3$-MeOH mixture and the solvent was evaporated to dryness. Absorption spectra of this residue in EtOH exhibited two bands at 270 and 217 mμ.

Acetylation of 20 mg. of (X) in the usual way with Ac_2O and pyridine yielded 20 mg. of (XI). The analytical sample recrystallized from Me_2CO-Et_2O-petr. ether melted at 218~221°. The melting point of the mixture of the sample prepared from (IX) showed no depression. $[\alpha]_D^{20}$ +32°($CHCl_3$). *Anal.* Calcd. for $C_{29}H_{40}O_9$: C, 65.39; H, 7.57. Found : C, 65.74; H, 7.49.

Although the presence of (VII) was observed by using paper chromatography in the fractions eluted with $CHCl_3$ and $CHCl_3$ containing 1% MeOH, from which (VIII) and (X) were obtained, (VII) was not obtained in crystalline form after repeated chromatography on Florisil. The fraction eluted with $CHCl_3$ containing 5% MeOH gave 15 mg. of (IX), m.p. 153~156°, after repeated crystallizations from H_2O. Its mobility on paper was the same as that of (IX) obtained from (VII).

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Summary

Bioconversions of several cardiac aglycones by a strain of *Gibberella saubinettii* (MONT.) SACC. were reported. Digitoxigenin was converted to digoxigenin in a good yield. Digitoxigenone was transformed into 3-dehydrodigoxigenin, 3-epidigitoxigenin, and 3-epidigoxigenin. Diginatigenin was obtained in approximately 6% yield from gitoxigenin. Oleandrigenin gave 16-monoacetyldiginatigenin (ca. 15% yield) and diginatigenin (ca. 2% yield), but the formation of gitoxigenin from oleandrigenin by incubation was found to be quite small. Enzymatic hydrolysis of 16β-acetoxyl group of the cardenolide was discussed.

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