Rates of Color Development——At the wave lengths of maximum absorption, gradual changes of absorbances of these colored solutions described above were measured against the ethanolic solutions of polynitrodiphenyl compounds with KOH. Fig. 2 indicates the changes of the apparent extinction coefficients which were given from the absorbances described above by dividing with the final concentrations of nitro compounds.

This work was supported in part by a Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education, for which the authors express their appreciation.

Summary

This work was undertaken to obtain a more satisfactory reagent for the assay of active methylene compounds than has been used. Biphenyl, diphenylmethane, diphenyl ether, diphenyl sulfide, diphenylsulfone and stilbene with nitro groups in 4,4′-, 2,4-, 2,2′,4,4′-positions were prepared and their color reactions with acetone and cyclohexanone in the presence of alkali were examined.

4,4′-Dinitro compounds gave negative Janovsky reaction. Bis(*p*-nitrophenyl)-(Ⅳ) and bis(2,4-dinitrophenyl)methane (Ⅴ) gave blue color in methylcellosolve with addition of alkali without active methylene compound.

2,4-Dinitro compounds showed bathochromic shift as compared with usual reagents such as *m*-dinitrobenzene, 1,3,5-trinitrobenzene and picric acid. The intensities of colors by these reagents were lower.

2,2′,4,4′-Tetranitro compounds showed higher intensities and sufficient stabilities of the color produced, but no bathochromic shifts. These tetranitro compounds also gave the specific color reactions for active methylene compounds such as acetone, cyclohexanone, 17-ketosteroids, creatinine and cardiac glycosides.

2,2′,4,4′-Tetranitrobiphenyl (Ⅲ) could be the reagent that has higher sensitivity, sufficient stability and specificity in color reaction of active methylene compounds with alkali.

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UDC 547.918 : 615.711.5

156. Tamotsu Okumura, Yoshio Nozaki, and Daisuke Satoh : Studies on Digitalis Glycosides. XIX.*1 Microbiological Transformation of Digitoxigenin Derivatives by *Absidia orchidis*.*2

(*Shionogi Research Laboratory, Shionogi & Co., Ltd.**3)

A previous paper[1] of this series reported the 1β-, 5β-, and 7β-hydroxylations of digitoxigenin (Ⅰ) by *Absidia orchidis*, a microorganism known to hydroxylate Reichstein's substance S (Ⅱ) at 6β-, 11α-, and 11β-positions.[2] Subsequently it was found that the

*1 Part XVⅢ : This Bulletin, **11**, 576 (1963).

*2 A part of this paper was presented at the 83rd Annual Meeting of the Pharmaceutical Society of Japan, November 2nd, 1963 at Tokyo College of Pharmacy.

*3 Fukushima-ku, Osaka (奥村 保, 野崎義雄, 佐藤大助).

1) Part XVⅡ. H. Ishii, Y. Nozaki, T. Okumura, D. Satoh : This Bulletin, **11**, 156 (1963).

2) O. Hanc, A. Capek, B. Kapac : Folia Microbiologica, **6**, 392 (1961).

same organism hydroxylated 3β,14,21-trihydroxy-14β-pregnan-20-one (Ⅲ) at 1β and 4,5-dehydrodigitoxigenone (Ⅳ) at 7β- and 12β-positions, and these results have been described briefly in the latest commuication[3] from this laboratory. The authors now report the details of bioconversions of 3β,14,21-trihydroxy-14β-pregnan-20-one and 4,5-dehydrodigitoxigenone by *A. orchidis*.

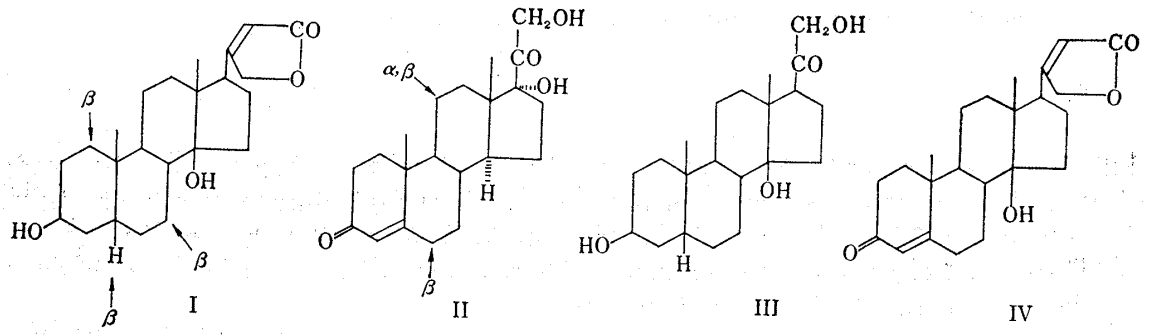


Chart 1.

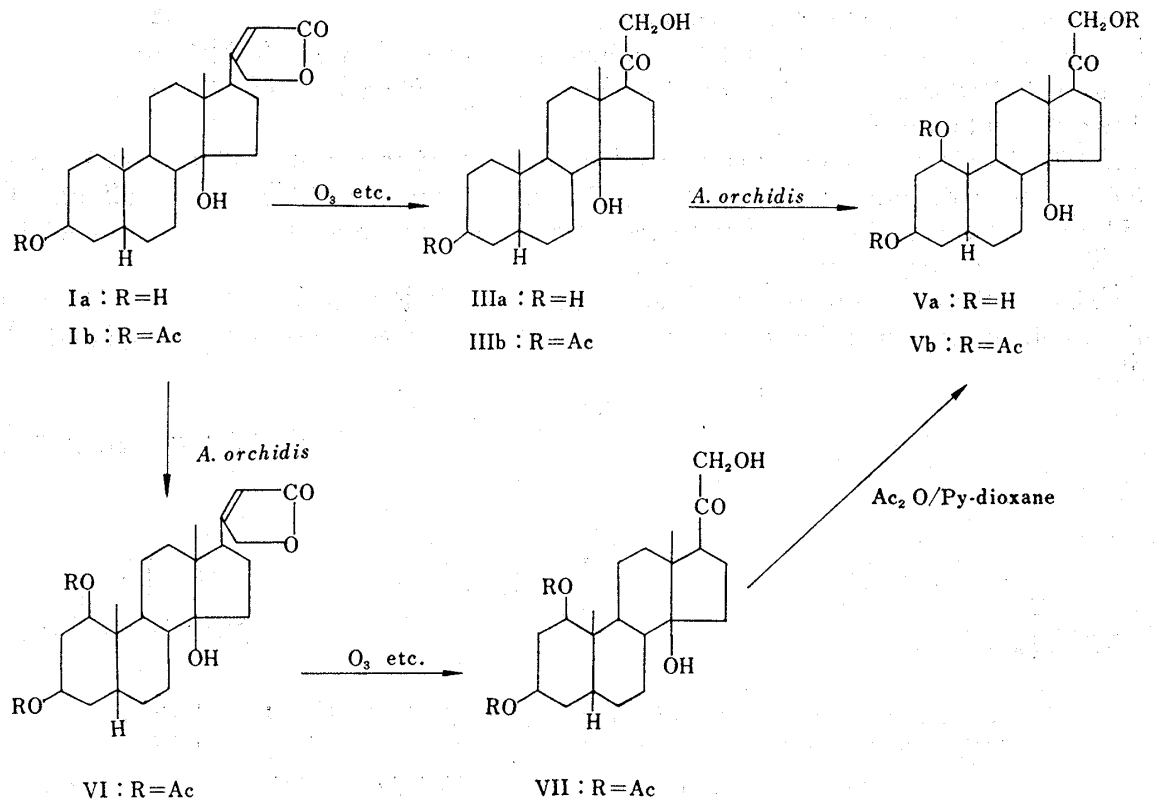


Chart 2.

A substrate (Ⅲb),*4 obtainable from digitoxigenin acetate (Ib) by ozonolysis followed by usual procedures,[4] was incubated with a mycelium suspension of *A. orchidis* for 48 hours. Fig. 1 shows a thin-layer chromatogram of the products. As a main product (Va), $C_{21}H_{34}O_5$, m.p. 252~263°, showed a positive triphenyl tetrazolium chloride test[5] and

*4 The same result was obtained when Ⅲa and Ⅲb were microbiologically transformed separately by *A. orchidis*. Hence the easily available Ⅲb was used as a substrate, the acetoxyl function of which was immediately hydrolized by the action of microbial esterase to give Ⅲa.

3) T. Okumura, Y. Nozaki, D. Satoh : This Bulletin, **11**, 1340 (1963).
4) K. Meyer, T. Reichstein : Helv. Chim. Acta, **30**, 1508 (1947).
5) R. B. Burton, A. Zaffroni, E. H. Keutmann : J. Biol. Chem., **188**, 763 (1951).

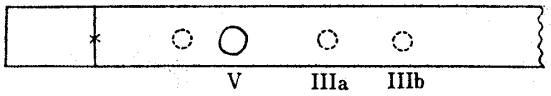


Fig. 1. Thin-layer Chromatogram of the Microbiological Transformation Products of Ⅲ
Adsorbent: Silica gel G (Merck)
Developing solvent: EtOAc
Spray reagents: TTC+NaOH and H_2SO_4

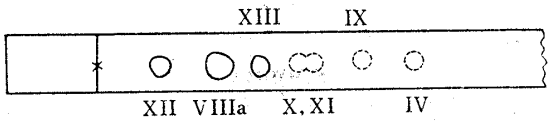


Fig. 2. Thin-layer Chromatogram of the Microbiological Transformation Products of Ⅳ
Adsorbent: Aluminum oxide G (Merck)
Solvent system: EtOAc-Me_2CO (3:2)
Spray reagents: *m*-dinitrobenzene+NaOH, 2,4-dinitrophenylhydrazine and H_2SO_4

gave a triacetate (Vb), $C_{27}H_{40}O_8$, m.p. 158~162° on acetylation with a mixture of acetic anhydride and pyridine, a new hydroxy group introduced microbiologically was assumed to be either a primary or a secondary one. Vb was identical with the ketol triacetate prepared from acovenosigenin A diacetate (Ⅵ), hence the newly introduced hydroxyl proved to be 1β-position.

Another substrate (Ⅳ) derived from dehydrogenation of digitoxigenone[6] was bioconverted into several kinds of products with the mycelium suspension of *A. orchidis* preincubated with progesterone.[7] Fig. 2 shows the thin-layer chromatogram of these

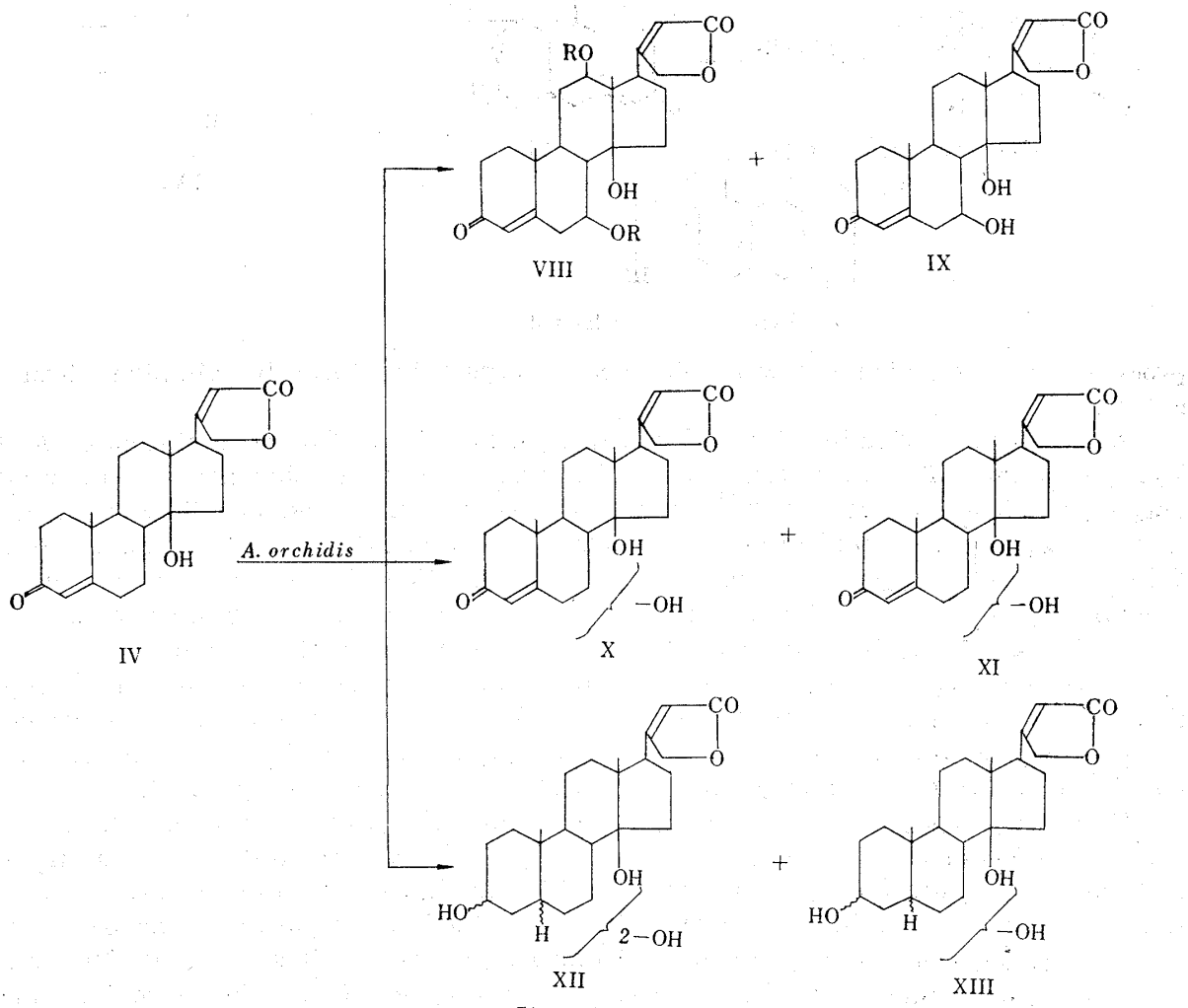


Chart 3.

6) D. Satoh, T. Wada: Yakugaku Zasshi, **80**, 1314 (1960).
7) Y. Nozaki, E. Masuo, D. Satoh: Agr. Biol. Chem., **26**, 399 (1962). While the usual bioconversion of Ⅳ performed without added progesterone produced six kinds of product in approximately equal amounts, the use of this culture gave Ⅷ preferentially.

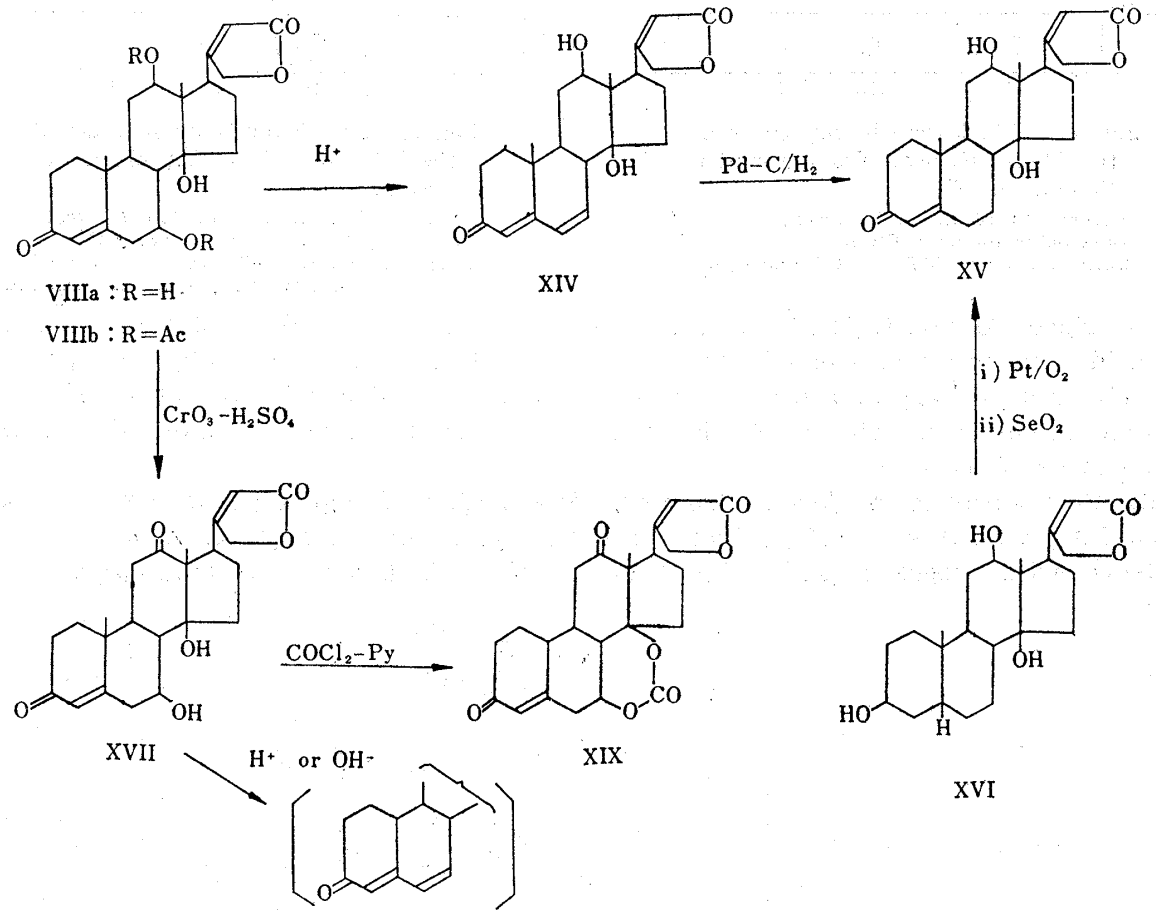


Chart 4.

products, among which six were obtained in crystalline form by alumina chromatography.

A dihydroxy derivatives (VIIIa), $C_{23}H_{30}O_6$, m.p. 287~290°, afforded a diacetate (VIII), $C_{27}H_{34}O_8$, m.p. 267~268°, with acetic anhydride and pyridine, so that both of newly introduced acylable hydroxyls were presumed to be primary or secondary. Treatment of VIIIa with 1% hydrochloric acid in acetone yielded a $\Delta^{4,6}$-3-one (XIV), $C_{23}H_{28}O_5$, m.p. 279~283°. When VIIIa was treated with zinc dust in glacial acetic acid at room temperature, no reaction took place. It is known that a hydroxyl group at 2- or 6-position of Δ^4-3-oxo-steroid is reductively liberated with zinc dust under the foregoing conditions.[8]

These facts suggested that one of the new hydroxyls had been introduced into 7-position and that no hydroxyl had existed in ring A. The positions 15 and 16 were eliminated from the infrared data of the diketone (XVII) described below. Therefore, the position 11α and 12 was thought to be probable for another new hydroxyl.

Partial hydrogenation of XIV with 1% paradium-charcoal[9] resulted in selective saturation of 6,7-double bond giving a Δ^4-3-one (XV). XV was in good agreement in their melting points, infrared spectra, and mobilities in thin-layer chromatography with the sample prepared from digoxigenin (XVI) by the method similar to that described above.[6] In this way the new hydroxyl introduced into ring C was clarified to be at 12β-position.

The configuration of C-7 hydroxyl in VIII was assigned β from the molecular rotatory studies. In general it is known that for saturated 7-hydroxysteroids,[10] the molecular

8) F. Sondheimer, *et al.*: J. Am. Chem. Soc., **75**, 4712 (1953).
9) K. Tsuda, *et al.*: This Bulletin, **6**, 388 (1958).
10) L. F. Fieser, M. Fieser: "Steroids," 179 (1959), Reinhold, New York.

rotatory contributions of 7β-hydroxy group are positive, while those of the 7α-group are negative. In contrast with this, the data reported by McAleer, *et al.*[11] show that both 7α- and 7β-hydroxyls of Δ^4-3-oxo-steroids cause negative shifts of about the same magnitude. Thus it appears to be impossible to assign the configuration to a 7-hydroxy-Δ^4-3-oxo-steroids on the basis of its molecular rotation differences. However, Tweit, *et al.*[12] reported that in some of the acetates of these hydroxy compounds the 7β-acetoxy-Δ^4-3-oxo-steroids have small ΔMD values, while the 7α-acetates have negatve values of $-300°$ or greater. Table I shows some of Tweit's examples for the rotatory contributions of 7-hydroxy-Δ^4-3-oxo-steroids. The authors assigned the configuration of C-7 hydroxyl in Ⅷ to β according to its small ΔMD values.

Table I. Contributions of Hydroxyls at C-7 to Molecular Rotations

	ΔMD [(7-OH)-(7-OH)]		ΔMD [(7-OAc)-(7-H)]	
Parent steroids	α	β	α	β
Progesterone	$-59°$	$-44°$	$-368°$	—
14α-Hydroxyprogesterone	$-71°$	$-144°$	$-433°$	$-52°$
21-Acetoxy-17α-progesterone	—	—	$-375°$	$-36°$
15β-Hydroxyprogesterone	—	$-28°$	—	$-21°$
4-Androstene-3,17-dione	$-71°$	$-29°$	$-298°$	—
Ⅷ		$-74°$		$-77°$

Oxidation of Ⅷa with chromium trioxide-sulfuric acid in acetone[13] at 0°*5 gave a diketone (XVII), $C_{23}H_{28}O_6$, m.p. 272~275°, the infrared spectrum of which exhibited a new absorption band for six-membered ring ketone in addition to those for conjugated ketone and that for still remaining hydroxyl groups. This diketone, when treated with dilute acid in a similar way described above or 0.06*N* tetramethylammonium hydroxide in 95% ethyl alcohol,[14] was converted to a compound (XVIII) having an absorption maximum at 283 mμ characteristic for $\Delta^{4,6}$-3-one. Treatment of XVII with phosgene in pyridine[15] yielded a 7β,14β-cyclocarbonate (XIX), $C_{24}H_{26}O_7$, m.p. 297~300° and this fact confirmed the β-configuration of C-7 hydroxyl in the molecule. Thus the structure of Ⅷ was assigned to 7β,12β-dihydroxy-4,5-dehydrodigitoxigenone.

One of the monohydroxylated microbiological products (Ⅸ), $C_{23}H_{30}O_5$, m.p. 281~284°, exhibited an ultraviolet maximum characteristic for dienone (XXI) when treated in the

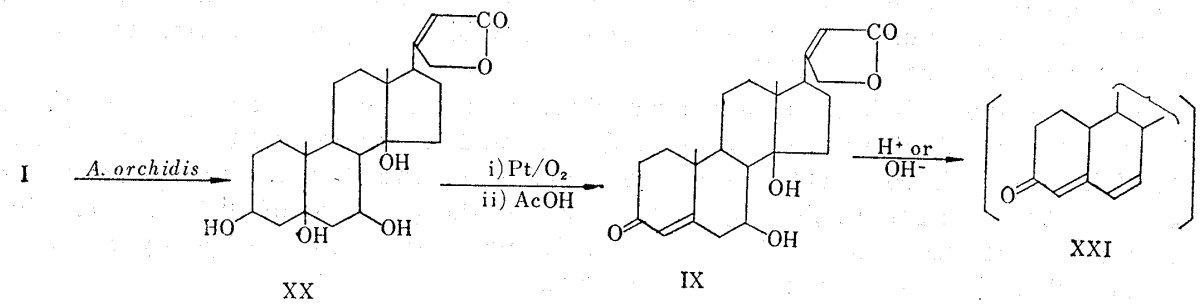


Chart 5.

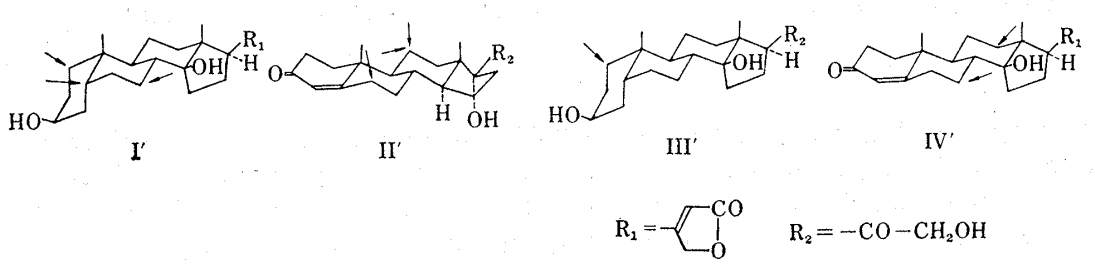
*5 Under this condition, the equatorial C-7-hydroxyl was not oxidized.

11) W. J. McAleer, *et al.*: J. Org. Chem., **23**, 958 (1958).
12) R. C. Tweit, A. H. Goldkanys, R. M. Dodson: J. Org. Chem., **26**, 2856 (1961).
13) R. Tschesche, *et al.*: Ann., **648**, 185 (1961).
14) A. S. Meyer: J. Org. Chem., **20**, 1240 (1955).
15) W. Schlegel, Ch. Tamm, T. Reichstein: Helv. Chim. Acta, **38**, 1013 (1955).

same way as Ⅷa, and a new hydroxyl was assumed to be at 7-position. This compound was identical with the 7β-hydroxy-Δ^4-3-one derived from 5β,7β-dihydroxydigitoxigenin (XX).[1] Thus the occurrence of 7β-monohydroxylation of Ⅳ was clarified.

The positions of new hydroxyls in other two kinds of monohydroxy compounds (X), $C_{23}H_{30}O_5$, m.p. 290~295°, and (Ⅺ), $C_{23}H_{30}O_5$, m.p. 267~273°, have not yet been characterized, although some experimental results eliminated 1, 2, 4, 7, and 12β-positions of X and Ⅺ as a point of microbiological hydroxylation.

The compounds (Ⅻ), $C_{23}H_{34}O_6$, m.p. 265~270° and (XIII), $C_{23}H_{34}O_5$, m.p. 228~230° were thought to be a dihydroxy and a monohydroxy derivatives of a 4,5-saturated-3-hydroxy compound respectively, judged from the analitical values and the disappearance of characteristic peaks for α,β-unsaturated ketones in their ultraviolet and infrared spectra which were observed in those of the starting material (Ⅳ). It may be considered that microbiological hydrogenation of carbonyl and double bonds transformed Ⅳ into Ⅻ and XIII having 5ξH-3ξOH-functions respectively. The positions of these hydroxyl groups and the enzymatic course of reduction are now being investigated. This may be the first case of microbiological hydrogenation of Δ^4-3-oxo grouping of a cardiac aglycone, although a various kinds of microorganisms[16~18] have been reported to hydrogenate the Δ^4-3-keto groupings of corticosteroid.



I′ II′ III′ IV′

$R_1 =$ $R_2 = -CO-CH_2OH$

Chart 6.

In Chart 6 are shown the perspective formulae of substrate steroids and the positions where *Absidia orchidis* introduces hydroxyl groups. The first point which arises from comparison of these results is that this organism is able to hydroxylate I′ at positions 1β, 5β, and 7β, but Ⅲ′ only at 1β. Hence it should be considered that the difference of the side chain at C-17 between the substrates (I′) and (Ⅲ′) does not influence the 1β-hydroxylation of these substrates.

Secondly, there is no resemblance in the type of bioconversion between the substrates (Ⅱ′) and (Ⅲ′) which differ in the A/B and C/D ring junctures but possess the similar α-ketol side chain at C-17.

The compound (Ⅳ′) has the structure similar in part to I′ and in part to Ⅱ′. Ⅳ′ undergoes the microbial hydroxylations at 7β on the B ring and at 12β on the C ring, while Ⅱ′ is hydroxylated at 6β on the B ring and 11 on the C ring. Therefore, it may be probable that the same A/B ring fusion, that is, the presence of Δ^4-3-oxo groupings in Ⅱ′ and Ⅳ′ give rise to some similarities in the point of microbiological hydroxylation of the B and C rings of these steroidal substrates.

Clarification of the relationship between the structures of substrates and the positions to be hydroxylated by microorganism is of course the problem rather difficult to solve and much further works should, the authors think, be performed for more detailed considerations.

16) H.R. Barkemeyer, *et al.*: Applied Microbiology, 8, 237 (1960).
17) M. Shirasaka, M. Ozaki: Nippon Nogei Kagakukaishi, 35, 200 (1961).
18) C.J. Sih, *et al.*: J. Org. Chem., 28, 854 (1963).

Experimental*6

Fermentation Condition and Separation Procedure——*A. orchidis* was grown for 66 hr. with shaking on a nutrient medium containing 3.5% glucose, 3.0% peptone and 1.0% corn steep liquor, the mycelium was harvested, washed and resuspended in distilled H_2O. Each substrate dissolved in MeOH was added to this mycelium suspension and incubation was continued for a further 48 hr. The fermentation filtrate and mycelium were extracted separately with EtOAc and then with $CHCl_3$. The whole extracts were submitted to column chromatography and preparative thin-layer chromatography on neutral alumina (Merck).

1β,3β,14,21-Tetrahydroxy-14β-pregnan-20-one (Va)——Substrate (IIIb), 520 mg., was transformed into Va, 54 mg., m.p. 252~263°, as plates after recrystallization from MeOH. *Anal.* Calcd. for $C_{21}H_{34}O_5$: C, 68.82; H, 9.35. Found: C, 69.10; H, 9.48. IR λ_{max}^{Nujol} cm^{-1}: 3425, 3360, 3205, 1700, 1061. $[\alpha]_D^{24}$ −0.4° (c=0.934, pyridine).

1β,3β,14,21-Tetrahydroxy-14β-pregnan-20-one 1β,3β,21-Triacetate (Vb) from Va——Va, 26 mg., was acetylated in the usual way with Ac_2O and pyridine to give the triacetate (Vb), m.p. 158~162°, as plates from CCl_4-hexane and Et_2O. *Anal.* Calcd. for $C_{27}H_{40}O_8$: C, 65.83; H, 8.19; CH_3CO, 26.21. Found: C, 66.23; H, 8.33; CH_3CO, 28.21. IR λ_{max}^{Nujol} cm^{-1}: 3420, 1747, 1718, 1264, 1234, 1058.

1β,3β,14,21-Tetrahydroxy-14β-pregnan-20-one 1β,3β,21-Triacetate (Vb) from VI——1β,3β-Diacetoxyacovenosigenin A, 400 mg., m.p. 218~221°, was dissolved in 50 ml. of abs. EtOAc and O_2 containing ca. 3% dried O_3 (ca. 80~100 ml./min.) was passed through the solution at −80° for 40 min. The resultant blue-violet solution was left to stand at −80° for 15 min. and then at room temperature for 20 min. After removal of the solvent *in vacuo*, the crude ozonide was reductively decomposed to a glycolic acid ester with Zn dust in glacial AcOH, and this ester was dissolved in $CHCl_3$ and washed 3 times with ice and 2% $NaHCO_3$ and then once with ice and H_2O. The ester, 430 mg., dissolved in 25 ml. of MeOH and 10 ml. of H_2O was saponified with 55 mg. of $KHCO_3$ at 6~8° for 17 hr., neutralized with dil. HCl, extracted with $CHCl_3$ and the ketol, 352 mg., was recrystallized from Et_2O, Me_2CO-Et_2O to give 278 mg. of plates (VII), m.p. 160~169°. *Anal.* Calcd. for $C_{25}H_{38}O_7$: C, 66.64; H, 8.50. Found: C, 66.61; H, 8.52. IR λ_{max}^{Nujol} cm^{-1}: 3380, 1724, 1716, 1264, 1237, 1052. $[\alpha]_D^{24.5}$ +9.5° (c=1.047, $CHCl_3$).

This ketol, 87 mg., dissolved in 0.3 ml. of pyridine and 0.3 ml. of dioxane was acetylated with 0.03 ml. of Ac_2O to afford the triacetate (Vb), 81 mg., m.p. 156~158°, as plates. *Anal.* Calcd. for $C_{27}H_{40}O_8$: C, 65.83; H, 8.19; CH_3CO, 26.21. Found: C, 65.57; H, 8.13; CH_3CO, 27.11. IR λ_{max}^{Nujol} cm^{-1}: 3415, 1748, 1723, 1263, 1231, 1059. $[\alpha]_D^{26.5}$ +15.7° (c=1.029, $CHCl_3$).

The melting points of both triacetates were not depressed by admixture and their IR spectra were shown to be superimposable.

7β,12β-Dihydroxy-4,5-dehydrodigitoxigenone (VIIIa)——Substrate (IV), 1 g., was transformed into VIIIa, 145 mg., m.p. 287~290°, recrystallized as prisms from MeOH. *Anal.* Calcd. for $C_{23}H_{30}O_6$: C, 68.63; H, 7.51. Found: C, 68.66; H, 7.61. IR λ_{max}^{Nujol} cm^{-1}: 3385, 3275, 1800, 1779, 1721, 1683, 1613, 1026, 1011. UV: λ_{max}^{EtOH} 226 mμ*7 (ε 21,100). $[\alpha]_D^{25}$ +66.7° (c=1.014, pyridine).

7β,12β-Diacetoxy-4,5-dehydrodigitoxigenone (VIIIb)——VIIIa, 46 mg., was acetylated with Ac_2O-pyridine (1:1) in a usual way to give the diacetate (VIIIb), 34 mg., m.p. 265~268°, as needles from benzene. *Anal.* Calcd. for $C_{27}H_{34}O_8$: C, 66.65; H, 7.04; Ac, 17.69. Found: C, 66.89; H, 7.14; Ac, 17.82. IR λ_{max}^{Nujol} cm^{-1}: 3573, 1788, 1739, 1681, 1626, 1242, 1041, 1022. $[\alpha]_D^{26}$ +52° (c=1.029, pyridine).

12β-Hydroxy-4,5:6,7-didehydrodigitoxigenone (XIV)——VIIIa, 148 mg., was dissolved in 150 ml. of HCl-Me_2CO (1:100), allowed to stand at room temperature for 2 hr., poured into an aq. solution of 100 ml. of NaOAc, 5 g., extracted with $CHCl_3$, washed with 2% $NaHCO_3$ and H_2O, and dried over anhyd. Na_2SO_4. After removal of the solvents *in vacuo*, 141 mg. of residue was recrystallized from $CHCl_3$ to give 105 mg. of XIV, m.p. 279~283° (decomp.), as prisms. *Anal.* Calcd. for $C_{23}H_{28}O_5$: C, 71.85; H, 7.43. Found: C, 71.45; H, 7.43. IR λ_{max}^{Nujol} cm^{-1}: 3470, 3366, 1788, 1746, 1644, 1610, 1578. UV λ_{max}^{EtOH} mμ (ε): 217 (16,700), 283 (25,200).

12β-Hydroxy-4,5-dehydrodigitoxigenone (XV) from XIV——A solution of 73 mg. of XIV in 9 ml. of MeOH was catalytically reduced with 1% Pd-C (15 mg.). After about 5.7 ml. (1.23 moles) of H_2 was absorbed, the hydrogenation was stopped. The catalyst was filtered off, MeOH removed *in vacuo*, and the residue (73 mg.) was purified by preparative thin-layer chromatography (alumina), and recrystallized from MeOH-Me_2CO to XV (26 mg.) as plates, m.p. 263~269°. *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 70.87; H, 7.90. IR λ_{max}^{Nujol} cm^{-1}: 3520, 3440, 1810, 1730, 1658, 1615. UV: λ_{max}^{EtOH} 226 mμ*7 (ε 20,700).

12β-Hydroxy-4,5-dehydrodigitoxigenone from XVI——A solution of XVI, 390 mg., m.p. 209~222°, dissolved in 60 ml. of Me_2CO and 32 ml. of distilled H_2O was stirred in O_2 stream for 2.5 hr. with added

*6 All melting points were measured on a Kofler block and are uncorrected.

*7 The appearance of absorption band at this wave length is due to overlapping of absorption for α,β-unsaturated ketone at 241 mμ with that of unsaturated lactone at 217 mμ.

Pt catalyst which had been prepared from 270 mg. of $PtO_2 \cdot 2H_2O$ by hydrogenation. After removal of the catalyst, the solution was extracted with $CHCl_3$, dried over anhyd. Na_2SO_4, the solvents were distilled off *in vacuo* and the residue (370 mg.) so obtained was recrystallized from Me_2CO to 3-dehydrodigoxigenin (286 mg.), m.p. 253~257°, as plates. To a solution of 286 mg. of this 3-dehydro compound dissolved in 30 ml. of *t*-BuOH and 5 ml. of AcOH, 157 mg. (1.9 moles) of SeO_2 was added, the mixture was refluxed for 2 hr. at 100°. After removal of precipitated Se black, the solvents were distilled off, and the residue (488 mg.) dissolved in $CHCl_3$-MeOH (9:1) was washed successively with 2% $NaHCO_3$, 2% NaS 5 times, 0.5% HCl, and H_2O, purified by preparative thin-layer chromatography on alumina, and was recrystallized from Me_2CO-MeOH to XV (48 mg.), m.p. 266~271°, as plates. *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.38; H, 8.00. IR λ_{max}^{Nujol} cm^{-1}: 3520, 3440, 1810, 1730, 1658, 1615. UV: λ_{max}^{EtOH} 229 mμ*7 (ε 22,900). $[\alpha]_D^{25.5}$ +88.6° (c=0.992, pyridine).

These Δ^4-3-ones were shown to be identical by mixed melting point determination and direct IR spectral comparison.

7β-Hydroxy-4,5-dehydrodigoxigenone (XVII)——To a solution of 28 mg. of VIIIa in 30 ml. of Me_2CO, 0.09 ml. of CrO_3-H_2SO_4 solution (13.3 g. CrO_3 in 20 ml. H_2O plus 23 ml. conc. H_2SO_4, H_2O added to 50 ml.) was added dropwise at 0°. After stirring for 4 min., the solution was poured into an aq. solution of 1 g. of NaOAc, the product extracted with $CHCl_3$, dried over anhyd. Na_2SO_4, the solvents distilled off and the residue, 27 mg., was recrystallized from Me_2CO-Et_2O to XVII, 14 mg., m.p. 272~275°, as needles. *Anal.* Calcd. for $C_{23}H_{28}O_6$: C, 68.98; H, 7.05. Found: C, 68.79; H, 7.13. IR $\lambda_{max}^{CHCl_3}$ cm^{-1}: 3450, 1779, 1742, 1710, 1670, 1625. UV: λ_{max}^{EtOH} 225 mμ*7 (ε 22,900). $[\alpha]_D^{26.5}$ +111.8° (c=0.532, pyridine).

7β-Hydroxy-4,5-dehydrodigoxigenone-7β,14β-cyclocarbonate (XIX)——XVII (50 mg.) was suspended in alcohol-free $CHCl_3$ (10 ml.) and about 2 ml. of the $CHCl_3$ was removed by distillation. To this suspension 4 ml. of pyridine was added to dissolve XVII completely. The mixture was cooled to −20° and 10 ml. of 10% $COCl_2$-toluene solution was added dropwise. The reaction mixture was allowed to stand at room temperature for 3 hr. After decomposing an excess of $COCl_2$ with ice, H_2O and $CHCl_3$ were added. The $CHCl_3$ layer was washed successively with dil. HCl, dil. $NaHCO_3$ solution and H_2O, dried over anhyd. Na_2SO_4, and concentrated to dryness *in vacuo*. The residue (50 mg.) was recrystallized from $CHCl_3$-MeOH (1:1) to give plates of XIX (32 mg.), m.p. 297~300° (decomp.). *Anal.* Calcd. for $C_{24}H_{26}O_7$: C, 67.59; H, 6.15. Found: C, 67.37; H, 6.31. IR λ_{max}^{Nujol} cm^{-1}: 1780, 1749, 1732, 1713, 1671, 1619, 1238, 1087, 1082. $[\alpha]_D^{26.5}$ +69.1° (c=1.025, pyridine).

Treatment of XVII with Tetramethylammonium Hydroxide (TMAH)——XVII (1.092 mg.) was dissolved in 6.0 ml. of 10% TMAH and 94 ml. of 95% EtOH. The solution was allowed to stand at room temperature for 22 hr. and its UV spectrum measured. The absorption maximum was recognized at 283 mμ (ε 21,900).

7β-Hydroxy-4,5-dehydrodigitoxigenone (IX)——Substrate (IV), 1 g., was transformed into 14 mg. of IX, m.p. 281~284° as needles crystallized from MeOH. *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.35; H, 7.81. IR λ_{max}^{Nujol} cm^{-1}: 3380, 1810, 1732, 1679, 1670, 1631, 1616. UV: λ_{max}^{EtOH} 223.5 mμ*7 (ε 22,800). $[\alpha]_D^{26}$ +82.8° (c=1.059, pyridine). IX was shown to be identical with the authentic sample of Δ^4-3-dehydro-7β-hydroxydigitoxigenin[1] by mixed melting point determination and direct IR comparison.

Treatment IX with 1% Hydrochloric Acid-Acetone——About 1 mg. of IX was dissolved in 100 ml. of this reagent, left to stand at room temperature for 1.5 hr. and its UV spectrum was measured. The absorption peak was obtained at 283 mμ (ε 23,600) and at 218 mμ (ε 14,600).

Bioconversion Products (X, XI, XII, and XIII)——Substrate (IV), 1 g., was transformed into X, 12 mg., XI, 13 mg., XII, 15 mg., XIII, 12 mg., respectively.

X, m.p. 290~295°. *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 70.89; H, 7.66. IR λ_{max}^{Nujol} cm^{-1}: 3350, 1809, 1727, 1665, 1632, 1612, 1020. UV: λ_{max}^{EtOH} 220 mμ (ε 23,000). XI, m.p. 267~273°. *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.79; H, 7.90. IR λ_{max}^{Nujol} cm^{-1}: 3495, 1802, 1724, 1667, 1614. UV: λ_{max}^{EtOH} 235 mμ (ε 22,000), XII, m.p. 265~270°. *Anal.* Calcd. for $C_{23}H_{34}O_6$: C, 67.95; H, 8.43. Found: C, 68.48; H, 8.02. IR λ_{max}^{Nujol} cm^{-1}: 3420, 1808, 1734, 1613, 1048, 1027. UV: λ_{max}^{Nujol} 217 mμ (ε 16,400). $[\alpha]_D^{24.5}$ −41.5° (c=0.715, pyridine). XIII, m.p. 228~230°. *Anal.* Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.78. Found: C, 71.47; H, 8.30. IR λ_{max}^{Nujol} cm^{-1}: 3490, 1794, 1775, 1728, 1632, 1280, 1266, 1040. UV: λ_{max}^{EtOH} 218 mμ (ε 16,600).

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Summary

Through the microbiological transformation of 3β,14,21-trihydroxy-14β-pregnan-20-one (III) and 4,5-dehydrodigitoxigenone (IV) by *Absidia orchidis*, several kinds of products

were isolated, and the structure and configuration of three hydroxylated products, one from Ⅲ and two from Ⅳ, were clarified; 1β,3β,14,21-tetrahydroxy-14β-pregnan-20-one (Ⅴ), 7β,12β-dihydroxy-4,5-dehydrodigitoxigenone (Ⅷ), and 7β-hydroxy-4,5-dehydrodigitoxigenone (Ⅸ).

Based on these experimental results, the relationships between the structure of substrate and the positions to be hydroxylated in the microbiological transformation of cardiac aglycone derivatives by *A. orchidis* were discussed.

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157. Tadashi Watabe, Hidetoshi Yoshimura, and Hisao Tsukamoto: Metabolism of Drugs. L.*1 The *In Vitro* Study on Metabolism of Brucine and 4-Substituted Veratroles.

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In the previous papers of this series, it was found that one of two adjacent methoxyl groups of brucine and 4-substituted veratroles were selectively removed in rabbits.[1,2] Interesting finding in that study was that the selective demethylation of brucine occurred at the *meta*-position of its lactam group, while of 4-substituted veratroles at the *para*-position of their 4-substituents.

The enzyme systems which catalyze O-demethylation of various foreign compounds have been known to locate in liver microsomes and to require both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen for their activity.[3] It, therefore, seemed to be of interest to examine whether or not the microsomal enzyme systems were also responsible for the demethylation of above compounds which have two adjacent methoxyl groups attached to an aromatic ring.

The present paper deals with *in vitro* demethylation of brucine and 4-substituted veratroles, using 9000×g. supernatant fractions of rabbit liver homogenates. In addition, demethylation of 4-nitroveratrole has been further studied by the microsomal fractions.

It will be shown that these demethylations are also catalyzed by the microsomal enzyme systems, and that not only NADPH but also reduced nicotinamide adenine dinucleotide (NADH) is an effective cofactor for demethylation of 4-nitroveratrole.

Materials and Methods

Materials——Brucine·HCl was prepared from a commercial sample of brucine, and MDB-I (2-methoxy-3-hydroxystrychnine) and MDB-Ⅱ (2-hydroxy-3-methoxystrychnine) were obtained from the urine of rabbits administered brucine and by partial hydrolysis of brucine, respectively, as described previously.[1,2] 4AV (4-acetamidoveratrole),[4] 4AG (4-acetamidoguaiacol),[5] 5AG (5-acetamidoguaiacol),[6] 4-aminoveratrole,[7]

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