Transformations of Cardio-active Steroids by Microorganisms.

Part I. Microbial Dehydrogenation of 3-Hydroxyl Group of Digitalis Cardiac Aglycone*

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Microbial dehydrogenations of 3β -hydroxyl groups of Digitalis cardiac aglycones by *Calonectria decora* and *Nigrospora sphaerica* were studied, both of which exhibited similar transformation pattern. Digitoxigenin was transformed exclusively into 3-dehydrodigitoxigenin by *C. decora* and into digoxigenin and 3-dehydro-digoxigenin by *N. sphaerica*. Both gitoxigenin and 3-acetyl-gitoxigenin were dehydrogenated to 3-dehydrogitoxigenin. 3-Dehydro-16-acetylgitoxigenin was also detected among the bioconversion products of 16-acetylgitoxigenin and 3,16-diacetylgitoxigenin by these two species.

Since the successful establishment of the application of microorganisms to the synthesis of biologically active C_{19} and C_{21} steroids, interest has also been awakened in the field of microbial metabolism of cardio-active steroids. Microbial attacks hitherto known towards this group of steroid are: hydrolysis of glycoside linkage and of acyl group attached to the hydroxyl present in the steroid nucleus or in the sugar moiety^{1~3}; introduction of hydroxyl groups at 5β ⁴, 7β ⁵, 11α ⁶, 12β ^{3,7} and 16β ⁸ positions of steroid aglycones; and reduction of 3-ketones to the 3-*epi*-hydroxyl groups².

In this paper are described the microbial

dehydrogenations of 3β -hydroxyl groups of Digitalis cardiac aglycones with or without the concomitant introduction of hydroxyl group at 12β position. Concerning the dehydrogenation reactions of 3-hydroxyl groups of steroids by microorganisms, pregnenolone⁹, androsterone and related C_{19} steroids¹⁰, bile acids¹¹ and cholesterol¹² have been reported to be dehydrogenated to their 3-ketones.

In the course of studies on microbial metabolism of cardiac glycosides and aglycones in this laboratory, a considerable number of microorganisms tested were found to convert cardiac aglycones into substances which were reactive with 2,4-dinitrophenylhydrazine reagent on paper chromatograms. As the tests for the reactivity of authentic samples of 3-dehydrogenated cardiac aglycones with this reagent were positive, dehydrogenation of 3-

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hydroxyl group to 3-ketone was suspected to occur by microbial activity.

Thus, from the transformation products of digitoxigenin (3 β ,14-dihydroxy-5 β -card-20(22)-enolide) (I) by *Nigrospora sphaerica* (Sacc.) Mason (order Moniliales, Fungi Imperfecti)*, 3-dehydrodigitoxigenin (3-dehydro-12 β -hydroxydigitoxigenin) (III) and a small amount of digoxigenin (12 β -hydroxydigitoxigenin) (IV) were isolated and identified by comparison with authentic samples.

Another microorganism, *Calonectria decora* (Wallr.) Sacc. (order Hypocreales, Ascomycetes) seems to have more potent dehydrogenating activity. When incubated with this fungus which has been reported to be a 12 β ,15 α -hydroxylator of progesterone¹³⁾, digitoxigenin was converted exclusively into 3-

dehydrodigitoxigenin under the same experimental conditions used as in the case of *N. sphaerica*. Bioconversions of 3-acetyldigitoxigenin (II), 3-dehydrodigitoxigenin (V) and digoxigenin by this microorganism gave the same transformation product, 3-dehydrodigitoxigenin, as shown in Table I.

The marked dehydrogenating activity of *C. decora* was exhibited more clearly in the transformations of gitoxigenin (16 β -hydroxydigitoxigenin) (VI) and its acetyl derivatives. Gitoxigenin was converted into 3-dehydrogitoxigenin (X) without concomitant introduction of hydroxyl group into steroid nucleus. 3-Acetylgitoxigenin (VII) was also transformed into the same compound after enzymatic hydrolysis of acetyl group. Moreover, 16-acetylgitoxigenin (oleandrigenin) (VIII) and 3,16-diacetylgitoxigenin (IX) were found to be dehydrogenated by *C. decora* to 3-dehydro-16-acetylgitoxigenin (XI). In addition to this compound the presence of

* The microorganisms belonging to the genus *Nigrospora*, especially *Nigrospora oryzae*, have been reported to hydroxylate progesterone at 11 α and 15 β positions. (R.D. Muir and R.M. Dodson, *U.S. Patent*, 2,823, 170, Feb. 11, 1958)

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TABLE I. R_F VALUES OF BIOCONVERSION PRODUCTS OF DIGITOXIGENIN AND ITS DERIVATIVES

System	A					B	C			
	II	V	I	III	IV	I	I	II	V	IV
I	0.92	0.84	0.77	0.34*	0.32	0.34*	0.34*	0.36*	0.35*	0.34*
	0.99	0.97	0.87	0.24*	0.06	0.25*	0.24*	0.24*	0.26*	0.25*
II						0.31				
						0.06				

System I. Toluene-butanol (3:1)-, II. Benzene-ethylene dichloride (3:7)-formamide-acetone (1:4), ascending method.

A. Authentic samples. B. Aglycones incubated with *N. sphaerica*. C. Aglycones incubated with *C. decora*.

* These spots gave yellow color with 2,4-dinitrophenylhydrazine reagent.

TABLE II. R_F VALUES OF BIOCONVERSION PRODUCTS OF GITOXIGENIN AND ITS DERIVATIVES

System	A						B			
	IX	XI	VII	VIII	X	VI	VI	VII	VIII	IX
I	0.96	0.85*	0.75	0.67	0.25*	0.08	0.24*	0.25*	0.68	0.95
							0.08	0.08	0.25*	0.85*
II	0.00	0.07*	0.14	0.26	0.45*	0.73	0.00	0.00	0.07	0.67
							0.07*	0.08*	0.07*	0.24*
									0.15	0.08
								0.25	0.15	0.00
									0.47*	0.07*
									0.75	0.15

System I. Benzene-ethylene dichloride (7:3)-formamide-acetone (1:4), ascending method. II. Toluene-chloroform (7:3)-propylene glycol-methanol (1:1), descending method.

A. Authentic samples. B. Aglycones incubated with *C. decora*.

* These spots gave yellow color with 2,4-dinitrophenylhydrazine reagent.

gitoxigenin, 3-dehydrogitoxigenin, 16-acetyl-gitoxigenin and the starting material, 3,16-diacetylgitoxigenin was demonstrated by paper chromatographic analysis of bioconversion products of 3,16-diacetylgitoxigenin (Table II).

N. sphaerica exhibited the same transformation pattern towards gitoxigenin and its acetyl derivatives, although the amounts of 3-dehydrogenated compounds formed were always less than those observed in transformations with *C. decora*.

EXPERIMENTAL*

Paper Chromatography. For the paper chromato-

* All melting points are uncorrected; cardiac aglycones of Digitalis used as starting materials in this work were prepared and generously donated to the author by Mr. H. Ishii and Mr. T. Okumura of this Laboratory.

graphic analyses of bioconversion products of cardiac steroids, the following solvent systems were used: i) toluene-butanol (3:1)-formamide-acetone (1:4)¹⁴, ii) benzene-ethylene dichloride (7:3) or (3:7)-formamide-acetone (1:4)¹⁴, iii) toluene-chloroform (7:3)-propylene glycol-methanol (1:1)¹⁵. The steroids were developed on Tôyô filter paper No. 51 at 26°C and were located using i) alkaline *m*-dinitrobenzene¹⁴, ii) trichloroacetic acid in chloroform¹⁶, iii) antimony trichloride in nitrobenzene¹⁷ or iv) 2,4-dinitrophenylhydrazine¹⁸.

Transformation of Digitoxigenin by *Nigrospora sphaerica*. *Nigrospora sphaerica* (Sacc.) Mason (culture collection of Nagao Institute, Tokyo) was grown

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15) D.R. Colingsworth, et al., *J. Biol. Chem.*, **203**, 807 (1953).

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17) H. Rosenkranz, *Arch. Biochem. Biophys.*, **44**, 1 (1953).

18) L.R. Axelrod, *J. Biol. Chem.*, **205**, 173 (1953).

in cotton-plugged 500 ml shake flasks containing 100 ml of medium. The medium used in this experiment contained 35 g of glucose, 20 g of peptone, 5 g of corn steep liquor and 1 l of distilled water. After inoculation the flasks were incubated at 26°C on a reciprocal shaker (140 r.p.m.; 7 cm amplitude) for 4 days and 20 mg of digitoxigenin in 1 ml of ethanol was charged to each flask. After 10 days' reincubation, the contents of 10 flasks were pooled (pH 7.6) and acidified to pH 3.2 with 10% HCl solution. The mycelium was filtered and extracted with acetone and chloroform successively. The filtrates of fermentation broths were extracted three times using 1 l of chloroform each time. The chloroform extracts were washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. Paper chromatographic analysis indicated the disappearance of the starting material and the presence of two transformation products, one of which gave a yellow spot with 2,4-dinitrophenylhydrazine (Table I).

The residual solids thus obtained (704 mg) were dissolved in benzene-chloroform (9:1) and chromatographed over 21 g of alumina. The column was eluted by successive elution with benzene, chloroform, and methanol using 30 ml portions of each eluant. The solids obtained from chloroform-methanol (100:1) eluate were recrystallized from acetone-ether, yielding 25 mg of plates melting at 254~256°C. The admixture of this crystal with an authentic sample of 3-dehydrodigoxigenin^{7,19)} did not depress the m.p. *Anal.* Found: C, 70.79; H, 8.40. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. $\lambda_{\max}^{EtOH}$ 220 μ ($\log \epsilon$ 4.17), 275 μ ($\log \epsilon$ 2.00). $\lambda_{\max}^{Nujol}$ 5.64, 5.74, 5.91, 6.13 μ .

From chloroform-methanol (50:1) eluates was obtained a small amount of crystals melting at 197~208°C, which did not show depression of mixed m.p. with an authentic sample of digoxigenin^{2,20)}.

3-Dehydrodigoxigenin from Digitoxigenin by *Calonectria decora*. 200 mg of digitoxigenin were added to the 6-days' culture of *C. decora* (culture collection of Centraalbureau voor Schimmelfcultures, Baarn, Holland) grown in Czapek-Dox solution supplemented with 0.5% corn steep liquor. After shaking for 10 days at 26°C, the broths (pH 8.4) were extracted as mentioned above. Evaporation of the solvent gave

233 mg of residual solids which were chromatographed over alumina. The solids from chloroform-methanol (100:1) eluate were recrystallized from ethyl acetate, yielding 32.3 mg of crystals, m.p. 234~246°C. Recrystallization from acetone-ether gave 20 mg of plates melting at 242~253°C. Mixed fusion with an authentic sample of 3-dehydrodigoxigenin did not show m.p. depression.

Paper chromatographic analysis showed the presence of a very small amount of digoxigenin in the fractions eluted with chloroform-methanol (50:1).

Bioconversion of Gitoxigenin and 3-Acetylgitoxigenin by *C. decora*. 180 mg of gitoxigenin were incubated for 10 days with 5 days' culture of *C. decora* (20 mg of gitoxigenin per 100 ml of medium). Papergram analysis of the broths showed the presence of the residual starting material and of the less polar bioconversion product reactive with 2,4-dinitrophenylhydrazine as shown in Table II. The solids obtained by chloroform extraction were chromatographed over 6 g of alumina. Chloroform-methanol (50:1) eluates gave after evaporation of the solvent 31 mg of crystals. Recrystallization from acetone-ether afforded 18 mg of prisms, m.p. 201~209°C. Admixture with an authentic sample of 3-dehydrogitoxigenin^{2,19)} prepared from gitoxigenin by N-bromosuccinimide oxidation (m.p. 202~207°C) did not show depression of the m.p. *Anal.* Found: C, 71.17; H, 8.38. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30.

The acetylation of mother liquor with pyridine and acetic anhydride gave 3 mg of plates, m.p. 242~247°C, which showed no depression on admixture with an authentic sample of 3-dehydro-16-acetylgitoxigenin²¹⁾.

Biooxidation of 200 mg of 3-acetylgitoxigenin in a similar way to the process described above gave 240 mg of steroidal extracts. By chromatographic separation were obtained 29 mg of crystals melting at 190~203°C which yielded, after recrystallization from acetone-ether, 16 mg of prisms, m.p. 195~198°C. Mixed fusion with an authentic sample did not show depression of the m.p. *Anal.* Found: C, 70.73; H, 8.35. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. $\lambda_{\max}^{Nujol}$ 5.61, 5.78, 5.83, 6.13 μ .

3-Dehydro-16-acetylgitoxigenin from 16-Acetylgitoxigenin by *C. decora*. Incubation of 300 mg of 16-acetylgitoxigenin with *C. decora* gave 305 mg of residual solids, which were dissolved in 3 ml of benzene and

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20) S. Pataki, K. Meyer and T. Reichstein, *Helv. Chim. Acta*, **36**, 1295 (1953).

21) A. Hunger and T. Reichstein, *Helv. Chim. Acta*, **33**, 76 (1950); Ch. Tamm and A. Gubler, *Helv. Chim. Acta*, **41**, 1762 (1958).

chromatographed over 6 g of alumina. Fractions eluted with benzene-chloroform (2:1) and (1:1) gave, after evaporation of the solvent, 31 mg of crystals which were recrystallized twice from acetone-ether yielding 6 mg of plates, m.p. 242~249°C. Admixture with an authentic sample of 3-dehydro-16-acetylgitoxigenin did not show m.p. depression. *Anal.* Found: C, 69.43; H, 7.93. Calcd. for $C_{26}H_{34}O_6$: C, 69.74; H, 7.96.

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