Tetrahedron. Vol. 29, pp. 823 to 826. Pergamon Press 1973. Printed in Great Britain

MICROBIOLOGICAL SYNTHESIS OF 16-KETOPREGNANES FROM STEROIDAL SAPOGENINS

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(*Received in Japan* 1 *September* 1972; *Received in the UK for publication* 10 *October* 1972)

Abstract—Incubation of diosgenone (1, 25D-spirost-4-en-3-one) with *Verticillium theobromae* (Turconi) Mason et Hughes (CBS) afforded 20α-hydroxypregn-4-ene-3,16-dione (3) and 3α,11β,20α-trihydroxy-5α-pregnan-16-one (4). The same transformation products were also obtained by the use of *Stachylidium bicolor* Link (IFO 6647). Isolation and identification processes of the products are discussed.

As far as the microbial transformation of steroidal sapogenins is concerned, we have already reported the new degradation of a spiroketal side ring in diosgenin or diosgenone (1, 25D-spirost-4-en-3-one) to give androsta-1,4-diene-3,16-dione (2), 16α-hydroxyandrosta-1,4-dien-3-one, and 16β-hydroxyandrosta-1,4-dien-3-one.[1] This finding stimulated us to establish the degradation pathway from 1 to 2, and to investigate the universality of the degradation pattern in a variety of micro-organisms (Fungi).

Here we report another type of transformation product from 1 by the use of *Verticillium theobromae* (Turconi) Mason et Hughes (CBS) and *Stachylidium bicolor* Link (IFO 6647).[2] The 16-keto-20α-hydroxypregnane of side chain in the products obtained here has been conjectured as an obligatory intermediary in the course of 1 to 2.[1]

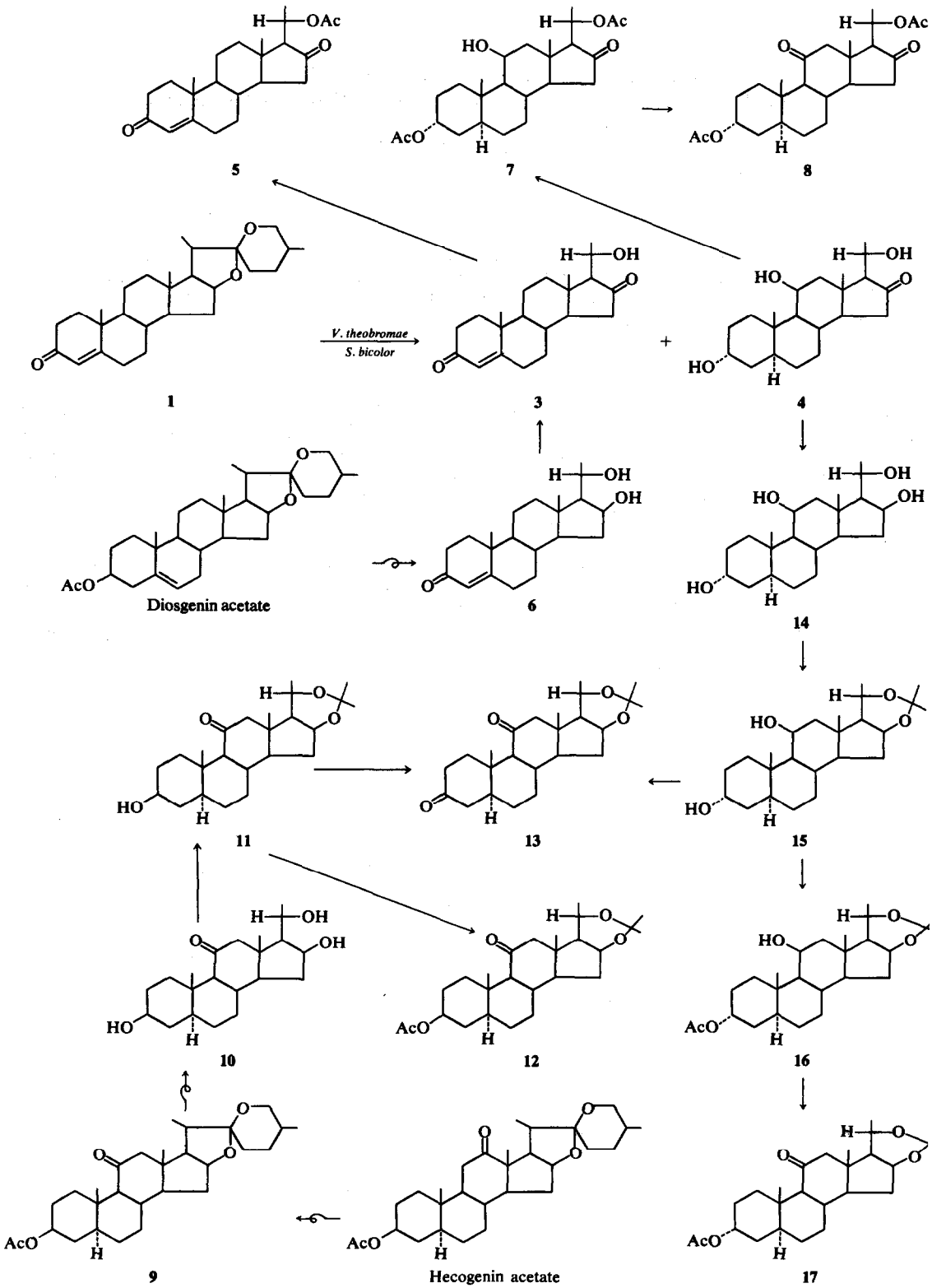
Incubation of 1 with *V. theobromae* afforded several metabolites, from which two crystalline compounds (3 and 4) were isolated. These were also obtained, but in much smaller quantities by using *S. bicolor*. Diosgenin was not such an effective substrate for these microorganisms in yielding the products.

Acetylation of 3 with Ac_2O/py gave a monoacetate (5), whose IR spectrum showed no hydroxyl groups. C—H analyses of 3 and 5 afforded figures in good agreement with $C_{21}H_{30}O_3$ and $C_{23}H_{32}O_4$, respectively. The NMR spectrum[a] of 3 exhibited bands corresponding to a secondary Me group [8·78 τ, d (J = 6 Hz)] and two angular Me groups (9·15 τ for 18-Me and 8·78 τ for 19-Me). The IR spectrum of 3 showed the bands at 3498 (OH), 1724 (5-membered ring ketone), 1666 and 1619 (Δ⁴-3-ketone) cm^{-1}. The CD spectrum[b] of 3 ($[\theta]_{302}$ = −22,533) indicated the 5-membered ring ketone in 3 should be in position 16.[3] From these results, 3 was assigned the structure of 20-hydroxypregn-4-ene-3,16-dione. To establish the configuration of the 20-OH group, 20α,16β-dihydroxypregn-4-en-3-one (6) was tentatively synthesized from diosgenin acetate according to the procedure of Morita *et al.*[4,5] Selective oxidation of the OH groups in 6 with sodium dichromate and H_2SO_4 gave 20α-hydroxypregn-4-ene-3,16-dione identical in all respects to 3 obtained microbiologically.

Treatment of 4 ($C_{21}H_{34}O_4$) with Ac_2O/py at room temperature afforded a diacetate (7, $C_{25}H_{28}O_6$) containing a 5-membered ring ketone (1721 cm^{-1}) and a OH group (3469 cm^{-1}). Mild oxidation of 7 with CrO_3 in acetone gave a diacetoxydiketone (8), whose IR spectrum showed no OH group. These results suggest that the OH group in 7, being resistant to mild acetylation, should be secondary. Among the secondary OH groups in the steroidal nucleus, an 11β-OH group is likely to have the characteristics described above, *i.e.* resistant to acetylation but not to oxidation. Thus it was supposed that 4 and 7 have an 11β-OH group and 8 has an 11-keto group. CD and NMR spectra of 4 indicated the presence of a 16-keto group ($[\theta]_{302}$ = −20,168)[3] and a secondary Me group [8·8 τ, d (J = 6·5 Hz)] besides two angular Me groups (8·96 τ for both 18-Me and 19-Me). Furthermore, the partial structure of saturated ring A with a 3-OH group was proposed from IR and UV spectra. To establish the structure of 4, a series of related compounds were synthesized. 11-Ketotigogenin acetate (9, m.p. 213–216°) was prepared from hecogenin acetate by a well-known chemical procedure.[6,7] Degradation of the spiroketal ring of 9 afforded 3β,16β,20α-trihydroxy-5α-pregnan-11-one (10), which was converted into 3β-acetoxy-16β,20α-isopropylidenedioxy-5α-pregnan-11-one (12) through 3β-hydroxy-16β,20α-isopropylidenedioxy-5α-pregnan-11-one (11). $NaBH_4$ reduction of 4 gave a tetrahydroxy compound (14), from which

[a]NMR spectra were taken with a Varian HA-60 spectrometer on $CDCl_3$ with TMS as an internal reference.

[b]CD spectra were taken in $CHCl_3$ containing 1% EtOH with Jasco ORD/UV-5.


5
7
8
1
V. theobromae
S. bicolor
3
4
Diosgenin acetate
6
14
11
13
15
10
12
16
9
Hecogenin acetate
17

SCHEME 1

acetonide **15** was obtained. CrO_3 oxidation of **15** afforded a diketone **13** identical in all respects to 16β,20α-isopropylidenedioxy-5α-pregnane-3,11-dione derived from **11**. Mild oxidation of **16**, obtained from **15** by acetylation, afforded 3-acetoxy-16β,20α-isopropylidenedioxy-5α-pregnan-11-one (**17**) whose physical properties were different from those of **12** (3β-OAc). From these results it was concluded that **4** should be 3α,11β,20α-trihydroxy-5α-pregnan-16-one.

EXPERIMENTAL*

Fermentation and isolation of products. (a) Twenty 500 ml shaking flasks, each containing 100 ml of sterilized medium composed of 3·5% glucose, 2% peptone, and 0·3% corn steep liquor (pH 6·8–7·0), were incubated with *Verticillium theobromae* (Turconi) Mason et Hughes (CBS) for 4 days at 28° on a reciprocal shaker. Mycelia were harvested by filtration and washed with distilled water divided into 20 flasks, each containing 100 ml of distilled water and 20 mg of diosgenone (**1**), and the flasks shaken for 4 days. The total reaction mixtures were EtOAc extracted. The solvent layer was water washed and evaporated under reduced pressure to yield a residue (825 mg), which was chromatographed on 50 g of silica gel. Elution with $CHCl_3$ ether (9:1 v/v) afforded oils, which were rechromatographed on Florisil yielding 63·8 mg of **3**; m.p. 126–127°, $[\alpha]_D^{24} - 49{\cdot}5°$ (± 1·4°), $\lambda_{max}^{alc.}$ 241·5 mμ (ε = 16,300), $\nu_{max}^{CHCl_3}$ 3498, 1724, 1666, 1619 cm^{-1}. (Found: C, 76·07; H, 9·52. $C_{21}H_{30}O_3$ requires: C, 76·32; H, 9·15%). Elution with $CHCl_3$ ether (7:3 v/v) gave the fractions containing **4** dominantly. These fractions were combined and rechromatographed by using Hyflo Super-Cel. Analytical sample of **4** (38·8 mg) showed the following constants; m.p. 231–232°, $[\alpha]_D^{24} - 105{\cdot}2°$ (± 2·5°), ν_{max}^{Nujol} 3433, 1724 1724 cm^{-1}. (Found: C, 72·15; H, 9·86. $C_{21}H_{34}O_4$ requires: C, 71·96; H, 9·78%).

(b) **1** (200 mg) was treated with wet mycelia (80 g) of *Stachylidium bicolor* Link (IFO 6647) as described in (a) using 10 flasks. Similar product extraction and isolation, afforded 21·3 mg of **3** and 17·8 mg of **4**.

20α-*Acetoxypregn-4-ene*-3,16-*dione* (**5**). A solution of **3** (10 mg) in 1 ml of pyridine and 1 ml of Ac_2O was allowed to stand at room temp overnight. Usual treatment of the mixture afforded 7·5 mg of **5**; m.p. 182–183°, $[\alpha]_D^{24}$ −52·7° (± 1·5°), $\lambda_{max}^{alc.}$ 240·5 mμ (ε = 15,890), ν_{max}^{Nujol} 1733, 1672, 1611 cm^{-1}. (Found: C, 74·11; H, 8·76. $C_{23}H_{32}O_4$ requires: C, 74·16; H, 8·66%).

16β,20α-*Dihydroxypregn-4-en*-3-*one* (**6**). Diosgenin acetate was converted into **6** using the procedure of Morita *et al.*[4,5] An analytical sample of **6** had m.p. 188–189°, $[\alpha]_D^{24} + 122{\cdot}5°$ (± 2·9°), $\lambda_{max}^{alc.}$ 241·5 mμ (ε = 17,100), ν_{max}^{Nujol} 3538, 3386, 1644, 1608 cm^{-1}. (Found: C, 75·84; H, 9·82. $C_{21}H_{32}O_3$ requires: C, 75·86; H, 9·70%).

Oxidation of **6**. To a stirring solution of **6** (50 mg), dissolved in 0·75 ml THF, 0·75 ml of benzene and 10 ml of ether, an 0·18 ml of sodium dichromate soln† was added dropwise over a 40-min at room temp and stirring continued for 2 h. After the addition of a small amount of water, the mixture was extracted with CH_2Cl_2, the organic layer washed with $NaHCO_3$aq and water, dried (Na_2SO_4) and concentrated under red. pres. to give 37·5 mg. Purification of TLC (silica gel; $CHCl_3$-ether 1:1 v/v) afforded a crystalline product (24·8 mg) identical to **3**, (TLC, IR and m.m.p.).

3α,20α-*Diacetoxy*-11β-*hydroxy*-5α-*pregnan*-16-*one* (**7**). To a soln of **4** (70 mg) in 1 ml of pyridine was added 1 ml Ac_2O. After standing at room temp overnight, the mixture was treated as usual yielding 74 mg of **7**; m.p. 228–229°, $[\alpha]_D^{23} - 87{\cdot}3°$ (± 2·1°), ν_{max}^{Nujol} 3469, 1741, 1721, 1243 cm^{-1}. (Found: C, 69·30; H, 8·93. $C_{25}H_{28}O_6$ requires C, 69·09; H, 8·81%).

3α,20α-*Diacetoxy*-5α-*pregnane*-11,16-*dione* (**8**). To a soln of **7** (40 mg) in 8 ml of acetone, 0·102 ml of chromic acid solution‡ was added dropwise with stirring and the mixture allowed to stand at room temp for 20 min. After addition of water, the mixture was $CHCl_3$ extracted. The $CHCl_3$ layer was washed with $NaHCO_3$aq and water, dried (Na_2SO_4), and evaporated to dryness, giving 36·6 mg of **8**, m.p. 184–186·5°, ν_{max}^{Nujol} 1752, 1732, 1711, 1251, 1235 cm^{-1}. (Found: C, 69·82; H, 8·20. $C_{25}H_{36}O_6$ requires C, 69·42; H, 8·39%).

11-*Ketotigogenin acetate* (**9**). This was obtained from hecogenin acetate as previously reported.[6,7] An analytical sample of **9** has m.p. 213–216°, ν_{max}^{Nujol} 1740, 1699, 1248 cm^{-1}. (Found: C, 73·65; H, 9·27. $C_{29}H_{44}O_5$ requires: C, 73·69; H, 9·38%).

3β,16β,20α-*Trihydroxy*-5α-*pregnan*-11-*one* (**10**). 3 g of **9** was converted into 1·84 g of **10** by the method of Morita *et al.*[4,5] An analytical sample of **10** had m.p. 222–223°, $[\alpha]_D^{22} + 67{\cdot}1°$ (± 1·1°) (EtOH), ν_{max}^{Nujol} 3327, 3234, 1697 cm^{-1}. (Found: C, 71·68; H, 9·74. $C_{21}H_{34}O_4$ requires: C, 71·96; H, 9·78%).

3β-*Hydroxy*-16β,20α-*isopropylidenedioxy*-5α-*pregnan*-11-*one*. (**11**). To 1 g of **10**, suspended in 60 ml acetone, was added 7 drops of BF_3-ether (b.p. 126–128°) over 5 min and the mixture allowed to stand for 90 min (stirring) at room temp. After addition of a few drops of pyridine, acetone was removed. Addition of water (70 ml) afforded the crude **11** (0·89 g). Recrystallization (MeOH) gave an analytical sample; m.p. 237–238°, $[\alpha]_D^{23} + 20{\cdot}9°$ (± 0·6°), ν_{max}^{Nujol} 3500, 1704, 1239, 1222 cm^{-1}. (Found: C, 73·72; H, 9·73. $C_{24}H_{38}O_4$ requires: C, 73·80; H, 9·81%).

3β-*Acetoxy*-16β,20α-*isopropylidenedioxy*-5α-*pregnan*-11-*one* (**12**). Acetylation of 200 mg of **11** with 2 ml of pyridine and 2 ml Ac_2O at room temp afforded 158 mg **12**; m.p. 201–203°, $[\alpha]_D^{23} + 7{\cdot}1°$ (± 0·5°), ν_{max}^{Nujol} 1740, 1699, 1255, 1246 cm^{-1}. (Found: C, 72·21; H, 9·56. $C_{26}H_{40}O_5$ requires: C, 72·19; H, 9·32%).

16β,20α-*Isopropylidenedioxy*-5α-*pregnane*-3,11-*dione* (**13**). (a) To a soln of **11** (200 mg), dissolved in 2 ml of pyridine, 2·5 ml of pyridine-chromic acid complex containing 250 mg CrO_3 was added dropwise with stirring and the mixture allowed to stand at room temp overnight. After addition of water (80 ml), the mixture was benzene extracted. The benzene layer was water washed, dried (Na_2SO_4) and evaporated to dryness, giving 151 mg of **13**. An analytical sample had m.p. 235–237°, $[\alpha]_D^{23} + 39{\cdot}4°$ (± 0·8°), ν_{max}^{Nujol} 1712, 1700, 1244, 1223 cm^{-1}. (Found: C, 74·08; H, 9·33. $C_{24}H_{36}O_4$ requires: C, 74·19; H, 9·34%).

(b) To 12 mg of **15** (described below), dissolved in 0·1 ml of pyridine, 0·4 ml of pyridine-chromic acid complex containing 40 mg CrO_3 was added dropwise and the mix-

*M.p.s were determined on a Yanagimoto micro apparatus and are corrected. Unless otherwise noted, optical rotations were determined in $CHCl_3$ containing 1% EtOH with Rudolph polarimeter Model 200.

†Sodium dichromate solution was 2·5 g $Na_2Cr_2O_7 \times 2H_2O$ and 1·875 ml conc. H_2SO_4 in 25 ml water.

‡The chromic acid solution contained 668 mg CrO_3 and 0·575 ml conc. H_2SO_4 in 5 ml water.

ture allowed to stand at room temp overnight. Similar treatment of the mixture as above gave 5·4 mg of **13**.

3α,11β,16β,20α-Tetrahydroxy-5α-pregnane (**14**). To an iced soln of **4** (130 mg), dissolved in 50 ml MeOH, 27 mg of $NaBH_4$ was added. After standing at 0–3° for 1 h, the mixture was diluted with 80 ml of water, adjusted to pH 5·5 with AcOH, evaporated to remove MeOH and then $CHCl_3$ extracted. The organic layer was washed with $NaHCO_3$aq and water, dried (Na_2SO_4) and evaporated to yield crystalline residue (145 mg) mainly composed of **14**. Further purification was not performed.

3α,11β-Dihydroxy-16β,20α-isopropylidenedioxy-5α-pregnane (**15**). To a soln of the residue (145 mg) composed of **14**, dissolved in 12 ml of acetone, two drops of BF_3-ether (b.p. 126–128°) were added and the mixture allowed to stand at room temp for 2 h (stirring). After addition of pyridine (1 drop), the mixture was evaporated to yield (after prep. TLC, silical gel, EtOAc) **15** (128 mg). Recrystallization from ether-pet. ether afforded 101 mg of an analytical sample, m.p. 234–235°, $[\alpha]_D^{25}$ +1·9° (±1·0°), ν_{max}^{Nujol} 3455, 1235, 1226 cm^{-1}. (Found: C, 73·70; H, 10·33. $C_{24}H_{40}O_4$ requires: C, 73·43; H, 10·27%).

3α-Acetoxy-11β-hydroxy-16β,20α-isopropyridenedioxy-5α-pregnane (**16**). Acetylation of **15** (50 mg) with 1 ml pyridine and 1 ml Ac_2O at 80–90° for 30 min afforded 43·8 mg of **16**, m.p. 193–194°, $[\alpha]_D^{24}$ +7·0° (±0·9°), ν_{max}^{Nujol} 3594, 1728, 1255, 1236, 1218 cm^{-1}. (Found: C, 71·93; H, 9·60. $C_{26}H_{42}O_5$ requires: C, 71·85; H, 9·74%).

3α-Acetoxy-16β,20α-isopropyridenedioxy-5α-pregnan-11-one (**17**). Forty mg of **16** dissolved in 0·5 ml of pyridine was oxidized with 1·2 ml of pyridine–CrO_3 complex (120 mg of CrO_3) at room temp for 19 h. Similar treatment of the mixture as described above afforded 32·3 mg of **17**; m.p. 206–207°, $[\alpha]_D^{23}$ +25·7° (±1·3°), ν_{max}^{Nujol} 1738, 1701, 1256, 1238 cm^{-1}. (Found: C, 72·25; H, 9·23. $C_{26}H_{40}O_5$ requires: C, 72·19; H, 9·32%).

Acknowledgement—We are greatly indebted to Dr. K. Takeda, Director of this laboratory, for his helpful advice and encouragement.

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