ELSEVIER

# Preparation of 3-ketodesogestrel metabolites by microbial transformation and chemical synthesis

**Helmut Groh,* Renate Schön,† Michael Ritzau,† Helmut Kasch,† Katrin Undisz,† and Gerhard Hobe†**

**Institute of Pharmacy of the Friedrich-Schiller-University, Jena, Germany; and †Hans-Knöll-Institute for Natural Product Research, Jena, Germany*

*Specific microbial reactions were used for the preparation of metabolites of 3-ketodesogestrel (13-ethyl-17β-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one, the active form of the progestagen desogestrel.* Clostridium paraputrificum *transformed 3-ketodesogestrel (KDG) to the 5β-dihydro and tetrahydro metabolites 13-ethyl-17β-hydroxy-11-methylene-18,19-dinor-5β,17α-pregnan-20-yn-3-one and 13-ethyl-11-methylene-18,19-dinor-5β,17α-pregnan-20-yne-3α,17β-diol, respectively. The epimeric compound 13-ethyl-11-methylene-18,19-dinor-5β, 17α-pregnan-20-yne-3β, 17β-diol was obtained by chemical reduction of the 3-oxo compound.* Mycobacterium smegmatis *converted KDG to metabolites of the 5αH-series: 13-ethyl-17β-hydroxy-11-methylene-18,19-dinor-5α, 17α-pregnan-20-yn-3-one, 13-ethyl-11-methylene-18,19-dinor-5α, 17α-pregnan-20-yne-3α,17β-diol and 13-ethyl-11-methylene-18,19-dinor-5α,17α-pregnan-20-yne-3β,17β-diol. The ring A-aromatized analog of KDG 13-ethyl-11-methylene-18,19-dinor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol was obtained by microbial 1-dehydrogenation with* Rhodococcus rhodochrous. *Additionally, chemical syntheses of the microbially obtained KDG metabolites listed above were carried out. These included Birch reduction, reduction of KDG with sodium borohydride in aqueous pyridine and in methanol, reduction of KDG with potassium selectride in tetrahydrofuran, and dehydrogenation of KDG with cupric-II bromide in acetonitrile. The problems encountered in chemical syntheses favor the microbial procedures. The compounds were characterized by mass spectra (MS), IR, and circular dichroism (CD). Complete assignments of $^{1}H$ and $^{13}C$ chemical shifts were made using homo- and heteronuclear 2-DN-NMR spectroscopy. Chromatographic [gas-liquid chromatography (GLC), high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC)] data of all the prepared KDG metabolites are presented.* (*Steroids* **62**:437–443, 1997) © *1997 by Elsevier Science Inc.*

**Keywords:** 3-ketodesogestrel; metabolite; microbial transformation; chemical synthesis; NMR

## Introduction

Metabolites of 3-ketodesogestrel (13-ethyl-17β-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one, KDG) the active form of the progestagen desogestrel, are of interest for various reasons. The availability of metabolites will support studies on KDG biotransformation and facilitate the isolation of other metabolites. KDG metabolites are required for testing the crossreactions of KDG antisera, which will be necessary when a radioimmunoassay for KDG is established. Furthermore, there is a growing interest in potential biologic effects of drug metabolites, including those of KDG.

An important pathway in the metabolism of steroid compounds with a 3-oxo-4-en structure is the reduction to saturated 3-hydroxy compounds. Synthetic progestagens of the 17α-substituted 19-nortestosterone type, such as norethisterone and levonorgestrel, have been reported to be preferentially reduced to metabolites of the 5βH-series.[1] Metabolites of KDG, like the allylic compounds 3α-OH- and 3β-OH-desogestrel and the saturated 5α-dihydro and tetrahydro metabolites, have been mentioned in biotransformation studies as compounds for comparison.[2–4] There is, however, little information in the literature on the preparation of these compounds. To our knowledge, metabolites of the 5βH-series as well as the ring A-aromatized analog of KDG, which may be potential products of biotransformation, have not been described until now.

In previous studies, we have used specifically hydrogenating microorganisms such as the anaerobic intestinal bacterium *Clostridium paraputrificum*, the yeast *Rhodotorula glutinis*, and the aerobic actinomycete *Mycobacterium*

In remembrance of Prof. Dr. Cläre Hörhold-Schubert, former head of the Division of Steroid Research of the Central Institute of Microbiology and Experimental Therapy, Jena who died in July 1996.

Address reprint requests to Dr. Gerhard Hobe, Hans-Knöll-Institute for Natural Product Research, Beutenbergstr. 11, D-07745 Jena, Germany.

Received October 9, 1996; accepted December 2, 1996.

Steroids 62:437–443, 1997

655 Avenue of the Americas, New York, NY 10010

0039-128X/97/$17.00
PII S0039-128X(97)00014-7

*smegmatis* for the preparation of metabolites of endogenous steroids and those of steroid drugs.[5–9] We have also studied the microbial aromatization of 19-norsteroids by bacterial flora[10] and by the steroid-1-dehydrogenase of *Nocardia* species.[11] In the present study, we make use of the specific steroid-converting enzymes of these microorganisms for the preparation of KDG metabolites. With the microbially obtained compounds for comparison, we also studied some chemical reactions for the preparation of KDG metabolites.

## Experimental

### *Substances*

3-Ketodesogestrel was kindly provided by Prof. Dr. S. Schwarz, Jenapharm GmbH. [16(n)-$^3$H]-3-ketodesogestrel was supplied by Wyeth Ayerst Research Laboratories, Princeton, New Jersey, USA. The reference compounds 5α- and 5β-dihydro levonorgestrel were obtained from the Steroid Collection of the former Central Institute of Microbiology and Experimental Therapy (CIMET) Jena, Germany. Solvents were of analytical grade and were used like all chemical reagents as supplied from Merck (Darmstadt, Germany) and Sigma-Aldrich Chemie, Deisenhofen, Germany. The nutrient compounds and salts needed for media preparation were obtained from Difco (Detroit, MI) and Merck.

### *Culture media*

**Medium 1.** 1 L filtered infusion from 500 g trimmed fresh beef liver, 10 g Bacto-Casitone, 5 g glucose, 5 g NaCl, pH 7.4; 5 pieces of approximately 8 × 8 mm boiled liver were added to each 10 mL of infusion medium.

**Medium 2.** 15 g Bacto-Casitone, 5 g yeast extract, 5.5 g glucose, 2.5 g NaCl, 0.5 g cysteine, 0.07 g sodium thioglycolate, and 0.75 g agar were dissolved in 1 L of water, and the pH was adjusted to 6.6.

**Medium 3.** 1 volume egg yolk mixed with 1 volume of the following solution: 4 g L-asparagine, 60 g glycerol, 2 g citric acid, 0.5 g $K_2HPO_4$, 0.5 g $MgSO_4 \times 7\ H_2O$, and 0.05 g ferric ammonium citrate per 1 L of water, pH 7.0.

**Medium 4.** 10 g peptone, 10 g glucose, 1 g casamino acids, 2 g yeast extract, and 6 g NaCl per 1 L of water, pH 7.0.

**Medium 5.** Medium 4 with 15 g agar per 1 L. All media were autoclaved at 120°C for 20 min.

### *Microorganisms*

*Clostridium paraputrificum* (IMET H2/10865 = DSM 46280) stored at 4°C in medium 1, *Mycobacterium smegmatis* (IMET H 124/SG 98) stored at 4°C in medium 3, and *Rhodococcus rhodochrous* (invalid name *Nocardia opaca,* IMET 7030)[22] stored at 4°C in medium 5 were obtained from the Type Culture Collection of the former CIMET, Jena, Germany.

### *General procedures*

Gas-liquid chromatography (GLC) analyses were performed on a HP 5890 Series II Plus apparatus equipped with a HP 7673 automatic injector and an Ultra 1 capillary column (50 m × 0.32 mm × 0.52 μm). Column temperature 260°C, injector 230°C, FID 310°C, hydrogen flow rate 2.41 mL/min, split ratio 20:1. High-performance liquid chromatography (HPLC) was carried out on a Shimadzu apparatus equipped with a Lichrosorb® RP 18 column (250 × 4.6 mm) and a photodiode array ultraviolet-VIS detector (205–350 nm) and using acetonitrile/water (60:40 v/v, 1 mL/min) as the eluent. Thin-layer chromatography (TLC) was carried out on silica gel $GF_{254}$ (Merck) and on alumina (Merck) with the solvent systems described in Table 1. The compounds were observed under ultraviolet light, by staining with tungstophosphoric acid, and when using a radioactive substrate, with a radio thin-layer scanner (Linear Analyzer Berthold, Wildbad, Germany). Column chromatography was performed on silica gel (60–120 μm) with cyclohexane/ethyl acetate mixtures as the eluent. Melting points (mp) were determined on a Reichler–Kofler microscope and were not corrected. Circular dichroism (CD) spectra were measured in dioxane using a spectro polarimeter Jasco J-720. High-resolution mass spectra (HRMS) were recorded on a Joel JMS-D 100 and the IR spectra (KBr) on a Galaxy 4020 Mattson instrument. NMR spectra ($^1$H, $^{13}$C, distortionless enhancement by polarization transfer (DEPT), correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser spectroscopy (NOESY), J-resolved spectroscopy (2-D-J), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) were measured on a Bruker Avance DRX 500 spectrometer with sample concentrations of 0.005 mM in $CDCl_3$. Chemical shifts are given in ppm relative to the internal TMS standard. TOCSY (mixing time 70 ms), NOESY (mixing time 0.7 s), and HSQC spectra were obtained in phase sensitive mode using time proportional phase increments (TPPI). The HMBC spectra were optimized on a long-range coupling of 7 Hz. To determine the KDG conversion rate and the ratio of the metabolites formed in the fermentations, representative experiments were carried out with 2–5 μCi $^3$H KDG added to the nonlabeled substrate.

### *Metabolites obtained by microbial transformation of KDG by* C. paraputrificum*: 13-Ethyl-17β-hydroxy-11-methylene-18,19-dinor-5β,17α-pregnan-20-yn-3-one (1) and 13-ethyl-11-methylene-18,19-dinor-5β,17α-pregnan-20-yne-3α,17β-diol (2)*

*C. paraputrificum* H2 was grown anaerobically under reduced atmospheric pressure (30–40 mm Hg) in 80 mL of medium 1 at 37°C for 24 h and inoculated into 400 mL of medium 2. A solution of 20 mg KDG in 4 mL of acetone was added, and fermentation was carried out under reduced atmospheric pressure at 37°C for 24–48 h. The cultures were extracted three times with $CHCl_3$, and the combined extracts were washed with water, dried with anhydrous $Na_2SO_4$, and evaporated in vacuo. Column chromatography of the extract on silica gel with cyclohexane/ethyl acetate (4:1 v/v) as the eluent yielded metabolite **1** with small amounts of KDG. Compound **1** was separated using TLC in system C (see Table 1). Yields of 60 and 20% were obtained, dependent upon the fermentation time of 24 and 48 hours, respectively. Metabolite 1 was recrystallized from acetone/cyclohexane: mp 193–198°C. HRMS: 326.2240 ($M^+$), calc. for $C_{22}H_{30}O_2$ 326.2246. IR [$\nu_{max}$]: 3400 (R-OH), 3255 (−C ≡ CH), 1702 (>C = O), 1640 (>C = $CH_2$), 899 (>C = $CH_2$) $cm^{-1}$. CD [$\Delta\varepsilon$ ($M^{-1}$ $cm^{-1}$)/λ (nm)]: 0/220; 0/(250); −0.17/280; −0.31/300; −0.08/320; 0/350; (c = 0.67 mg/mL). $^1$H and $^{13}$C NMR data (see Tables 2 and 3).

During column chromatography, the KDG metabolite **2** was eluted following **1** with cyclohexane/ethyl acetate (3:1 v/v) as the eluent, with yields of 5 and 50%, dependent upon the fermentation time of 24 and 48 hours, respectively. mp 177–179°C. HRMS: 328.2408 ($M^+$), calc. for $C_{22}H_{32}O_2$ 328.2402. IR [$\nu_{max}$]: 3400 (R-OH), 3305 (−C ≡ CH), 1642 (>C = $CH_2$), 895 (>C = $CH_2$) $cm^{-1}$. $^1$H and $^{13}$C NMR data (see Tables 2 and 3).

### *Chemical reduction of **1** to 13-ethyl-17β-hydroxy-11-methylene-18,19-dinor-5β,17α-pregnan-20-yne-3β,17β-diol (**3**)*

Reduction of the 3-oxo compound **1** with potassium selectride in tetrahydrofuran according to Wiebe et al.[13] yielded the 5βH-3β-OH tetrahydro metabolite **3** as a main product (40%), which was separated from byproducts by TLC using systems A and D. **3:** not crystalline. HRMS: 328.2398 ($M^+$), calc. for $C_{22}H_{32}O_2$ 328.2402. $^1H$ and $^{13}C$ NMR data (see Tables 2 and 3).

### *Metabolites obtained by microbial transformation of KDG with* M. smegmatis*: 13-Ethyl-17β-hydroxy-11-methylene-18,19-dinor-5α,17α-pregnan-20-yn-3-one (**4**), 13-ethyl-11-methylene-18,19-dinor-5α,17α-pregnan-20-yne-3α,17β-diol (**5**), and 13-ethyl-11-methylene-18,19-dinor-5α,17α-pregnan-20-yne-3β,17β-diol (**6**)*

5 mL of a *M. smegmatis* SG 98 culture grown in medium 4 for 3 days at 30°C were inoculated into each 50 mL of the same medium in 500 mL flasks and incubated for 3 days at 30°C on a gyratory shaker (200 rpm). Subsequently, the cells (about 2.5 g/50 mL) were harvested by centrifugation and washed three times with 20 mL of 0.1 M phosphate buffer, pH 7.0. The cells were resuspended in buffer (2 g/20 mL), and a solution of KDG in acetone (5 mg/mL) was added (10 μL/mL medium). During the fermentation at 30°C, samples were drawn for GLC and TLC analysis every 2 hours. A complete conversion of KDG and four transformation products **4, 5, 6, and 7** in a ratio of 1:5:2:1, respectively, were found after 12 h incubation. After fermentation, the culture was repeatedly extracted with ethyl acetate, and the combined extracts were separated by TLC using systems B and C, yielding the single compounds **4** (5%), **5** (50%), **6** (10%), and **7** (6%). To obtain a larger amount of the dihydro metabolite **4,** the tetrahydro compounds **5** and **6** were oxidized with pyridinium chlorochromate.[12]

**4:** mp 176–182°C. HRMS: 326.2251 ($M^+$), calculated for $C_{22}H_{30}O_2$ 326.2246. IR [$\nu_{max}$]: 3400 (R-OH), 3280 (−C ≡ CH), 1700 (>C = O), 1645 (>C = $CH_2$), 901 (>C = $CH_2$) $cm^{-1}$. CD [$\Delta\varepsilon$ ($M^{-1}$ $cm^{-1}$)/λ(nm)]: 0/220; 0/250; +0.25/280; +0.43/300; +0.13/320; 0/350; (c = 1.03 mg/mL).

**5:** not crystalline. HRMS: 328.2408 ($M^+$), calc. for $C_{22}H_{32}O_2$ 328.2402. IR [$\nu_{max}$]: 3412 (R-OH), 3305 (−C ≡ CH), 1641 (>C = $CH_2$), 897 (>C = $CH_2$) $cm^{-1}$. $^1H$ and $^{13}C$ NMR data (see Tables 2 and 3).

**6:** mp 84–87°C. HRMS: 328.2406 ($M^+$), calc. for $C_{22}H_{32}O_2$ 328.2402. IR [$\nu_{max}$]: 3421 (R-OH), 3308 (−C ≡ CH), 1641 (>C = $CH_2$), 900 (>C = $CH_2$) $cm^{-1}$. $^1H$ and $^{13}C$ NMR data (see Tables 2 and 3).

### *Transformation of KDG by* R. rhodochrous *to 13-Ethyl-11-methylene-18,19-dinor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol (**7**)*

10 mL of a *R. rhodochrous* 7030 culture grown in medium 4 at 30°C for 24 h were inoculated into 500 mL flasks each containing 100 mL of medium 4, and 1 mg KDG in 1 mL of acetone was added to each flask. Fermentation proceeded for 24 h at 30°C on a gyratory shaker (180 rpm). After centrifugation, the supernatant was extracted three times with benzene. Column chromatography on silica gel with cyclohexane/ethyl acetate (85:15 v/v) as the eluent yielded **7** in about 60% yield. 7: mp 168–171°C. UV [$\lambda_{max}$]: 279 nm. HRMS: 322.1940 ($M^+$), calculated for $C_{22}H_{26}O_2$ 322.1933. IR [$\nu_{max}$]: 3390 (R-OH), 3303 (−C ≡ CH), 1645 (>C = $CH_2$), 1611, 1580, 1499 (aromatic ring A), 902 (>C = $CH_2$) $cm^{-1}$. $^1H$ and $^{13}C$ NMR data (see Tables 2 and 3).

Following reaction with pyridine acetanhydride (1:1 v/v) for 5 h at room temperature, compound **7** was completely transformed to two less polar compounds with a ratio of 10:1. These compounds had $R_f$ values of 0.49 and 0.63, respectively (in system C), and a $\lambda_{max}$ of 269(274) nm. Taken together, these data characterized the two compounds as the mono and diacetates of **7,** respectively.

### *Chemical reactions*

Oxidation of 3-hydroxy metabolites to 3-oxo compounds was conducted with pyridinium chlorochromate in dichlormethane.[12] 3-Oxo compounds were reduced to 3-hydroxy metabolites by reaction with sodium borohydride in methanol and with potassium selectride in tetrahydrofuran.[13]

### *Chemical reduction of KDG to 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yne-3α,17β-diol (**8**) and 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yne-3β,17β-diol (**9**)*

Reduction of KDG with $NaBH_4$ in methanol for 2 h on ice resulted in complete conversion and formation of **8** and **9** at a ratio of 1:10. With potassium selectride in tetrahydrofuran as the reducing agent, only 40% of KDG reacted to forms **8** and **9** in a 1:1 ratio. The isomers were separated by repeated TLC in systems B and C. **8:** mp 148–152°C. HRMS: 326.2251 ($M^+$), calc. for $C_{22}H_{30}O_2$ 326.2246. IR [$\nu_{max}$]: 3400 (R-OH), 3282 (−C ≡ CH), 1641 (>C = $CH_2$), 895 (>C = $CH_2$) $cm^{-1}$. **9:** mp 153–156°C. HRMS: 326.2250 ($M^+$), calc. for $C_{22}H_{30}O_2$ 326.2246. IR [$\nu_{max}$]: 3400 (R-OH), 3302 (−C ≡ CH), 1642 (>C = $CH_2$), 904 (>C = $CH_2$) $cm^{-1}$. $^1H$ and $^{13}C$ NMR data (see Tables 2 and 3).

### *Birch-reduction of KDG*[14]

18.9 mg (0.058 mmol) KDG dissolved in 0.5 mL of tetrahydrofuran (THF) were added to a Birch solution prepared by reaction of 7.8 mg (1.1 mmol) lithium with 4.5 mL of liquid ammonia and 0.5 mL of THF at a temperature of about −50°C and under argon gas atmosphere. After 3 min, 20 mg solid ferric nitrate were added to destroy excess reducing agent. After evaporation of the ammonia, water was added, and the reaction mixture was extracted with $CH_2Cl_2$. The reaction product, containing the 5α-dihydro metabolite **4,** and the corresponding 17α-vinyl compound in a 4:1 ratio was separated by HPLC on Lichrosorb® Si 60 (10 μm) using acetonitrile/water (60:40 v/v) as the eluent.

### *Reduction of KDG with $NaBH_4$ in aqueous pyridine*[15] *to **2, 3, 5, and 6***

A suspension of 16.7 mg (0.44 mmol) $NaBH_4$ in 0.5 mL pyridine was added under shaking (300 rpm) to a solution of 100 mg KDG (0.31 mmol) in 1 mL of pyridine and 0.07 mL of water. After shaking for 2 h at room temperature, the reaction product was poured into a mixture of 22 mL of water and 2.5 mL of 1 M HCl, left for 2 h in a refrigerator, and then extracted with $CHCl_3$. Separation of the complex mixture of KDG tetrahydro metabolites was achieved by repeated TLC in system A yielding metabolites **2** (16%), **3** (2.5%), **5** (5%), and **6** (31%) in addition to 11% of the starting material KDG.

### *Reaction of KDG with cupric-II-bromide in acetonitrile*

KDG was reacted in anhydrous acetonitrile with $CuBr_2$ (molar ratio 1:1.2 and 1:0.9) for 16 h at room temperature, according to the procedure described by Rao et al.[16] The reaction product was

separated by TLC in system A, cyclohexane/$CHCl_3$/ethanol (46:46:8 v/v/v), and $CHCl_3$/ether (87:13 v/v). Only a small amount of **7** could be obtained (<10%) when 0.9 mol $CuBr_2$ was used. The main product in both reactions was a mixture of compounds with a strong ultraviolet absorption and high mol masses (399, 417, 467, and 496).

## Results and Discussion

The present study on the microbial hydrogenation and dehydrogenation of KDG demonstrates once more that microbial procedures are useful in the preparation of steroid metabolites because of their high specificity. When compared to chemical synthesis, undesired side reactions occur less frequently with microbial procedures, which is especially important if there are sensitive areas in the steroid molecule. The radioactive labeling of part of the substrate allows for convenient follow-up of the course of transformation. In Figure 1, the structural formulas of all prepared KDG metabolites are presented. Table 1 displays chromatographic data of the metabolites obtained by GLC, TLC, and HPLC. The complete $^1H$ and $^{13}C$ NMR assignments, as derived from 2D experiments, are compiled in Tables 2 and 3, respectively.

### *5βH- Metabolites of KDG obtained by* Clostridium paraputrificum

This anaerobic strain converted KDG stereospecifically to the 5β-dihydro and tetrahydro metabolites **1** and **2,** respectively. After 24 h of fermentation, metabolite **1** dominated; whereas, after 48 h, **2** was the main product, indicating the precursor function of **1.** The conversion rate of KDG to **1** and **2** was about 90% in 48 h. The 5βH configuration in **1** was proved by circular dichroism. The measured negative values at 300 nm are characteristic for a ring A/B cis-junction[17] and were also found with the reference compound 5β-dihydro levonorgestrel. The axial position of the 3βH in **2** was proved by the presence of a big di-axial coupling constant (10.0 Hz) in its multiplet at 3.65 ppm.[5,6,18] Additionally, the $^1H$ and $^{13}C$ NMR data demonstrate that the 11-methylene and the 17α-ethinyl group remained unchanged in the microbial transformation.

For completion of the 5βH KDG metabolites for comparison, the dihydro compound **1** was reduced with potassium selectride in tetrahydrofuran[13] yielding the 5βH-3β-OH-tetrahydro metabolite **3** in addition to compound **2.** The structure of **3** was proved by MS, IR, and NMR. In contrast to **2,** the 3H proton of **3** appears at 4.11 ppm as a pentet with a coupling constant of 2.9 Hz. Thus, no di-axial coupling constant is present, and the position of the 3H must be equatorial (3αH). In addition to the coupling constant, the $^1H$ chemical shift of 3H in **2** and **3** is characteristic for a 3βH and a 3αH, respectively.[18] As far as we know, 5βH metabolites of KDG have not been described until now. The formation of these compounds as products of KDG biotransformation in man is conceivable, because 5βH metabolites have been found as urinary metabolites of levonorgestrel and norethisterone.[1]

### *5αH-KDG metabolites obtained by* Mycobacterium smegmatis

For the microbial preparation of 5αH-metabolites of KDG, we first tried the yeast *Rhodotorula glutinis,* which has been found to transform endogenous and also synthetic 4-en-3-oxo-steroids to metabolites of the 5αH series.[7,9] Although levonorgestrel was extensively converted to predominantly 5α-tetrahydro metabolites with time, KDG was only poorly transformed (<10%), even after 88 h fermentation. With *M. smegmatis,* a rapid and nearly complete conversion of KDG was found after 12 h fermentation. The 5α-dihydro metabolite **(4)** and both tetrahydro metabolites **(5, 6)** were formed, but **4** was produced in only small quantities. Larger amounts of the 3-oxo compound **4** were prepared by oxidation of **5** and **6** with pyridinium chlorochromate in $CH_2Cl_2$. The 5αH configuration in **4** was demonstrated by the positive values of CD at 300 nm,[17] as has been found with the reference compound 5α-dihydro levonorgestrel. Metabolite **5** was the main product of the KDG transformation with *M. smegmatis.* The $^1H$ NMR chemical shifts and coupling constants of

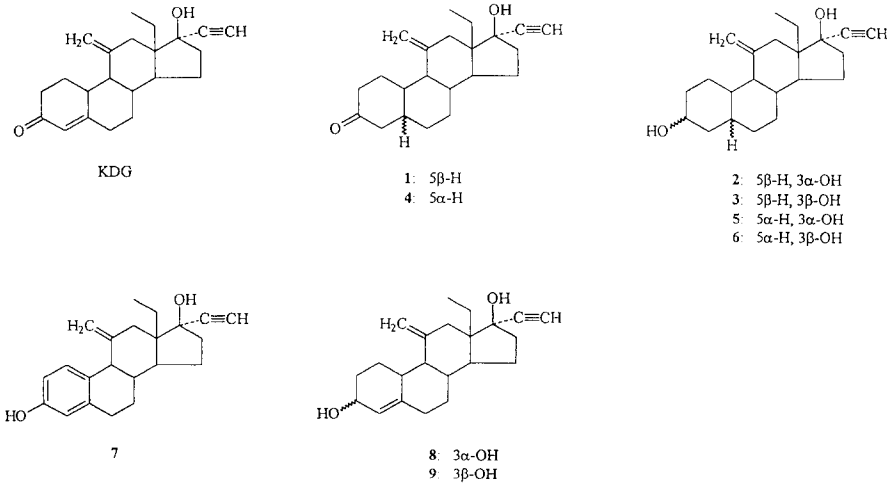


**Figure 1** Structures of KDG and metabolites **1–9.**

**Table 1** TLC, GLC, and HPLC data of KDG metabolites **1–9** as compared to KDG

| Metabolite | 1<br>5βH-<br>3-oxo | 2<br>5βH-3αOH | 3<br>5βH-3βOH | 4<br>5αH-3-oxo | 5<br>5αH-3αOH | 6<br>5αH-3βOH | 7<br>Arom.<br>A-ring | 8<br>Δ4-3αOH | 9<br>Δ4-3βOH | KDG |
|---|---|---|---|---|---|---|---|---|---|---|
| TLC[a] | | | | | | | | | | |
| $R_F$ in system | | | | | | | | | | |
| A | 0.79 | 0.48 | 0.62 | 0.81 | 0.64 | 0.54 | 0.79 | 0.57 | 0.59 | 0.68 |
| B | 0.71 | 0.44 | 0.54 | 0.75 | 0.54 | 0.49 | 0.65 | 0.49 | 0.53 | 0.66 |
| C | 0.72 | 0.35 | 0.50 | 0.77 | 0.52 | 0.43 | 0.63 | 0.43 | 0.50 | 0.66 |
| D | 0.75 | 0.25 | 0.48 | 0.76 | 0.45 | 0.34 | 0.28 | 0.35 | 0.39 | 0.69 |
| GLC[a] | | | | | | | | | | |
| $t_R$ (min) | 12.74 | 11.88 | 12.20 | 12.55 | 11.88 | 12.04 | 16.30 | 12.41 | 12.74 | 16.03 |
| HPLC[a] | | | | | | | | | | |
| $T_R$ (min) | 10.31 | 9.76 | 8.33 | 10.17 | 10.66 | 8.50 | 6.81 | 8.65 | 7.01 | 7.71 |

[a]For conditions see Experimental section.
TLC in solvent systems A, B, and C on silica gel, and in system D on alumina. Solvent systems: A: benzene/ether (1:1 v/v 2×); B: $CHC_3$/ethyl acetate (85:15 v/v 2×); C: $CHCl_3$/ether 9:1 v/v 2×); D: benzene/ethanol 98:2 v/v 2×).

**Table 2** $^1$H NMR chemical shifts of KDG metabolites **1–9** (in ppm relative to the internal standard TMS)

| Metabolite proton | 1<br>5βH-<br>3-oxo | 2<br>5βH-<br>3αOH | 3<br>5βH-<br>3βOH | 4<br>5αH-<br>3-oxo | 5<br>5αH-<br>3αOH | 6<br>5αH-<br>3βOH | 7<br>Arom.<br>A-ring | 8<br>Δ4-3αOH | 9<br>Δ4-3βOH |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.76 ddt | 1.33 m | 1.75 m | 1.14 m | 1.13 m | 0.75 m | 7.21 d | 1.00 m | 0.95 m |
| | 2.46 m | 2.27 dt | 1.97 m | 2.57 m | 2.06 dq | 2.28 m | | 1.70 m | 1.70 m |
| 2 | 2.16 ddd | 1.24 m | 1.47 m | 2.32 dt | 1.58 m | 1.27 m | 6.63 dd | 2.00 m | 1.38 m |
| | 2.27 dt | 1.66 m | 1.55 m | 2.39 m | 1.77 m | 1.99 m | | 2.24 dt | 1.99 m |
| 3 | — — | 3.65 sep | 4.11 p | — — | 4.12 p | 3.65 p | — — | 4.13 m | 4.19 m |
| 4 | 2.06 dd | 1.57 m | 1.38 m | 2.18 t | 1.35 m | 1.11 m | 6.57 d | 5.60 m | 5.47 s |
| | 2.61 dd | 1.62 m | 1.86 dt | 2.37 m | 1.73 m | 1.93 m | | | |
| 5 | 2.33 m | 1.89 m | 2.23 m | 1.48 m | 1.48 m | 1.15 m | — — | — — | — — |
| 6 | 1.53 m | 1.56 m | 1.48 m | 1.14 m | 0.97 m | 1.08 m | 2.68 m | 1.64 m | 1.99 m |
| | 1.67 ddt | 1.60 m | 1.58 m | 1.65 m | 1.51 m | 1.57 dt | 2.81 dt | 1.67 m | 2.22 dt |
| 7 | 1.21 dq | 1.21 m | 1.18 m | 1.02 m | 1.01 m | 0.95 m | 1.33 m | 1.41 m | 1.12 m |
| | 1.48 m | 1.38 m | 1.42 m | 1.68 dq | 1.64 dq | 1.64 m | 1.80 dq | 2.08 m | 2.30 m |
| 8 | 1.30 m | 1.86 m | 1.97 m | 1.31 dq | 1.22 dq | 1.22 dq | 1.62 dq | 1.36 m | 1.35 m |
| 9 | 1.93 t | 1.87 t | 1.78 t | 1.39 m | 1.36 m | 1.25 m | 3.04 d | 1.44 dt | 1.33 m |
| 10 | 2.08 m | 1.24 m | 1.19 m | 1.62 dq | 1.20 m | 1.09 m- | — — | 2.19 m | 2.23 m |
| 12 | 2.30 d | 2.27 d | 2.27 d | 2.29 d | 2.27 d | 2.26 d | 2.46 d | 2.28 d | 2.24 d |
| | 2.65 d | 2.59 d | 2.61 d | 2.64 d | 2.60 d | 2.60 d | 2.77 d | 2.63 d | 2.62 d |
| 14 | 1.86 m | 1.84 ddd | 1.82 m | 1.83 ddd | 1.80 ddd | 1.77 ddd | 2.02 ddd | 1.79 ddd | 1.77 ddd |
| 15 | 1.35 m | 1.33 m | 1.33 m | 1.37 m | 1.34 m | 1.34 m | 1.46 m | 1.33 m | 1.34 m |
| | 1.64 m | 1.63 m | 1.62 m | 1.66 m | 1.62 m | 1.63 m | 1.73 m | 1.62 m | 1.62 m |
| 16 | 2.11 ddd | 2.09 ddd | 2.09 ddd | 2.11 ddd | 2.09 ddd | 2.09 ddd | 2.16 ddd | 2.09 ddd | 2.10 ddd |
| | 2.37 ddd | 2.35 ddd | 2.35 ddd | 2.34 m | 2.35 ddd | 2.35 ddd | 2.42 ddd | 2.35 ddd | 2.34 ddd |
| 18 | 1.41 m | 1.39 m | 1.40 m | 1.41 m | 1.40 m | 1.40 m | 1.47 m | 1.40 m | 1.41 m |
| | 1.45 m | 1.43 m | 1.43 m | 1.47 m | 1.44 m | 1.44 m | 1.50 m | 1.43 m | 1.44 m |
| 18a | 1.06 t | 1.03 t | 1.04 s | 1.05 t | 1.03 t | 1.02 t | 1.06 t | 1.04 s | 1.03 s |
| 21 | 2.64 s | 2.65 s | 2.63 s | 2.63 s | 2.62 s | 2.61 s | 2.65 s | 2.63 s | 2.60 s |
| 11-$CH_2$ | 4.79 q | 4.73 q | 4.76 q | 4.75 q | 4.67 q | 4.68 q | 4.89 q | 4.78 q | 4.78 q |
| | 5.01 q | 4.95 q | 4.96 q | 4.99 q | 4.94 q | 4.95 q | 5.00 q | 5.00 q | 5.00 q |

3-H in **5** and **6** (4.12 ppm, p, $J$ = 2.8 Hz, and 3.65 ppm, p, $J$ = 9.9 Hz, respectively) are in conformity with an axial 3α-OH group in **5** and an equatorial 3β-OH-group in **6**.[5,6,18] MS, IR, $^1$H and $^{13}$C NMR data demonstrate again that in this microbial transformation, the 11-methylene group and the 17α-ethinyl side chain remain intact. 5αH-metabolites of KDG have been found as products of desogestrel and KDG biotransformation in vitro.[2–4]

The 5α-hydrogenase of *R. glutinis* obviously has a higher substrate specificity and was inhibited by the 11-methylene group of KDG. Inhibition of this enzyme has also been found by an additional 1- and 9-double bond and a 11β-hydroxy group in the steroid molecule.[5,6] On the other hand, the 5β-hydrogenase of *C. paraputrificum* demonstrated only a moderate substrate specificity. KDG, with its 11-methylene group and like other steroids with a 11β-hydroxy group or additional double bonds in the 1-, 6-, and 9-positions, was smoothly transformed to 5βH-metabo-

**Table 3** $^{13}C$ NMR chemical shifts of KDG metabolites **1–9 (in ppm relative to the internal standard TMS)**

| Metabolite carbon | 1<br>5βH-<br>3-oxo | 2<br>5βH-<br>3αOH | 3<br>5βH-<br>3βOH | 4<br>5αH-<br>3-oxo | 5<br>5αH-<br>3αOH | 6<br>5αH-<br>3βOH | 7<br>Arom.<br>A-ring | 8<br>Δ4-<br>3αOH | 9<br>Δ4-<br>3βOH |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 28.2 | 26.2 | 21.5 | 31.2 | 25.2 | 29.3 | 131.3 | 31.3 | 31.4 |
| 2 | 36.8 | 30.4 | 27.9 | 41.4 | 33.3 | 35.6 | 112.5 | 35.2 | 32.0 |
| 3 | 213.1 | 71.7 | 66.9 | 211.4 | 66.7 | 70.6 | 153.2 | 65.0 | 67.4 |
| 4 | 42.7 | 36.4 | 33.7 | 49.0 | 41.1 | 43.4 | 115.3 | 123.5 | 125.2 |
| 5 | 37.7 | 35.2 | 29.3 | 43.4 | 36.4 | 41.3 | 139.5 | 144.6 | 142.9 |
| 6 | 30.2 | 31.9 | 30.8 | 32.9 | 32.7 | 32.4 | 30.7 | 30.9 | 35.1 |
| 7 | 24.5 | 25.1 | 25.1 | 30.2 | 30.5 | 30.4 | 26.9 | 23.6 | 26.4 |
| 8 | 42.6 | 33.7 | 34.4 | 42.4 | 42.7 | 42.4 | 42.0 | 42.4 | 42.5 |
| 9 | 44.3 | 43.9 | 43.6 | 52.1 | 52.6 | 52.2 | 50.9 | 54.2 | 54.6 |
| 10 | 33.8 | 49.0 | 42.6 | 41.0 | 42.4 | 41.0 | 127.8 | 36.8 | 36.7 |
| 11 | 147.5 | 147.6 | 147.6 | 147.3 | 147.5 | 147.2 | 147.8 | 147.4 | 147.0 |
| 12 | 40.9 | 40.8 | 40.9 | 40.5 | 40.6 | 40.3 | 40.4 | 40.6 | 40.6 |
| 13 | 50.4 | 50.7 | 50.7 | 50.5 | 50.5 | 50.3 | 50.9 | 50.4 | 50.3 |
| 14 | 52.8 | 52.7 | 52.9 | 52.4 | 52.7 | 52.4 | 52.0 | 52.3 | 52.3 |
| 15 | 21.9 | 21.8 | 21.8 | 21.8 | 21.8 | 21.6 | 21.7 | 21.9 | 21.9 |
| 16 | 40.0 | 39.9 | 40.0 | 39.8 | 39.9 | 39.7 | 40.0 | 39.8 | 39.8 |
| 17 | 81.0 | 81.1 | 81.2 | 81.0 | 81.2 | 80.9 | 81.1 | 81.1 | 81.8 |
| 18 | 19.9 | 19.9 | 19.9 | 19.9 | 19.9 | 19.6 | 20.0 | 19.0 | 19.8 |
| 18a | 9.1 | 9.2 | 9.2 | 9.2 | 9.2 | 9.0 | 9.3 | 9.1 | 9.1 |
| 20 | 87.7 | 87.8 | 88.0 | 87.8 | 87.9 | 87.7 | 87.9 | 87.8 | 87.8 |
| 21 | 74.1 | 74.2 | 74.1 | 74.2 | 74.1 | 73.8 | 74.2 | 74.1 | 74.1 |
| 11 = $CH_2$ | 109.1 | 108.6 | 108.7 | 108.5 | 108.3 | 108.3 | 109.2 | 108.5 | 108.8 |

lites.[5–7] The 5α-hydrogenase of *M. smegmatis* was not inhibited by the 11-methylene group of KDG.

The inherent 1-dehydrogenase of this microorganism becomes evident by the concomitant aromatization of KDG to metabolite **7.** The aromatization of ring A can constitute, in some cases, the main reaction in the transformation of 19-norsteroids by this strain.[8]

### *Dehydrogenation of KDG with* Rhodococcus rhodochrous

For a more favorable preparation of metabolite **7,** we microbially converted KDG with *Rhodococcus rhodochrous,* which is known to have a potent steroid 1-dehydrogenase.[19] With this strain, a complete transformation of KDG was achieved within 24 h. The structure of **7,** consisting of an aromatic ring A, was proved by MS, UV, IR, and NMR. Formation of **7** in KDG biotransformation in vivo is conceivable; for example, by splitting off water from a metabolite hydroxylated at ring A. Analogous biotransformation reactions have been reported with other steroid drugs.[20,21] To our knowledge, metabolite **7** has not been described until now.

### *Preliminary studies on the chemical synthesis of KDG metabolites*

To complete the series of hydrogenated KDG metabolites for chromatographic comparison, compounds **8** and **9,** which have a 3-OH-4-en-structure, were prepared by chemical reduction of KDG with sodium borohydride and potassium selectride according to Wiebe et al.[13] The 3α-OH-and the 3β-OH-isomers were formed in a 1:1 ratio when prepared with potassium selectride in tetrahydrofuran; whereas, a 1:10 ratio was found when $NaBH_4$ in MeOH was used. Metabolites **8** and **9** are considered to be intermediates in the biotransformation of desogestrel to 3-ketodesogestrel, but no complete NMR assignments have previously been made.

The availability of microbially prepared KDG metabolites gave rise to some further studies of the chemical synthesis of KDG metabolites. Birch reduction with lithium in liquid ammonia resulted in a very rapid and complete 5α-hydrogenation of the 4-double bond of the KDG molecule. Simultaneously, however, extensive hydrogenation of the 17α-ethinyl group to 17α-vinyl also occurred. By decreasing the amount of lithium and the reaction time, the vinyl component was diminished. The 17α-ethinyl-5α-dihydro compound obtained with this procedure was identical to metabolite **4** prepared by microbial transformation.

Hübner et al.[15] used reduction with $NaBH_4$ in aqueous pyridine for the preparation of hydrogenated metabolites of 17α-cyanomethyl-19-nortestosterone. Analogous to his findings, KDG was also transformed to predominantly tetrahydro metabolites of the 5αH- and 5βH-series. The reaction mixture was rather complex, because the metabolites **2, 3, 5,** and **6** are simultaneously formed in addition to a small amount of a compound with a 4-en-3-OH structure. Separation of this mixture by repeated TLC is possible, but rather laborious. As already proposed by Hübner, it seems more favorable to oxidize the crude reaction product with pyridinium chlorochromate to a less complex mixture of dihydro metabolites and to separate the resulting 5αH- and 5βH-compounds. Subsequently, reduction to the 3-hydroxy metabolites can be carried out. There is no evidence that the 11-methylene or 17α-ethylene group is altered in this reaction.

To introduce a 1-double bond in $C_{19}$ and $C_{21}$ steroids, DDQ (2,3-dichloro-5,6-dicyano-p-benzoquinone) and p-chloranil (tetrachloro-p-benzoquinone) have frequently

been used. Introduction of a 1-double bond in 19-norsteroids should result in spontaneous aromatization. We did not succeed in transforming KDG to **7** by refluxing with DDQ in $CH_2Cl_2$, benzene, or dioxane. Recently, the transformation of levonorgestrel to 17α-ethinylestradiol with $CuBr_2$ in acetonitrile with a high yield has been described.[16] It was not possible to apply this reaction to KDG. With an excess of $CuBr_2$ and a longer reaction time, complete transformation of KDG to products with strong ultraviolet absorption and with high mass numbers (>400) was found, but **7** was not formed. Obviously, $CuBr_2$ reacts with the 11-methylene group. When using a submolar amount of $CuBr_2$, the conversion was incomplete, and only a small amount of **7** could be obtained.

The original aim of this study was the preparation of small amounts of KDG metabolites in conjunction with the establishment of a KDG radioimmunoassay. Because we found no data on the preparation of the metabolites in the literature, we tried microbial conversions and, later on, chemical syntheses to prepare the compounds and to evaluate physicochemical and chromatographic properties. The procedures described above were not optimized and point out possible ways for the preparation of KDG metabolites.

The data presented in Table 1 demonstrate that no single chromatographic method on its own is capable of separating all KDG metabolites. Some metabolite pairs withstand separation. By changing the chromatographic method, most of the separation problems can be solved. NMR spectroscopy data in Tables 2 and 3 show that this method can efficiently contribute to the elucidation of the ring A/B junction and the configuration of the 3-hydroxy group of hydrogenated steroid metabolites.

The study presented here demonstrates that KDG metabolites can be prepared by microbial transformation and also by chemical synthesis. Because of their high specificity, microbial conversions have considerable benefits. The preliminary studies on the chemical synthesis point to some problems associated with these methods. The availability of the 5βH-metabolites and of the KDG analog with an aromatic ring A will facilitate studies on KDG biotransformation in vivo and on the potential biologic effects of the metabolites described in this paper.

## Acknowledgments

We are most grateful to Dr. Günther Burckhardt and Mrs. Waltraud Scheiding, Institute of Molecular Biology of the Friedrich-Schiller University, for recording the CD and IR spectra, respectively, and to Mrs. Renate Koch, Analytical Department of the Hans-Knöll-Institute, for recording the mass spectra. We also thank Mr. Albin Henker and Mrs. Uta Tinney for their competent technical assistance. The generous financial support by the "Fonds der Chemischen Industrie" to the Institute of Pharmacy (Director: Prof. Dr. G. Reuter) is also gratefully acknowledged.

## References

1. Gerhards E, Hecker W, Hitze H, Nieuweboer B, Bellmann B (1971). Zum Stoffwechsel von Norethisteron (17α-Äthinyl-4-östren-17β-ol-3-on) und DL-sowie D-Norgestrel (18-Methyl-17α-äthinyl-4-östren-17β-ol-3-on) beim Menschen. *Acta Endocr Copenh* **68**:219–248.
2. Viinikka L (1979). Metabolism of a new synthetic progestagen, Org 2969, by human liver in vitro. *J Steroid Biochem* **10**:353–357.
3. Madden S, Back DJ, Orme M L'E (1990). Metabolism of the contraceptive steroid desogestrel by human liver in vitro. *J Steroid Biochem* **35**:281–288.
4. Wild MJ, Rudland PS, Back DJ (1993). Metabolism of the oral contraceptive steroids ethynylestradiol, norgestimate and 3-ketodesogestrel by a human cancer cell line (HEC-1A) and endometrial tissue in vitro. *J Steroid Biochem* **45**:407–420.
5. Kaufmann G, Schumann G, Hörhold C (1986). Influence of 1-double bond and 11β-hydroxy group on stereospecific microbial reductions of 4-en-3-oxo-steroids. *J Steroid Biochem* **25**:561–566.
6. Schubert K, Schumann G, Kaufmann G (1983). Influence of a 9-double bond on stereospecific microbial 4,5-reductions. *J Steroid Biochem* **18**:75–80.
7. Schubert K, Schlegel J, Hörhold C, Frischleder H (1970). Metaboliten von 4-Chlor-17α-methyl-17β-hydroxy-1,4-androstadien-3-on durch mikrobielle Hydrierung. *J Steroid Biochem* **1**:229–236.
8. Hobe G, Schön R, Hörhold C, Hübner M, Schade W, Schubert K (1982). Microbial transformation of 17α-cyanomethyl-17-hydroxy-4,9-estradien-3-one (STS 557) and 17α-cyanomethyl-19-nortestosterone by *Mycobacterium smegmatis*. *Steroids* **39**:399–410.
9. Schubert K, Schlegel J, Böhme KH, Hörhold C (1967). Mikrobielle Hydrierungs-und Dehydrierungs-Reaktionen bei Δ4-3-Ketosteroiden mit einer 6-Hydroxy-gruppe. *Biochim Biophys Acta* **144**:132–138.
10. Hobe G, Dänhardt S, Busch W (1979). Umwandlung von 19-Norgestagenen in Östrogene durch Mikroorganismen des Rinderpansens. *Arch exper Vet med* **33**:495–498.
11. Groh H, Komel R, Deppmeyer V, Schade W, Hörhold C (1980). Steroid transforming enzymes from microorganisms: The reverse reaction of the steroid 1-dehydrogenase from *Nocardia*. *J Steroid Biochem* **13**:1413–1415.
12. Corey EJ, Suggs JW (1975). Pyridinium chlorochromate, an efficient reagent for oxidation of primary and secondary alcohols to carbonyl compounds. *Tetrahedron Lett* **31**:2647–2650.
13. Wiebe JP, Deline C, Buckingham KD (1985). Synthesis of the allylic gonadal steroids, 3α-hydroxy-4-pregnen-20-one and 3α-hydroxy-4-androsten-17-one, and of 3α-hydroxy-5α-pregnan-20-one. *Steroids* **45**:39–51.
14. Junghans K (1976). Selektive elektrochemische Reduktionen von Δ8-Estratetraen-Derivaten in flüssigem Ammoniak: Stereoselektivität der Birch-Reduktion in Gegenwart von Anilin. *Chem Ber* **109**:395–402.
15. Hübner M, Füssel I, Ponsold K (1984). Synthese potentieller Metaboliten der STS 557 (Dienogest). Teil I: Metaboliten mit einem hydrierten A/B-Ringsystem. *Pharmazie* **39**:462–463.
16. Rao PN, Cessa JW, Kim HK (1994). Preparative chemical methods for aromatization of 19-nor-Δ4-3-oxo steroids. *Steroids* **59**:621–627.
17. Velluz L, Legrand M (1961). Zirkulardichroismus und Raumstruktur. Neue Ergebnisse im Steroidgebiet. *Angew Chem* **73**:601–611.
18. Bridgeman JE, Cherry PC, Clegg AS, Evans JM, Jones ERH, Kasal A, Kumar V, Meakins GD, Morisawa Y, Richards EE, Woodgate PD (1970). Microbiological hydroxylation of steroids. Part I. Proton magnetic resonance spectra of ketones, alcohols, and acetates in the androstane, pregnane, and estrane series. *J Chem Soc* (C): 250–257.
19. Hörhold C, Atrat P, Deppmeyer V, Garbert A, Flemming CH, Schubert K (1976). Reinigung einer 3-Oxo-steroid:[2,6-Dichlorphenolindophenol]-Δ1-oxidoreductase aus *Nocardia opaca* mit Hilfe der Affinitätschromatographie. *Zeitschr Allg Mikrobiol* **16**: 559–561.
20. Hobe G, Schön R, Wehrberger K, Ritter P, Schade W, Hillesheim HG (1989). Studies on the biotransformation of the progestagen Dienogest in the rabbit. *Exper Clin Endocrinol* **94**:203–210.
21. Raynaud JP, Cousty C, Salmon J (1974). Metabolic studies of R 2453, a highly potent progestin. *J Steroid Biochem* **5**:324.
22. Kaufmann G, Schumann G, Wollweber L, Hüller L, Atrat, P (1990). Steroid-dehydrogenases in nocardioform bacteria studied by electrophoresis and immuno blotting techniques. *J Basic Microbiol* **30**:415–423.