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Production of malodorous steroids from androsta-5,16-dienes and androsta-4,16-dienes by *Corynebacteria* and other human axillary bacteria

Richard A. Decréau [a,b], Charles M. Marson [a,*], Kelvin E. Smith [b], John M. Behan [c]

[a] *Christopher Ingold Laboratories, Department of Chemistry, University College London, 20 Gordon Street, London WC1H 0AJ, UK*
[b] *School of Biological Sciences, Queen Mary College, Mile End Road, London E1 4NS, UK*
[c] *Quest International, Ashford, Kent TN24 0LT, UK*

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Abstract

The biotransformations of a number of steroids, chiefly 5,6,16,17-tetradehydro-androstanes, are reported. The strains investigated were *Corynebacteria* sp. G38, G40, G41, B, *Brevis* sp. CW5 and *Micrococcus* sp. M-DH2. *Corynebacterium* sp. G41 proved remarkably efficient in effecting oxidative isomerisation of 5-ene-3-sterols into the corresponding 4-en-3-ones. The main biochemical reactions involved were oxidation at C-3; no reduction processes were observed. Conversions of 3β-sterols into the C-3 oxo-steroids were high, but were correspondingly low for the 3α-sterol epimers. Androsta-4,16-dien-3-one and 5β-androsta-16-en-3-one are crucial to the formation of malodour. The rate of formation of these compounds was measured over 72 h incubation periods using three substrates: androsta-5,16-dien-3β-ol, androsta-4,16-dien-3β-ol and androsta-5,16-dien-3-one. Induction studies of the transformation of the androsta-5,16-dien-3β-ol into the very odorous compound androsta-4,16-dien-3-one showed that cells incubated with a mixture of antibiotics displayed the same extent of biotransformation as normal cells if the concentration of antibiotic was low (1, 3, 5 and 7 μg/ml), although at concentrations higher than 10 μg/ml, biotransformation yields were reduced. Pre-incubation with a 3β-fluoro-steroid inhibited the formation of the odorous androsta-4,16-dien-3-one.
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Keywords: Steroids; Androstadienones; *Corynebacteria*

1. Introduction

An understanding of the correlation of odour with steroid structure has been long sought [1]. Axillary (under-arm) malodour is known to be attributable in part to steroidal compounds [2–4], especially 16-androstenes [5,6]. Since freshly produced apocrine sweat is odourless [7,8], and was thought to contain little or no 16-androstenones [9], it had been supposed that a non-odourless steroid in freshly produced apocrine sweat undergoes transformation into odorous 16-androstenes catalysed by species in the axillary microflora. Axillary odour has been particularly associated aerobic Coryneform sp. [10,11] and only aerobic Coryneforms have been shown to produce axillary odour in vitro [10,11].

Gower et al. [13] identified all four of the 16,17-dehydrosteroids **5–8** (Scheme 1, below 'odorous' datum line) as being present in substantial quantity in male sweat. The four steroids **5–8** have been shown in laboratory controlled olfactory tests to have a powerful, sweaty, axillary odour [2–4]. The Gower group considered that 5α-androsta-5,16-en-3β-ol (3β-androstadienol) **1** and androsta-4,16-dien-3-one (androstadienone) (**6**), detected in apocrine sweat, might be important sources of highly odorous steroids such as 5α-androst-16-en-3-one (**7**), androst-16-en-3α-ol (**5**) and its β-epimer **5′** that are found in male body odour [13,14]. However, the details or sequence of the biotransformations were not clearly identified.

Odour has been correlated with stereochemistry of oxygenated moieties, principally keto and hydroxyl groups, on the steroid nucleus [4]. For example, sterol **1** (Scheme 1) is not malodorous, and has an equatorial 3-hydroxy group, whereas its epimer **3** is slightly malodorous. The axial configuration of the 3-hydroxy group in androsta-5,16-dien-3α-ol (**3**) is essential to its malodorous properties; olfactory detection of such 16,17-dehydro C_{19}-steroids is possible at concentrations as low as five parts per billion [4]. In

* Corresponding author. Tel.: +44-20-7679-4712; fax: +44-20-7679-7463.
E-mail address: c.m.marson@ucl.ac.uk (C.M. Marson).

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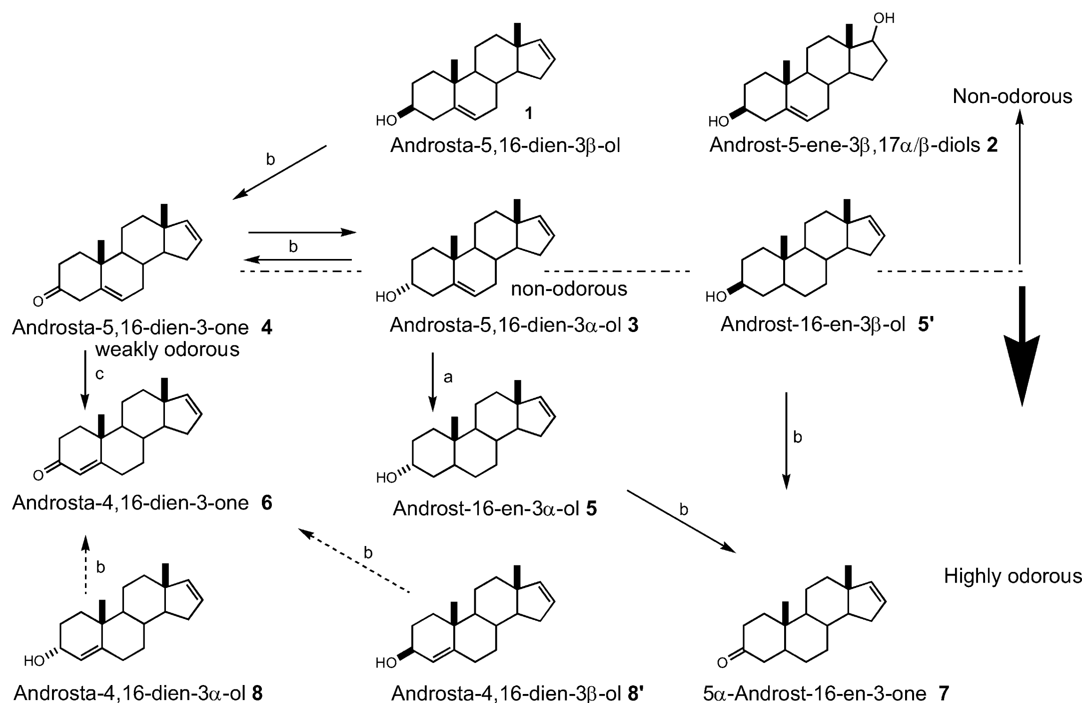


Scheme 1. Some proposed biotransformations effected by G41 Coryneform bacteria.

several other detailed studies, Ohloff's group and others have established that many 3α-sterols have intense odours, whereas the corresponding 3β-sterols usually have little or no detectable odour [4]. However, the steroidal precursors, and the relevance of their stereochemical configurations, have not been delineated.

Our findings provide some general rationales for correlating the stereochemistry of 16-androsten-3-ols with the production of malodour. We show here that of the two epimeric sterols **1** and **3**, the *non-odorous* β-hydroxyl isomer **1** leads predominantly to the highly malodorous androsta-4,16-dien-3-one (**6**). Additionally, we show that 5,6-dehydrosterols are central to malodour production, and importantly that their involvement is also consistent with the fact that Coryneform sp. F47 is known for its 5α-reductase activity. A consideration of likely biochemical pathways suggested to us that the non-odorous androsta-5,16-dien-3β-ol (**1**) (or a derivative) could be a central intermediate whose conversions are ultimately responsible for much of the malodour, especially by formation of steroids **4**–**8**. Indeed, Gower showed that androsta-5,16-dien-3β-ol (**1**) is frequently a substantial component in human axillae [13], although the biosynthetic precursors of sterol **1** have not yet been established.

2. Materials and methods

2.1. Bacteria

Bacteria were provided by Quest International (Ashford), including *Corynebacterium* spp. (G38, G40, G41, B), *Brevis* sp. CW5 and *Micrococcus* sp. DH2. These cells are human comensals and were isolated from axillae swabs. They were used in separate biotransformations or mixed cultures to mimic human axillary conditions [12,15].

2.2. Conditions of cultivation and biotransformation

2.2.1. Standard incubations

Bacterial cultures were grown aseptically for 16 h in tryptone soya broth (30 g/l) containing Tween 80 (0.1%, w/v) and yeast extract (0.1%, w/v) (Medium No. 1). Cells were harvested by centrifugation at 6000 rpm for 25 min in sealed 500 ml tubes, using a Europa 25 Centrifuge and then resuspension in a sterile semi-synthetic basal medium (potassium dihydrogen phosphate (1.6 g/l), ammonium hydrogen phosphate (5.0 g/l), sodium sulfate (0.38 g/l), yeast nitrogen base (3.35 g/l), yeast extract (10 g/l), Tween 80 (0.2 g/l), Triton X-100 (0.2 g/l) and magnesium chloride hexahydrate (0.5 g/l)) (Medium No. 2a) was conducted prior to addition of a stock solution of steroid (50 mg per ml of ethanol) was added to 100 ml of medium. In order to maximise the aerobic conditions required for vigorous growth of *Corynebacterium* cultures were placed in 250 ml baffled Erlenmeyer flasks and shaken at 220–250 rpm/min at 37 °C using a Lab-LINE (Jencons-PLS) shaker. Usually, 100 ml of culture was used. In all cases controls without bacteria were run in parallel with the test cultures.

2.2.2. Modified incubations

Some or all of the parameters described above for standard incubations were modified as follows:

1. Mixed culture experiments. Equal biomass of each bacterial strain (2 l) were mixed together prior to the addition of steroidal substrate.
2. Oxygen limitation experiments were performed as follows: anaerobic steroid transformation was performed by bubbling nitrogen through the broth medium. Semi-aerobic steroid transformation was performed in a plugged flask without shaking. Microaerobic steroid transformation was performed in a fully sealed flask with no exchange of air, and with shaking.
3. Effect of enriched medium. Cultures were supplemented with glucose at 10 mg/ml for a concentration in Medium No. 2b or Medium 1.
4. Effect of incubation times. Transformation times were usually from 16 to 72 h. However, one incubation of 504 h (21 days) was performed, as previously described [16–18] in order to examine any products of longstanding oxidation.

2.3. Extraction and purification of metabolites

After steroid transformation, cells and steroid-free controls were centrifuged at 6000 rpm for 25 min. The resulting pellets and supernatants were separated and separately processed as described below. Pellets were dried in air for 24 h then under vacuum for 6 h before being crushed into blocks and suspended in 100 ml of a mixture of diethyl ether, chloroform, ethanol, ethyl acetate and acetone (1:2:1:1:1, v/v) and stirred in a sealed flask for 16 h. After allowing to settle, the supernatant was decanted and reduced to half volume, then filtered through a C18-Bondapak Elut cartridge (VARIAN Sample Preparation products) and the resulting filtrate was evaporated at 30 °C and 15 mmHg to give a residue which was dried and weighed. The residue was redissolved in 5 ml of AR grade methanol. After sonication 2 μl of the solution was analysed by HPLC. For some extracts metabolites were isolated by preparative silica gel thin-layer chromatography on plates (20 cm × 20 cm and 0.25 mm thickness) cut to 10 cm × 10 cm. Steroids were eluted in chloroform–diethyl ether (1:1 v/v) or chloroform–ethanol (95:5, v/v) for non-polar metabolites and chloroform–methanol (v/v) for more polar compounds. A thin strip was cut from the TLC plate and stained with iodine to reveal the position of the metabolites. The metabolites on the remaining part of the plate were excised and extracted by stirring the silica with diethyl ether or methanol (2 ml × 10 ml) for 20 min.

The supernatant from the transformation incubations was extracted consecutively with diethyl ether (3 ml × 60 ml), ethyl acetate (2 ml × 50 ml) and chloroform (2 ml × 50 ml). The combined extracts were passed through a Set-Pak Bondelut cartridge. The solvent was evaporated and the extracts stored in virgin vials. The remaining solvent was allowed to evaporate to dryness in air.

2.4. HPLC and HPLC-MS of steroids and their metabolites

A Phenomenex Luna 5 μm, C18 reverse-phase HPLC column (250 mm × 4.60 mm) was coupled to a Millipore-Waters 600E System Controller. Eluate was passed through a Millipore-Waters 486 Tunable absorbance detector and relative amounts of individual metabolites were determined by a Hewlett-Packard HP 3396A Integrator printer. The wavelength of detection was set at 210–215 nm depending on the metabolite. The composition of the HPLC mobile phase was aqueous methanol, the percentage of which was varied depending of the polarity of the metabolites: either 1% (cholesterol and derivatives), 5% (non-polar androstene derivatives **1**–**8**), 20% (pregnenolone and derivatives) or 35% H_2O (polar androstene derivatives). The flow rate was 0.8 ml/min. For HPLC–MS, the above HPLC system was coupled to a Micromass LC QUATTRO Mass spectrometer.

2.5. Calibration curves for steroids and determination of yields of metabolites

Standard solutions of steroids considered to be potentially important as metabolites were prepared at several concentrations. For enone **6**: 1.0×10^{-4}, 3.0×10^{-4}, 5.0×10^{-4}, 7.0×10^{-4} and 1.0×10^{-3} M (in each case, a solution of 2 μl was injected). For steroids **5**, **5′**, **7** and **1**: 1.0×10^{-3}, 3.0×10^{-3}, 5.0×10^{-3}, 7.0×10^{-3} and 1.0×10^{-2} M (in each case, a solution of 2 μl was injected). Calibration curves were used to determine the molar quantities of pure steroid metabolites in biotransformed mixtures and hence the conversions.

2.6. Characterization of metabolites

Once an HPLC profile of the crude metabolite mixture had been obtained, an aliquot of that mixture was mixed with an approximately equal amount of an authentic steroid and the solution again analysed by HPLC. Where a single peak was obtained, the metabolite mixture was then submitted to HPLC–MS to determine the structure. Comparison by TLC with an authentic sample was also made, in order to confirm the HPLC–MS identification. Selected metabolites from mixtures analysed by HPLC were isolated and structures identified by ^{1}H and ^{13}C spectrometry (at 300 and 75 MHz, respectively). Spectra were obtained in deuteriated chloroform using a Bruker DRX 300 spectrometer, or for small quantities of metabolites NMR spectrometry was performed using a Bruker Avance 500.

2.7. Syntheses of steroid standards and an enzyme inhibitor

Steroids **1**–**8** were synthesised using literature procedures suitably modified to improve yields. Those compounds

Table 1
TLC mobility and HPLC retention times (using different mobile phases) of the steroidal substrates and metabolites involved in this study

Steroid	HPLC R_t (min) mobile phase composition (% H_2O in methanol)		
	5	20	35
1	10.1	–	–
3	9.7	–	–
4		–	–
5	10.6	–	–
5′	11.4	–	–
6	8.6	–	–
7	10.7	–	–
8	9.5	–	–
8′	9.7	–	–
2	4.2		25
DHA 11	4.4	8.4	27.7
Tes 9	4.5	7.7	23.4
AD 10	4.0	6.8	–

and their synthetic intermediates were characterised by ^{1}H, ^{13}C NMR and mass spectra, and melting point determination. NMR and mass spectral data were as reported in the literature [19–23]. The purity of authentic samples was also confirmed by HPLC and TLC (Table 1). 3β-Fluoro-androsta-5,16-diene was prepared and tested as an inhibitor of steroid transformation.

2.8. Enzyme inhibition experiments

Equal portions (60 ml) of fresh *Corynebacterium* sp. G41 cells in Medium No. 2a were added to three flasks: (a) control no. 1, (b) control no. 2, (c) incubation with 3β-fluoro-androsta-5,16-diene, for 1 h (procedure a) or for 9 h (procedure b) (19 mg dissolved in 0.4 ml of ethanol–ethyl acetate 1:2 (v/v) (see Section 2.2.1). The cells were harvested, centrifuged and resuspended in broth Medium No. 1 for 12 h. 3β-Fluoro-androsta-5,16-diene was then added (8 mg dissolved in 0.2 ml of ethanol–ethyl acetate 1:2, v/v). The molar ratio androsta-5,16-dien-3β-ol (**1**): 3β-fluoro-androsta-5,16-diene was 1:2. A fourth flask, using the same conditions as described above (27 mg of sterol **1** per 0.6 ml of ethanol–ethyl acetate 1:2 (v/v) per 60 ml of Medium No. 2) was used as a control experiment. Experimental conditions and work-up of metabolites are described in Section 2.3. The conversion of sterol **1** into enone **6** was determined using the calibration curves from HPLC measurements (method a) as described in Sections 2.4 and 2.5.

2.9. Time-course experiments

Biotransformations of steroids **1**, **4** and **8′** into **6** were conducted using 200 mg of steroid in 500 ml of Medium No. 2. An aliquot (10 ml) was taken at intervals of 16, 20, 30, 40, 50 and 72 h. These aliquots were processed as described in Section 2.3, and the concentration of **6** was determined as in Section 2.4-5. All biotransformations were repeated.

For the biotransformation of **4** into **6** a control experiment was performed at 37 °C in Medium No. 2 in the absence of cells. This was necessary since **4** was found to isomerise readily to **6** upon standing.

2.10. Induction experiments

Freshly grown pelleted cells were resuspended in Medium No. 2 (60 ml) and divided in 4 parts in baffled conical flasks (15 ml in each). In the first two flasks (set A), the inducer (sterol **1**, 5 mg) was incubated for 16 h, whereas no steroid was added to the two other flasks (set B). Cells of both sets A and B were harvested and washed thoroughly three times with a 10 mM phosphate buffer, pH 7.4. The cells were resuspended in fresh medium containing a mixture of the antibiotics streptomycin, erythromycin and rifampicin, each at concentrations of 1, 3, 5, 7, 10 and 20 μg/ml, respectively, and shaken at 37 °C for 12 h. After aseptically harvesting cells by centrifugation, pellets were washed with buffer, then resuspended in fresh Medium No. 2 and incubated with substrate (sterol **1**, 5 mg/0.25 ml ethanol per 25 ml Medium No. 2).s

2.11. Chemicals

Dehydroepiandrosterone, epiandrosterone, testosterone were purchased from Sigma (Poole, Dorset, UK), and androstenedione from Aldrich (Poole, Dorset, UK). Tryptone soya broth was purchased from Oxoid (Basingstoke, Hampshire, UK), and yeast extract and yeast nitrogen base from Difco (Detroit, Michigan, USA); Tween 80, Triton X-100, potassium dihydrogen phosphate, disodium hydrogen phosphate monohydrate and sodium dihydrogen phosphate were obtained from BDH (Poole, Dorset, UK), and ammonium dihydrogen phosphate and magnesium chloride hexahydrate from Sigma. Glassware was washed with 1:1 (v/v) water: concentrated hydrochloric acid, thoroughly rinsed with water, and then washed consecutively with ethyl acetate, chloroform and acetone. Virgin vials were used to store the biotransformation extracts.

3. Results

3.1. Synthesis of steroid substrates

3.1.1. Synthesis of 3α-sterols and 3β-sterols

For the syntheses of the 3α- and 3β-sterols, the literature methods [24,25] were modified as follows: To a solution of dehydroepiandrosterone (12.0 g) in 96% ethanol (120 ml) was added hydrazine hydrate (9.6 ml) and triethylamine (48 ml). The mixture was stirred and heated at reflux for 2 h. After cooling, water (700 ml) was added. Filtration, and washing with water (5 ml × 100 ml) afforded 3β-hydroxy-androst-5-en-17-one hydrazone (11.9 g, 95%) as prisms, mp

208–213 °C, lit. [24] mp 214–217 °C. 3β-Hydroxy-androst-5-en-17-one hydrazone (2.0 g) was dissolved in dry benzene (100 ml), triethylamine (21 ml) added, followed over 40 min by a solution of iodine (3.0 g) in benzene (32 ml), added dropwise with vigorous stirring at 20 °C. When the addition was complete, the solution was washed successively with 5% aqueous solutions (1 l) of 0.5 M HCl, sodium thiosulfate and sodium hydrogen carbonate, and finally with water. The solvent was removed under reduced pressure, and the residue was recrystallised twice from methanol, and dried to give 17β-iodo-androsta-5,16-dien-3β-ol (2.2 g, 85%), as needles, mp 174–176 °C, lit. [24] mp 173–174 °C. This sterol (1.0 g) was dissolved in dry ethanol (50 ml). The solution was heated at reflux and chips of sodium (6.0 g in total) were added in portions over 90 min. Soon after the last addition the mixture solidified. Ethanol (10 ml) was then added, the heating removed, and the mixture allowed to cool with constant stirring. Water (100 ml) was then added and the ethanol was removed under reduced pressure. Extraction of the steroid with methyl isobutyl ketone (2 ml × 150 ml), washing with water (2 ml × 150 ml) and evaporation under reduced pressure gave an oil that was recrystallised twice from aqueous methanol to give androsta-5,16-dien-3β-ol (**1**) (0.565 g, 83%) as prisms, mp 138–139 °C, lit. [26] mp 133–137 °C, lit. [24] mp 137–138 °C, lit. [2,3] mp 138–139 °C.

The above method was adapted for the synthesis of 5α-androst-16-en-3β-ol (**5′**) by using 3β-hydroxy-5α-androstan-17-one which was then converted successively into 3β-hydroxy-5α-androstan-17-one hydrazone (94%), mp 184–189 °C, lit. [27] 183–187 °C, 17α-iodo-5β-androst-16-en-3β-ol (58%), mp 150–152 °C, lit. [25] mp 147.5–149 °C and 5α-androsta-16-en-3β-ol **5′** (79%), mp 127–130 °C, lit. [25] mp 125–126, lit. [6,28] mp 125–127 °C.

Ohloff's procedures were used for the syntheses of 3α-sterols by reduction of 3-oxo steroids (**4**, **7** and **6**) with LS-Selectride at −55 °C under an atmosphere of nitrogen to give, respectively: androsta-5,16-dien-3α-ol **3** (50%), mp 137–138 °C, lit. [4] mp 138 °C, 5α-androst-16-en-3α-ol (**5**) (40%), mp 143–144 °C, lit. [6,29] mp 143.5–144 °C, lit. [30] mp 145 °C,], androsta-4,16-dien-3α-ol (**8**) (24%, mp 80–82 °C, lit. [4] mp 77–79 °C. Androsta-4,16-dien-3β-ol (**8′**) (40%), mp 114–117 °C, lit. [4,31] mp 116–118 °C) was similarly prepared. The ^{1}H NMR, ^{13}C NMR and mass spectra of these sterols were as reported in the literature [4,30,31].

Androst-5-ene-3β,17β-diol (**2**) (mp 174–176 °C, lit. [32] mp 175–176 °C) was prepared by reduction of dehydroepiandrosterone with $LiAlH_4$ in dry diethyl ether. ^{1}H NMR, ^{13}C NMR and mass spectra were as reported in the literature [21].

3.1.2. Synthesis of 3-oxo steroids

Androsta-5,16-dien-3-one (**4**) was either prepared [4] using Jones' reagent, or using pyridinium chlorochromate [33]. Androsta-5,16-dien-3β-ol (**1**) (0.5 g) was stirred in dichloromethane (10 ml) with pyridinium chlorochromate (on alumina) (4.85 g) for 2 h at 20 °C. The mixture was then filtered and washed with diethyl ether (2 ml × 20 ml). The solvent was evaporated and the oily mixture recrystallised from methanol to give androsta-5,16-dien-3-one (**4**) (0.238 g, 48%), mp 122–126 °C).

The method of Marx [24] was used to prepare androsta-4,16-dien-3-one (**6**) and 5α-androst-16-en-3-one (**7**) according to the following modification. A solution of androsta-5,16-dien-3β-ol (**1**) (0.5 g) in a dry mixture (15 ml) of 2:1 (v/v) toluene–cyclohexanol was heated in a Dean-Stark apparatus until 2 ml of distillate had been obtained. To the remaining solution was added aluminium isopropoxide (0.283 g) in solution in dry toluene (2.5 ml). The mixture was then heated for 30 min. After allowing to cool, the mixture was poured into water (50 ml), and the toluene was removed under reduced pressure. Steroids were extracted with methyl isobutyl ketone (2 ml × 40 ml), the extracts combined, and then washed successively with 10% hydrochloric acid (2 ml × 80 ml), then with water (3 ml × 80 ml). Evaporation under reduced pressure gave an oil from which crystals deposited on standing. The viscous yellow liquid was decanted and the remaining prisms recrystallised twice from 1:3 (v/v) acetone–methanol and lastly from pure acetone to give androsta-4,16-dien-3-one (**6**) (0.347 g, 70%) as prisms, mp 131–133 °C, lit. [24] mp 131–133 °C, lit. [4] mp 130–132 °C, lit. [6] mp 131.5–133.5 °C. ^{1}H NMR, ^{13}C NMR and mass spectra were as reported in the literature [4,34]. The above procedure was also used to prepare 5α-androst-16-en-3-one (**7**) from 5α-androst-16-en-3β-ol (**5′**) in 70% yield, mp 138–141 °C, lit. [25] mp 139–140 °C, lit. [6,28] mp 140–141 °C. ^{1}H NMR, ^{13}C NMR and mass spectra were also as described in [25,35,36].

3.1.3. Synthesis of the enzyme inhibitor 3β-fluoro-androsta-5,16-diene

To a solution in ice of **1** (0.340 g) and 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) (0.560 ml) in toluene (46 ml), *n*-perfluorobutanesulfonyl fluoride (0.335 ml) was introduced with constant stirring. After 1 h, the ice having melted, the mixture was evaporated under reduced pressure and the residue subjected to chromatography on silica (70 g) using hexane as eluent. An olefinic product eluted first, followed by 3α-fluoro-androsta-5,16-diene, and finally 3β-fluoro-androsta-5,16-diene (0.208 g, 61%) as needles, mp 83–84 °C. About 500 ml of hexane was required.

3.2. Determination of conversions

To determine the yield of conversion of substrates into metabolites, methods a (HPLC and calibration curves) and b (chromatographic separation and weighing) were compared for the transformation of three substrates (Table 2). The HPLC method was found to be reliable, and was much more straightforward than methods involving chromatographic separation.

Table 2
Comparison of the methods (a and b) checked for the determination of the yields of metabolites

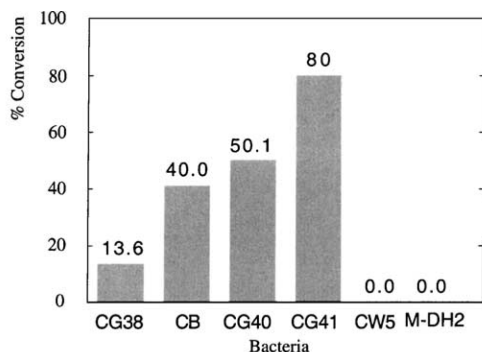
Method	Metabolite (%)		
	6		**7**
	From **1**	From **3**	From **5′**
a	80	8.5	27
b	75	10	25.2

3.3. Comparison of cell biotransformation activity

Cells were compared for their ability to convert **1** into **6**. Under identical conditions, most *Corynebacterium* species produced **1** efficiently, especially *Corynebacterium* sp. G41 (80%), sp. G40 (50%), sp. B (41%) and sp. G38 (13%). In contrast, neither *Brevis* sp. CW5 nor the *Micrococcus* sp. M-DH2 transformed **1** into **6** (Fig. 1). As a result of the very efficient conversion of **1** into **6** using *Corynebacterium* sp. G41, this species was used in all transformations involving steroidal substrates (Sections 3.5–3.8).

3.4. Modification of biotransformation conditions

Media: when broth Medium No. 1 (rich in nutrients), was used instead of Medium No. 2a for biotransformation, the conversion of **1** into **6** was about 92%; no other transformation product was found. Steroid **5′** was converted into **7** in 40% (Table 3). When a medium enriched in glucose was used (Medium No. 2b, 10 mg/ml), the conversion of **1** into **6** was 9%, and the conversion of **5′** into **7** was about 3%. *Oxygen*: limiting the supply of oxygen decreased the conversion of **1** into **6** (see Table 4). Incubation time: one experiment was run for 504 h (21 days). Even with this prolonged reaction time, no other products were detected, and there was no evidence of a reduction process.



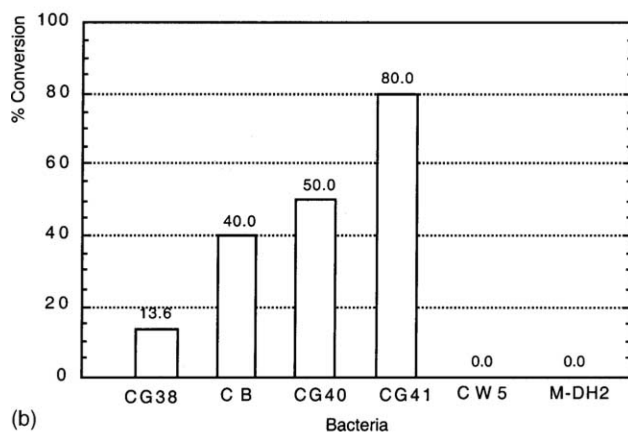


Fig. 1. Percentage conversion of androsta-5,16-diene-3β-ol **1** into androsta-4,16-diene-3-one **6** with different bacterial strains isolated from human axillae.

Table 3
Percentage conversions of metabolites depend upon the nature of the medium

Metabolites	Media		
	No. 1	No. 2	No. 2 + Glc
6	92	80	9
7	40	27	3

Medium No. 1: tryptone Soya Broth; Medium No. 2a: semi-synthetic basal medium; Medium No. 2b: 2a enriched in glucose at the concentration 1 mg/ml.

3.5. Time-course studies

The overall transformation time-course profile of the curve (Fig. 2a) shows that the conversion rate of **1** into **6** is biphasic. There was an induction period occurs lasting about 30 h and resulting in a conversion of only 5% of **1** into **6**. Biotransformation then proceeded rapidly from 40 to 72 h, in respective conversions of 20 and 80% (Fig. 2a).

The transformation of **4** into **6** occurs slowly on standing at 20 °C, more rapidly at 37 °C, being complete in approx. 30 h. This conversion also occurs throughout biotransformation runs and even in the period during which steroids are extracted from the pellet.

The conversion rate of **8′** into **6** (Fig. 2b). A lag period lasted for 50 h and gave a conversion of only 4%. Biotransformation proceeded slowly thereafter from 72 h (18%) to 168 h (94%).

3.6. Enzyme inhibition

Inhibition of conversion of **1** into **6** was shown by preincubation of 3β-fluoro-androsta-5,16-diene for 1 and 9 h, prior to the introduction of **1** into the transformation medium. The respective conversions were 37% and 10%. A control experiment with **1** alone gave **6** in 74% yield (see Sections 3.3 and 3.5). Thus, conversion was inhibited by

Table 4
Conversion of **1** into **6** requires an anaerobic medium

	Aerobic	Semi-aerobic No. 1	Semi-aerobic No. 1	Aerobic
Yield of **6** (%)	80	6	10	4

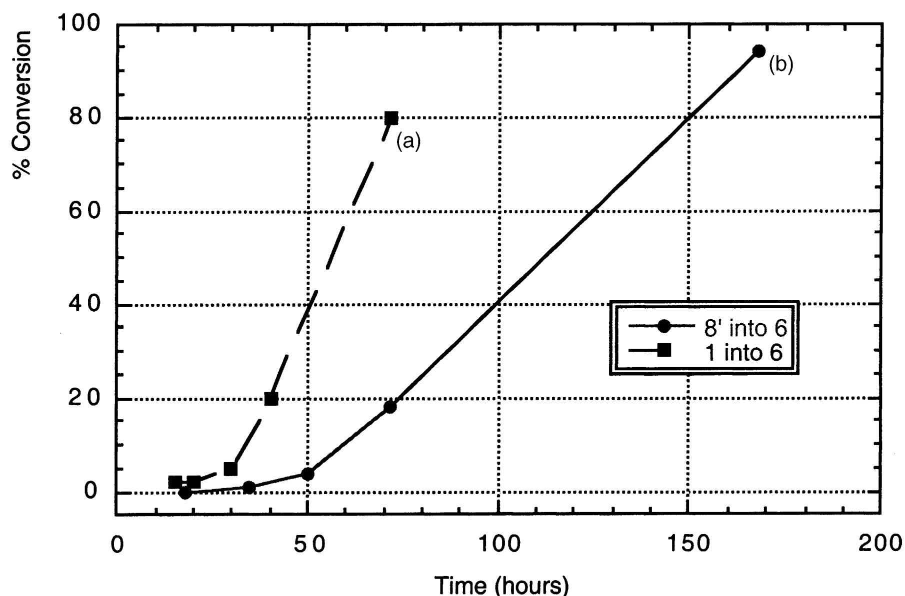


Fig. 2. Time courses. Conversions of: (a) **1** into **6** and (b) **8′** into **6**.

50% after 1 h and 86% after 9 h. 3β-Fluoro-androsta-5,16-diene was not cytotoxic to *Corynebacterium* sp. G41 because 89% of the control biomass was recovered after 9 h preincubation with 3β-fluoro-androsta-5,16-diene followed by 12 h incubation in Medium No. 1.

3.7. Biotransformation of sterols: stereochemical features

The production of the odorous 3-oxo-steroids **6** and **7** from six steroids were examined over a 72 h incubation time using three 3β-sterol substrates, compounds (**1**, **5′** and **8′**), and three 3α-sterol substrates (compounds **3**, **5** and **8**). For the androst-5,16-dien-3-ols, conversion was 10% for compound **3**, which possesses an axial hydroxy group, whereas it was 80% for compound **1** (equatorial hydroxy group) (C **1**:**3** = 8). Similar observations were made with the 5α-androst-16-en-3-ols: the β-epimer **5′** underwent conversion into **7** in 27%, whereas the α-epimer **5** was converted into **7** in only 3% (C **5′**:**5** = 8). For the conversions of androst-4,16-dien-3-ols into **6**, the α-epimer **8** gave only a trace amount but the β-epimer afforded 18% of **6** (Fig. 3). Androst-5-en-3β,17β-diol (**2**) underwent conversion into testosterone in 6% after 24 h.

3.8. Transformation of oxo-steroids

Transformations of 3-oxo and 17-oxo steroids were examined. Androsta-5,16-dien-3-one (**4**) underwent conversion into **6** in 95%. Conversely, no steroidal product was detected from **6** (90% recovery). The keto group of dehydroepiandrosterone was not reduced, whereas the hydroxy group at C-3 was oxidised, thereby affording androst-4-ene-3,17-dione in 20%.

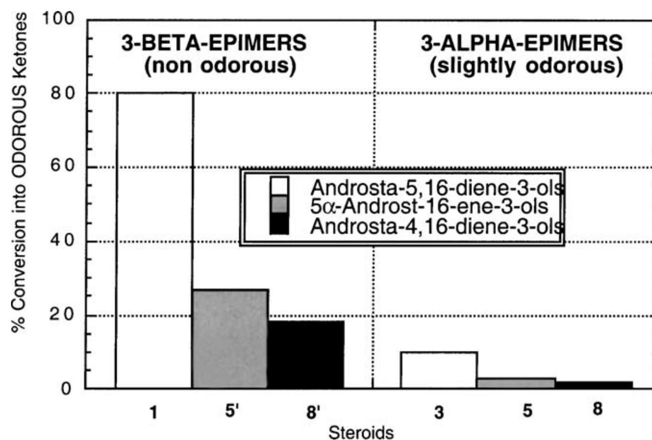


Fig. 3. Comparison of percentage conversion of 3α-sterols (**3**, **5**, **8**) vs. 3β-sterols (**1**, **5′**, **8′**) with *Corynebacterium* G41.

4. Discussion

Of the cells studied here, those in the genus *Corynebacteria* were found to be the most efficient in transforming 5α-androsta-5,16-dien-3β-ol (**1**) into the malodorous androsta-4,16-dien-3-one (**6**). Leyden et al. showed that there was indeed an association between axillary skin levels of aerobic Coryneform bacteria and under-arm odour intensity [12]. This is in accord with Rennie et al. who found that only the aerobic Coryneforms produced axillary odour [10]. We found that *Micrococcus* sp. M-DH2 did not metabolize sterol **1**. However, Rennie and co-workers reported that *Micrococcae* reduced 5,6-dehydro steroids to a mixture of the corresponding 5α- and 5β-reduced products [10].

Using modified experimental conditions, 16 h of incubation time was often sufficient to afford an appreciable yield

of a steroidal metabolite, e.g. as for the transformation of **2** into testosterone. However, most incubations were for periods of over 72 h (3 days), and in some cases an extended period (504 h) was required in order to obtain substantial yields of steroidal metabolite, as previously described Nixon [16]. A rich growth medium, e.g. Medium No. 1, gave 95% of conversion of **1** into **6** and 40% conversion of **5′** into **7**. In those experiments cells were still growing as shown by the increase in biomass of 44% compared to Medium No. 2a when was used. However, conversions using a medium enriched in glucose (1 g/l, Medium No. 2b) were about one tenth of those using Medium No. 2a.

Limiting the amount of oxygen led to a decrease in conversion of **1** into **6**; the lower the oxygen, the lower the yield. This demonstrates the mandatory aerobic nature of *Corynebacteria* G41. These data are in accord with Rennie's conclusions that only aerobic Coryneforms produced axillary odour [10], but contrast with those of Gower who reported complex transformations by Coryneforms F1 and F46 of a variety of steroid sulfates [37]. We found that *Corynebacterium* G41 afforded very few reactions, in essence only oxidation. Although we have not investigated the enzymology of these transformations, precedent would suggest the involvement of steroidal oxidoreductases.

In regard to enzyme inhibition, 3β-fluoro-androsta-5,16-diene was found to be non-cytotoxic towards *Corynebacterium* sp. G41, but efficiently inhibited the transformation of **1** into **6** when two mole-equivalents were incubated per mole-equivalent of **1**, and with pre-incubation times of 1 and 9 h. Those conditions led, respectively, to 50 and 86% inhibition of the conversion of **1** into **6**. Certain fluoro compounds are known to be efficient enzyme inhibitors [38–40].

Sterols **1**, **5′** and **8′** have an equatorial 3-hydroxy group and little odour, in contrast to the slightly odorous axial C-3-epimers **3**, **5** and **8**. This study demonstrates that the configuration at position-3 is crucial to the production of malodour; the slightly odorous 3α-sterols **3** and **5** gave only very low yields of odorous 3-oxo-steroids **6** and **7**. On the other hand, the non-odorous 3β-sterols **1** and **5′** were rapidly converted into the highly odorous steroids **6** and **7**. Evidently, 3β-sterol dehydrogenase activity was much greater than 3α-sterol dehydrogenase activity. After incubation for 72 h, sterol **8′** was converted into ketone **6** in 18%, whereas the 3α-ol epimer **8** afforded only a trace amount of ketone **6**. Our results are in keeping with Nixon's observations that 3α(β)-oxidoreduction occurs with aerobic *Corynebacteria*, except that we demonstrated oxidation but found no reduction. The formation of malodorous 3-oxo-steroids **6** and **7** from the non-odorous sterols **1**, **5′**, and **8′** is central to the production of under-arm odour. This is consistent with the observation that pure apocrine secretions are odourless until incubated with certain microfloral bacteria [7,8]. Furthermore, our results corroborate observations that 3-oxo-steroids **6** and **7** can be isolated from axillary sweat [13,35] whereas sterols **1**, **5**, and **5′** were detected in only very small amounts [13,14,35,41]. The 3-oxo-steroids **6** and **7** both display very low human olfactory thresholds, especially 5α-androst-16-en-3-one (**7**) which was found to have a powerful and urinous odour [5,23,42], and a human olfactory threshold of only 0.2 ppb [42].

Contrary to Nixon and Mallet's observations [16–18,43,44], neither **2** nor testosterone (**Tes 9**) were found to undergo oxidation at C-17 by *Corynebacterium* sp. G41, since no trace of androst-4-ene-3,17-dione was detected. Those authors demonstrated the formation of 5α-dihydrotestosterone and 5β-dihydrotestosterone. However, during the biotransformation of most steroidal substrates we observed no significant 5α- or 5β-reductase activity with *Corynebacterium* sp. G41, apart from the formation of **5** (1%) during the biotransformation of **3**. No reduction at C-17 was detected with either dehydroepiandrosterone or androst-4-ene-3,17-dione. We detected no Δ^1-steroid when using *Corynebacterium* sp. G41, although Charney found Δ^1-steroids when using *Coryne simplex* [45]. Although Gower originally linked malodour to the metabolism of testosterone, he later confirmed that Coryneform bacteria did not produce non-polar 16-androstenes from testosterone [18,46]. Our results also indicate testosterone is not a biosynthetic precursor of androsta-4,16-dien-3-one (**6**), as formed in the presence of *Corynebacterium* sp. G41. The use of a single Coryneform in the present work could be expected to give simpler and more clear-cut results than mixed cultures of Coryneform bacteria which have been shown to afford steroidal products derived by both oxidation and 4,5-reduction [45].

The formation of 3-oxo-steroids **6** and **7** is central to the production of malodour. The efficient conversion of the non-conjugated ketone **4** into the conjugated ketone **6** indicates that a 4,5-isomerase is present and active, and the yields are also consistent with a pathway that probably proceeds via **1** to **4** to **6**, rather by 4,5-isomerase action followed by oxidation. The narrow scope of transformation by *Corynebacterium* sp. G41 is delineated by the failure to detect any steroidal products derived from ketone **6** which was recovered in 90% yield even after prolonged incubation beyond 72 h. Evidently, enone **6** is a major end-product. Dienone **6** was not transformed into ketone **7**, consistent with the absence of 5α-reductase activity in *Corynebacterium* sp. G41. Nevertheless, ketone **7** has been detected in human axillary collections [42,47–50]. Gower considered the biotransformation of **1** into **3** to be a direct process. However, little evidence was provided, and we consider the alternative route: steroid **1** to **4** to **3** to be more likely.

Our observations can be understood in terms of the pathways outlined in Scheme 1. Biotransformation of each of the androstadienes **1**, **3** and **4** into dienone **6** (respective yields of 80, 10 and 95% after 12 h incubation) is consistent with our hypothesis that steroids of the androsta-5,16-diene series, and not those of the androsta-4,16-diene derivatives, are the major source of androsta-4,16-dien-3-one (**6**). Consequently, steroids of the androsta-5,16-diene series are likely to be early precursors of axillary malodour. The comparison

here of the 3α- and 3β-sterols under biotransformation with *Corynebacterium* sp. G41 shows that the β-epimer is much *more* readily transformed into enone **6** than is the α-epimer (80% versus 10%). The same also applies to the biotransformation into **7** (over 72 h) of the epimers **5′** (50%) and **5** (5%), the much *less* odorous 3β-epimer being transformed much more efficiently.

Rennie accounted for the formation of ketone **7** found in under-arm odour by assuming that it was derived from enone **6** by the action of 5α-reductase. However, we have shown here that ketone **7** is formed from **5′**, at least in vitro, and we conclude that this is an important pathway in the production of steroidal malodour in human axillae. The simplicity of products derived from biotransformations using *Corynebacterium* sp. G41 indicate that other axillary bacteria produce highly odorous steroids other than **6**, for example **5**, **7** and **8**, as detected by Gower et al. [13], and which require a reductase system, in accordance with Scheme 1. The biological origin of sterol **1** in axillae is not presently known, although generation of the 16,17-double bond from 17-substituted precursors requires a $C_{17,20}$-lyase. However, conclusions regarding the C_{19}-steroids can be drawn; in view of the highly efficient biotransformations described herein, we propose that the biotransformation of 3β-sterol **1** into androsta-4,16-dien-3-one **6** and of 3β-sterol **5′** into 5α-androst-16-en-3-one (**7**) under aerobic conditions may be the major pathways that lead to steroidal malodour in human axillae.

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