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Microbial pathways leading to steroidal malodour in the axilla

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Abstract

Odorous steroids, specifically the 16-androstenes, 5α-androstenol and 5α-androstenone, are widely accepted as being contributors to underarm odour, but the precursors and pathways to these odorous steroids were unclear. This study demonstrated that the axillary microflora could only generate odorous 16-androstenes from precursors that already contain the C16 double bond, such as 5,16-androstadien-3-ol and 4,16-androstadien-3-one. In incubations containing 5,16-androstadien-3-ol, mixed populations of *Corynebacterium* spp., isolated from the axilla, could generate many different 16-androstene metabolites, several of which were odorous. Isolation of individual *Corynebacterium* strains, followed by pure culture incubations with 5,16-androstadien-3-ol, revealed organisms capable of efficient, rapid reactions. However, no single isolate could carry out a full complement of the observed biotransformations. 16-Androstene metabolites were identified by gas chromatography–mass spectrometry (GC–MS), either by comparison with known standards, or by prediction from molecular ion and fragmentation patterns. Based on detection of these metabolites, a metabolic map for axillary corynebacterial 16-androstene biotransformations was proposed, detailing potential enzyme activities. In summary, the formerly implicated 4,16-androstadien-3-one, 5α-androstenone and 5α-androstenol were detected, along with previously unreported hydroxy- and keto-substituted 16-androstenes, 16-androstatrienones and 16-androstatrienols. Additionally, many other metabolites with steroidal fragmentation patterns were present, but have remained unidentified.

A key observation was that very low prevalences of microorganisms capable of biotransforming 16-androstenes were present on skin. For example, from a panel of 21 individuals, only 4 of 18 mixed populations of corynebacteria, and only 4 of 45 *Corynebacterium* isolates, could biotransform 5,16-androstadien-3-ol.

This study has increased understanding of the metabolic pathways involved in steroidal malodour formation, and has demonstrated that the biotransformations are more complex than previously anticipated. However, it is clear that further research is required, both to assess the level of contribution of 16-androstenes to underarm odour, and to further elucidate the pathways and odour molecules formed by corynebacteria.

Keywords: *Corynebacterium*; 16-Androstene; Axilla; Biotransformation; Steroid

1. Introduction

In the axilla, a large, permanent population of microorganisms thrive on secretions from skin glands, and it is widely accepted that odour is caused by the action of this microflora on key components within the secretions. The axilla supports a large population of bacteria ($\sim 10^6$ cells cm^{-2}), most of which are Gram-positive, belonging to the following groups: Micrococcaceae, mainly Staphylococcus species; anaerobic/microaerophilic Propionibacterium species; and aerobic coryneforms, primarily *Corynebacterium* species. The *Corynebacterium* genus can be split into two groups dependent on their ability to metabolise lipid, specifically free fatty acid (FFA). The groups have been named as corynebacteria (A), which have the ability to metabolise FFA, and corynebacteria (B), which are unable to utilise FFA as a carbon/energy source [1].

It is now accepted that odorous steroids are among the causal molecules of axillary odour. However, the precursors and the biochemical routes of production still remain matters for debate. Many steroidal components have been detected in axillary secretions; of particular interest are unsaturated steroids of the androstane (C19) family, the 16-androstenes, including 16-androsten-3-one (androstenone) and 16-androsten-3-ol (androstenol) [2]. Extensive studies have been carried out to elucidate the precursors and microbial biotransformations to these odorous steroids [3,4]. Initial findings concluded that aerobic coryneforms were responsible for generating androstenol

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and androstenone from unidentified odourless substrates in apocrine secretion. Axillary bacteria were unable to generate 16-androstenes from testosterone and pregnenolone, but the use of these substrates did give indications of enzyme activities possessed by aerobic coryneforms. Testosterone was converted to dihydrotestosterone (DHT), androstenedione and androstanedione, indicating 4-ene reductase and 3-hydroxysteroid dehydrogenase (HSD) activities [5–7]. Micrococci could only produce low levels of androstenedione, while staphylococci and propionibacteria were incapable of transforming either of the precursors. Whilst the odorous 16-androstenes were not generated from any substrates saturated at the 16-position, Rennie et al. [8] found that some coryneforms could generate androstenone and androstenol from the low odour 16-androstenes, 4,16-androstadien-3-one and 5,16-androstadien-3-ol. However, a weakness in these data was the low yields obtained despite the reaction time, with incubations being left for over 2 weeks before products were measurable.

This paper describes our research on 16-androstene biotransformations undertaken by axillary corynebacteria, with particular emphasis on the recently characterised (A) sub-group.

2. Materials and methods

2.1. General chemicals

All general laboratory chemicals were obtained from Sigma, UK, with the exception of 5,16-androstadien-3-ol and 4,16-androstadien-3-one, which were sourced from Steraloids, USA. Media components were obtained from either LabM Ltd., Oxoid Ltd. or Tissue Culture Services Ltd.

2.2. Organisms and growth media

Axillary coryneforms were identified as described by Taylor et al. [9] and James et al. [1], and isolated using the following selection media. Aerobic coryneforms from fresh axillary samples were isolated on ACP plates (39.5 g l^{-1} blood agar base No. 2; 3 g l^{-1} yeast extract; 2 g l^{-1} glucose; 5 ml l^{-1} Tween 80; 50 ml l^{-1} defibrinated horse blood; 500 mg l^{-1} phosphomycin), whereas coryneforms belonging to the corynebacteria (A) subgroup were isolated on ACFR plates (39.5 g l^{-1} blood agar base No. 2; 3 g l^{-1} yeast extract; 2 g l^{-1} glucose; 5 ml l^{-1} Tween 80; 50 ml l^{-1} defibrinated horse blood; 25 mg l^{-1} furazolidone). Pure isolates (corynebacteria (A), $n = 45$; corynebacteria (B), $n = 11$) were selected from 21 panellists and stored long-term at −80 °C on cryogenic beads. Mixed populations of corynebacteria (A) ($n = 18$) were obtained from 18 panellists. Using the appropriate selective plate, all colonies were combined and resuspended in biotransformation medium (1.0 g l^{-1} $NH_4H_2PO_4$; 1.0 g l^{-1} $(NH_4)_2HPO_4$; 2.0 g l^{-1} KH_2PO_4; 10 g l^{-1} yeast extract; 0.1 g l^{-1} Tween 80). The mixed populations were either used immediately, to prevent the risk of selecting the most vigorously growing organisms upon revival, or cryo-preserved at −80 °C, after addition of glycerol to a final concentration of 50% (v/v).

2.3. Steroid biotransformation studies

Starter cultures of pure bacterial isolates were grown in biotransformation medium (10 ml in a 30 ml Steralin vial) at 35 °C with agitation for either 24 or 48 h. The cells were pelleted by centrifugation at 2500 × *g* for 5 min, resuspended in 1 ml biotransformation medium and placed in 7 ml tall glass vials. Mixed populations were not grown as starter cultures, but resuspended directly into biotransformation medium. Appropriate steroid substrates (0.1–1.0 mg per assay) were filter sterilised in ethanol or methanol and added to the vials. The cultures were incubated for up to 48 h at 35 °C with agitation. Steroids were analysed by gas chromatography–mass spectrometry (GC–MS) following solvent extraction, as outlined further.

2.4. Extraction and analysis of steroids

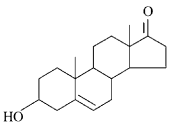
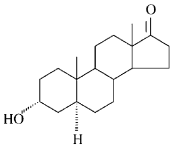
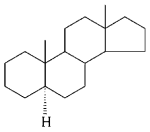
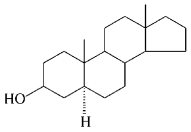
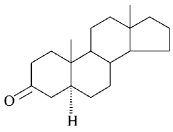
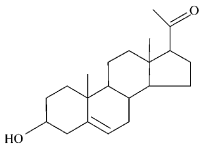
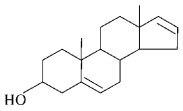
Total lipids were extracted using the chloroform/methanol extraction method of Folch [10]. Internal standard (androsterone or testosterone, 0.1–0.5 mg ml^{-1}) was added to each vial, followed by 4 ml chloroform, 2 ml methanol and 0.5 ml water, and the vial agitated. Once partitioned, the lower phase was removed and the upper layer re-extracted twice with chloroform:methanol:water (86:14:1). The lower layers were pooled, dried under nitrogen and resuspended in 2 ml heptane. Samples were analysed by GC–MS using an HP MSD 5972 instrument fitted with an HP-5 MS column (30 m × 0.32 mm (i.d.) × 0.25 μm film thickness). The temperature program was run from 80–200 °C at 10 °C min^{-1}, followed by 200–300 °C at 20 °C min^{-1}, and a final hold at 300 °C for 1 min (injector temperature, 280 °C; detector temperature, 300 °C). The mass spectrometer, with HP ChemStation data analysis, was set to EI+ ionisation, EM voltage 1694 mV, scanning *m/z* 40–550. Steroid metabolites were identified either by comparison with known standards, or by prediction from molecular ion and fragmentation patterns.

3. Results

3.1. Determination of potential precursors to steroidal odour

When a range of potential steroid precursors were incubated separately with a mixed population of corynebacteria (A), all, with the exception of androstane, were biotransformed to several metabolites. Hypothetical enzyme activities were assigned based on GC–MS identification

Table 1
Steroid biotransformations by a mixed population of corynebacteria (A) (Mix 1)

Substrate	Structure	Metabolites	Biotransformation[a] (%)	Postulated enzyme activity
Dehydroepi-androsterone (DHEA) (1 mg ml^{-1})		4-Androstenedione[b] 1,4-Androstadienedione[b] Hydroxysteroids (trace)[c] Androstanetrione (trace)[d]	32 1 1 1	3β-HSD Δ^{4-5} Isomerase 1-ene DH 6-HSD Hydration
Androsterone (1 mg ml^{-1})		Androstanedione[b] 1-Androstenedione[c] 4-Androstenedione[b] 1,4-Androstadienedione[b]	26 2 2.5 Trace	3β-HSD 1-ene DH 4-ene DH
Androstane (100 μg ml^{-1})		None	0	–
Androstanol (100 μg ml^{-1})		Androstanone[b] 4-Androsten-3-one[d] 1-Androsten-3-one[d] 1,4-Androstadien-3-one[c]	64 9 7 17	3β-HSD 1-ene DH 4-ene DH
Androstanone (100 μg ml^{-1})		Androstanol[b] 4-Androsten-3-one[b] 1,4-Androstadien-3-one[c]	2 1 6	3β-HSD 4-ene DH 1-ene DH
Pregnenolone (100 μg ml^{-1})		Progesterone[b] Pregnane-3,6,20-trione[d] Pregna-1,4-dien-3,20-dione[c] Pregna-4,6-dien-3,20-dione[d]	15 8 30 1.6	3β-HSD 6-ene DH 1-ene DH
5,16-Androstadien-3-ol (100 μg ml^{-1})		4,16-Androstadien-3-one[b] (odorous) 5β-Androst-16-en-3-ol[c] (odorous)	4 1	3β-HSD 4-ene reductase

Only the incubations giving the highest biotransformation levels and the greatest number of metabolites are presented here, hence the various starting substrate concentrations.

[a] Based on percentage substrate removal at 48 h; HSD, hydroxysteroid dehydrogenase; DH, dehydrogenase.

[b] Characterised by comparison with standards held by Unilever R&D Colworth.

[c] Characterised based on fragmentation pattern and molecular ion.

[d] Characterised by comparison to fragmentation patterns on Wiley/NIST library.

of the metabolites. These included hydroxysteroid dehydrogenases, and A and B ring dehydrogenases (Table 1). Additionally, incubations with dehydroepiandrosterone (DHEA) demonstrated either hydration or hydroxylation of the steroid B ring, with the double bond at C5 being reduced and a hydroxyl group inserted at C6 to produce a dihydroxy steroid; this biotransformation had not been reported previously. Despite the corynebacteria (A) mix being capable of various biotransformations, C16-ene bonds were not inserted into the steroid nucleus during any incubation. Odorous steroids were only generated during incubations with the odourless 16-androstene, 5,16-androstadien-3-ol, where the 16-ene bond is already present.

3.2. 16-Androstene biotransformations by axillary coryneforms

The generation of odorous 16-androstenes from the odourless 5,16-androstadien-3-ol was investigated with representatives of corynebacteria (A) and (B), using both mixed cultures and individual isolates (Table 2). Corynebacteria (A) were confirmed as being the main organisms capable of generating odorous steroids from 5,16-androstadien-3-ol. Mixed populations of corynebacteria (A) generated 4,16-androstadien-3-one as the major product, suggesting 3β-HSD activity was present. A diversity of products was generated by one of the corynebacteria (A) mixes, all of

Table 2
5,16-Androstadien-3-ol biotransformations by members of axillary microflora

Genus/subgroup	Culture (number)	Product(s)	Biotransformation (%)	Postulated enzyme activity
Corynebacteria (A)	Mix 1	4,16-Androstadien-3-one[a]	4	3β-HSD
		5β-Androst-16-en-3-ol[b]	1	4-ene
	Mix 2	4,16-Androstadien-3-one[a]	40	3β-HSD
		16-Androsten-3,6-diol[b]	7	Hydration
		16-Androsten-3-ol-6-one[b]	3	6-HSD
		1,16-Androstadien-3α/β-ol[b]	5	1-ene DH
	Mix 3	4,16-Androstadien-3-one[a]	8	3β-HSD
	Isolate 1	4,16-Androstadien-3-one[a]	32	3β-HSD
		6β-Hydroxy-4,16-Androstadien-3-one[a]	53	Hydration
		Hydroxy 16-androstenes[b]	12	4-ene
		5α-Androst-16-en-3-one[a]	Trace	Reductase
	Isolate 2	4,16-Androstadien-3-one[a]	15	3β-HSD
		5α-Androst-16-en-3-one[a]	2	4-ene
		6β-Hydroxy 4,16-androstadien-3-one[a]	65	Reductase
		Hydroxy-16-androstenes[b]	17	Hydration
	Isolate 3	4,16-Androstadien-3-one[a]	3	3β-HSD
		5α-Androst-16-en-3-one[a]	1	4-ene reductase
		6β-Hydroxy-4,16-Androstadien-3-one[a]	3	Hydration
		Keto-16-androstenes[b]	48	6-HSD
		1,4,16-Androstatrien-3-one[b]	45	1-ene DH
Corynebacteria (B)	Isolates 11	None	0	–

Start substrate, 100 μg ml^{-1}. Limit of detection, 100 pg. Number in parentheses denotes the number of cultures tested). (*) Based on percentage substrate removal at 48 h; HSD, hydroxysteroid dehydrogenase, DH, dehydrogenase. Characterised by comparison to fragmentation patterns on Wiley/NIST library.
[a] Characterised by comparison with standards held by Unilever R&D Colworth.
[b] Characterised based on fragmentation pattern and molecular ion.

which were 16-androstene metabolites. Corynebacteria (A) isolates could quickly biotransform all the substrate into a range of 16-androstene products. At least three molecular species of hydroxy 16-androstenes were detected, and the presence of keto-substituted 16-androstenes was also found.

3.3. Microbial biotransformation of other 16-androstene substrates

To investigate reversibility of the reactions, studies were extended to include additional 16-androstenes as substrates. Corynebacteria (A) were capable of biotransforming 4,16-androstadien-3-one, 5α-androstenone and 5α-androstenol. 4,16-Androstadien-3-one was biotransformed by the corynebacteria mixed population to 5α- and 5β-androstenone and 5β-androstenol, suggestive of 4-ene reductase and 3-HSD activities. When 5α-androstenone was provided as the substrate, both the 5α- and 5β-forms of androstenol were generated. However, in a separate incubation, 5α-androstenol was converted into 5α-androstenone, demonstrating the reversibility of the reaction.

3.4. Survey of 5,16-androstadien-3-ol biotransformations by corynebacteria (A) organisms

When corynebacteria (A) isolates and mixtures, from 21 panellists, were evaluated for their ability to biotransform 5,16-androstadien-3-ol, the prevalence of this activity was very low. 5,16-Androstadien-3-ol was biotransformed by only 4 of 18 mixtures and 4 of 45 isolates. The percentage biotransformation was low (2, 5, 8 or 55%) for mixed populations of corynebacteria (A); however, the efficiency increased when pure isolates were used in incubations. Individual isolates were capable of biotransforming 98–100% of 5,16-androstadien-3-ol.

4. Discussion

Corynebacteria (A) were identified as the being key steroid biotransforming organisms in the axilla, indicating the possession of a number of enzymes that can modify steroids. Evidence was found for A and B ring desaturation (suggesting 1-ene, 4-ene and 6-ene dehydrogenase activities), functional group oxidation and reduction (suggesting 3-, 6- and 7-hydroxysteroid dehydrogenase activities), 4-ene reduction, and preliminary evidence for hydration of steroid nuclear double bonds (suggesting 6-ene hydration). Evidence for hydroxysteroids also suggests that hydroxylases may be present in some isolates. Despite the corynebacteria (A) mix being capable of the above biotransformations, C16-ene bonds were not inserted into the steroid nucleus during any incubation.

This study has demonstrated that the biotransformation of axillary 16-androstenes by corynebacteria, in particular the recently characterised (A) sub-group, is more complex than

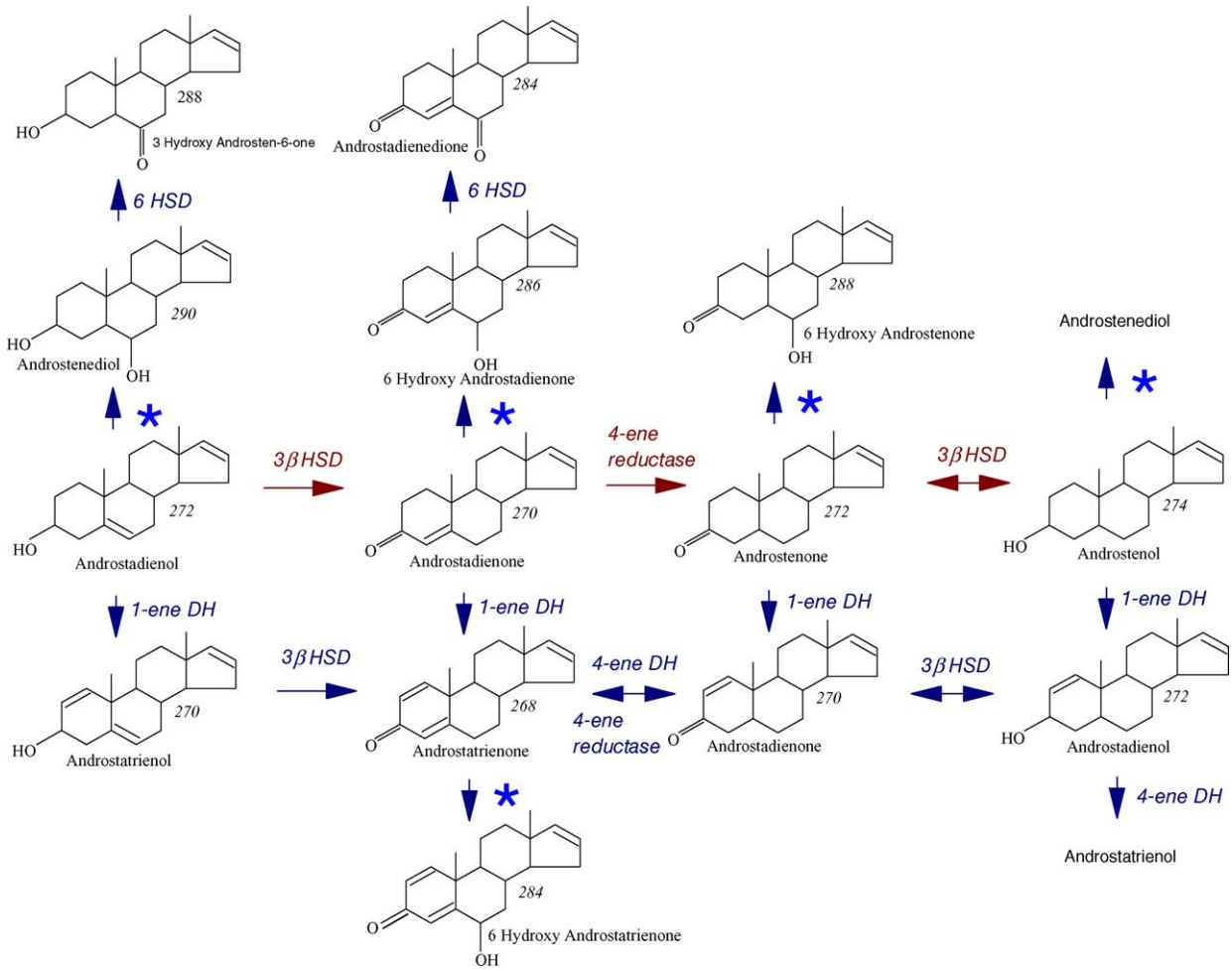


Fig. 1. Biotransformation of 16-androstenes by corynebacteria (A) axillary isolates. It is important to note that the extent of biotransformation of 16-androstene steroids is likely to be more complex than that presented in this figure, as both α- and β-forms of hydroxylated steroids are probably generated. Key: HSD, hydroxysteroid dehydrogenase; DH, dehydrogenase. The symbol (*) denotes biotransformations that may involve a number of enzymes (e.g. hydroxylase or dehydrogenase and hydratase activities).

previously anticipated, and a new metabolic map, based on GC–MS detection of microbial metabolites, has been proposed (Fig. 1). The entirety of biotransformations detailed in this metabolic map can only occur through interactions between a variety of corynebacteria (A) organisms; no single isolate is capable of carrying out the full complement of activities. The complex blend of 16-androstenes detected also indicates that a number of odorous steroids may contribute to axillary steroidal malodour.

The number of isolated corynebacteria (A) organisms capable of biotransforming 5,16-androstadien-3-ol was low. From the work carried out to date, all members of the corynebacteria (A) subgroup have, by definition, the ability to biotransform long chain fatty acids [1]. However, from this study, only a proportion of this sub-group have the ability to biotransform 16-androstenes. These organisms were slow growing and difficult to culture, accumulating in large clumps and adhering readily to culture glassware. These distinguishable characteristics, alongside their unique ability to generate odorous steroids, suggest that they are a sub-set of the corynebacteria (A) group. However, although this study has only detected low levels of organisms possessing the key enzyme activities, this could be due to isolation problems from axillae dominated by other corynebacteria and *Staphylococcus* species. These less fastidious organisms may out-compete the slow growing, steroid biotransforming corynebacteria (A) organisms, thus ensuring that the latter remain undetected.

This study has demonstrated the variety of 16-androstene biotransformations carried out by axillary corynebacteria (A). However, the relative contribution of steroids to underarm odour is still a matter of debate, especially as high levels of specific anosmia are reported for the 16-androstenes [2], while the prevalence of 16-androstene biotransforming organisms in the axillae of normal individuals appears very low. It is clear that further research is required in this area, both to assess the level of contribution of these steroids to underarm odour, and to further elucidate

the pathways and odour molecules formed by corynebacteria (A).

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