5α-Androst-16-en-3α-ol β-D-Glucuronide, Precursor of 5α-Androst-16-en-3α-ol in Human Sweat

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5α-Androst-16-en-3α-ol (α-androstenol) is an important contributor to human axilla sweat odor. It is assumed that α-andostenol is excreted from the apocrine glands *via* a H_2O-soluble conjugate, and this precursor was formally characterized in this study for the first time in human sweat. The possible H_2O-soluble precursors, sulfate and glucuronide derivatives, were synthesized as analytical standards, *i.e.*, α-androstenol, β-androstenol sulfates, 5α-androsta-5,16-dien-3β-ol (β-androstadienol) sulfate, α-androstenol β-glucuronide, α-androstenol α-glucuronide, β-androstadienol β-glucuronide, and α-androstenol β-glucuronide furanose. The occurrence of α-androstenol β-glucuronide was established by ultra performance liquid chromatography (UPLC)/MS (heated electrospray ionization (HESI)) in negative-ion mode in pooled human sweat, containing eccrine and apocrine secretions and collected from 25 female and 24 male underarms. Its concentration was of 79 ng/ml in female secretions and 241 ng/ml in male secretions. The release of α-androstenol was observed after incubation of the sterile human sweat or α-androstenol β-glucuronide with a commercial glucuronidase enzyme, the urine-isolated bacteria *Streptococcus agalactiae*, and the skin bacteria *Staphylococcus warneri* DSM 20316, *Staphylococcus haemolyticus* DSM 20263, and *Propionibacterium acnes* ATCC 6919, reported to have β-glucuronidase activities. We demonstrated that if α- and β-androstenols and androstadienol sulfates were present in human sweat, their concentrations would be too low to be considered as potential precursors of malodors; therefore, the H_2O-soluble precursor of α-androstenol in apocrine secretion should be a β-glucuronide.

Introduction. – Malodor counteraction is a basic concern in the fragrance industry, and, therefore, understanding the biological pathways leading to the formation of bad odors is of high importance. Both eccrine and apocrine glands are present in human underarms. Apocrine glands were reported to excrete odorless polar compounds, which are then bio-transformed by axillary microflora to odorous compounds [1]. The present work focused on understanding the formation of 5α-androst-16-en-3α-ol (α-androstenol), known to be involved in human underarm malodors [2][3].

The starting point of this work was a textbook on cosmetic science, edited in 1999 [4], a standard reference in the field of deodorants. The authors stated that apocrine secretions contain, among others, '*steroid conjugates such as androstenol sulfate and glycosides*'. The cited publications referred to 3α/β-hydroxy-5α-androstan-17-one (androsterone and epiandrosterone) sulfates, 3β-hydroxyandrost-5-en-17-one (= 'dehydroepiandrosterone'; DHEA) sulfate, and 5-androstene-3β,17β-diol 3-sulfate [5][6]. The occurrence of odoriferous, volatile α-androstenol in underarms was confirmed by the analysis of axillary pads [2][7]. The capacity of axillary-isolated coryneform bacteria to transform synthetic α-androstenol sulfate to odoriferous

steroids was also clearly demonstrated, but no final evidence of the natural occurrence of the precursor in underarms was provided [8]. Another biochemical pathway was proposed, starting with the 'necessary secretion' of androsta-4,16-dien-3β-ol (β-androstadienol) conjugates to initiate the metabolic sequence toward odoriferous steroids such as androsta-4,16-dien-3-one, 5α-androst-16-en-3-one (androstenone), and androstenols [4]. To date, no evidence of the occurrence of β-androstadienol sulfates or glucuronides in apocrine secretions is available from the abundant literature on this topic.

Thanks to new liquid chromatography (LC) and mass spectrometry (MS) systems, the analytical profiling of anabolic androgenic glucuronidated and sulfated steroids in body fluids has made huge progress [9–11]. We, therefore, decided to elucidate the natural precursor of α-androstenol in sterile human sweat using these systems.

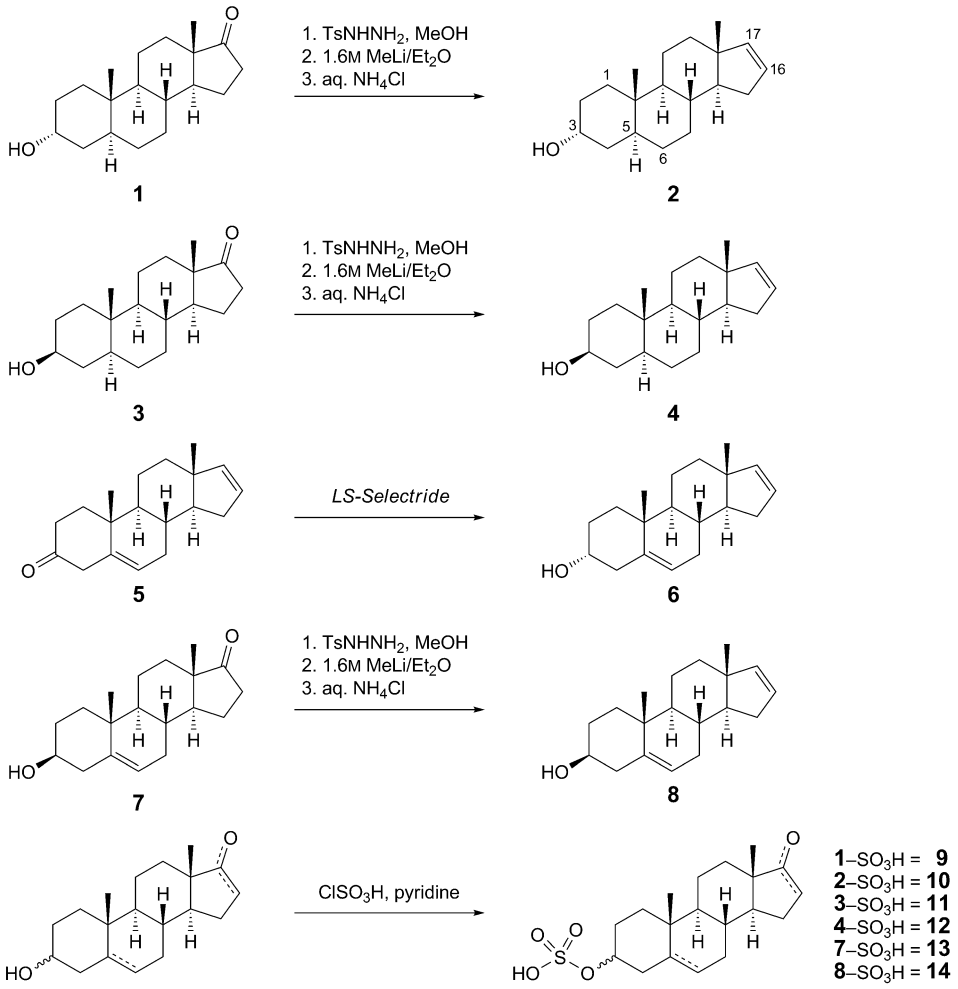
Results and Discussion. – *Preparation of Steroid Sulfates.* Androsterone (**1**) was reduced *via* the *Bamford–Stevens* reaction [12] to prepare pure α-androstenol (**2**). β-Androstenol (**4**) was also prepared *via* the *Bamford–Stevens* reaction from epiandrosterone (**3**). α-Androstadienol (**6**) was obtained by reduction with *LS-Selectride* [12] from androstadienone (**5**), and β-androstadienol (**8**) was prepared from DHEA (**7**) *via* the *Bamford–Stevens* reaction. The DHEA sulfate is commercially available; the other sulfates were prepared in pyridine with chlorosulfonic acid ($ClSO_3H$) from the corresponding alcohols (*Scheme 1*) [8].

LC/MS Analysis of Steroid Sulfates. The chromatographic conditions and the LC/MS method to separate and identify steroid sulfates were developed with commercial DHEA sulfate **13**. With HESI in negative-ion mode, the base-ion peak of **13** was at m/z 367, corresponding to $[M-1]^-$. In the MS/MS mode, only a unique fragment-ion peak at m/z 97 could be observed, corresponding to the mass of the sulfate moiety (HSO_4^-) and displaying a small isotopic ion peak (m/z 99) of 4.4% intensity, thereby excluding the possibility of the presence of a phosphate. The same fragmentation pattern was observed for all steroid sulfates prepared, *i.e.*, **9**–**12** and **14**. This observation allowed us to conduct an MS experiment, *i.e.*, parent-ion scanning, which can be performed on a triple quadrupole mass spectrometer to specifically detect compounds losing HSO_4^-.

The consequence of the formation of an ion peak at m/z 97 was that the MS/MS spectra were not very informative, revealing only the presence of a sulfate moiety. Therefore, the identification of the steroid could only rely on molecular mass and retention times.

Analysis of Steroid Sulfates in Axillary Sweat. The sterile sweat was diluted twice in MeOH prior to injection. The UPLC/MS *TSQ Quantum Ultra* trace displayed in *Fig. 1,a*, represents the total-ion scanning mode of the sterile sweat. The sterile sweat was then injected in parent-ion mass mode set at m/z 97. We observed that most of the peaks detected in scan mode correspond to sulfate derivatives (*Fig. 1,b*). The androsterone and epiandrosterone sulfates **9** and **11**, respectively, and the DHEA sulfate **13** were well-detected, as was the cholesterol sulfate. None of the peaks displayed in *Fig. 1,b*, could correspond to α- or β-androstenol sulfate derivatives **10** and **12** ($C_{19}H_{30}O_4S$; M_r 354) at retention times (t_R) of 4.68 and 4.73 min measured for the synthetic **10** and **12**, respectively. The same was observed for β-androstadienol sulfate (**14**; $C_{19}H_{28}O_4S$; M_r 352) at 4.59 min, which is the t_R for synthetic β-androstadienol

Scheme 1. *Preparation of the Steroid Sulfates Used as Authentic Standards*



sulfate (**14**). This offered us two options: β-androstadienol sulfate (**14**), and α- and β-androstenol sulfates **10** and **12**, resp., are not present in axillary sweat, or their concentrations are too low to be detected under our conditions.

Calibration curves were established, and the detection limits were as follows: α-androstenol sulfate **10** (SIM) *m*/*z* 352.8–353.8, (signal-to-noise ratio) S/N 3; 0.05 μg/ml), β-androstenol sulfate **12** (selected ion monitoring (SIM) *m*/*z* 352.8–353.8; S/N, 10; 0.0125 μg/ml) and β-androstadienol sulfate **14** (SIM, *m*/*z* 350.8–351.8; S/N 4; 0.025 μg/ml). From LC/MS in base-peak mode, we easily detected the presence of **9**, **11**, **13**, and the cholesterol sulfate in sweat (*Fig. 1*, *a*) without preconcentration. The concentration of DHEA sulfate **13** was estimated at 19 μg/ml ($\pm$0.9), which is in agreement with published data [13].

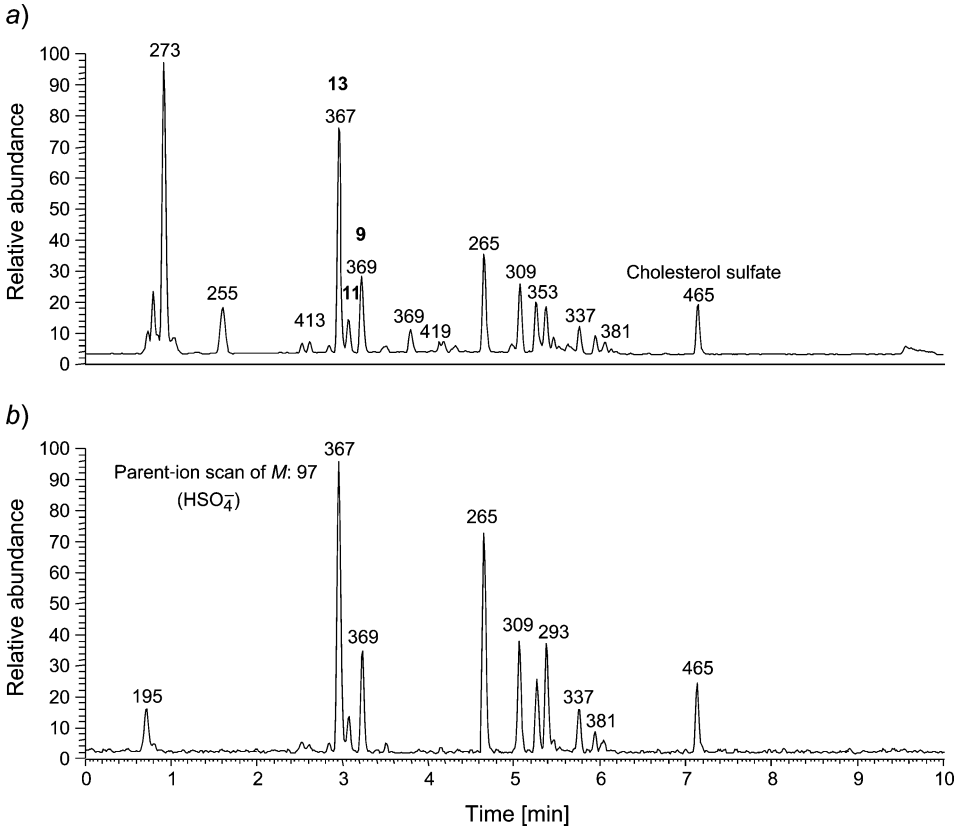
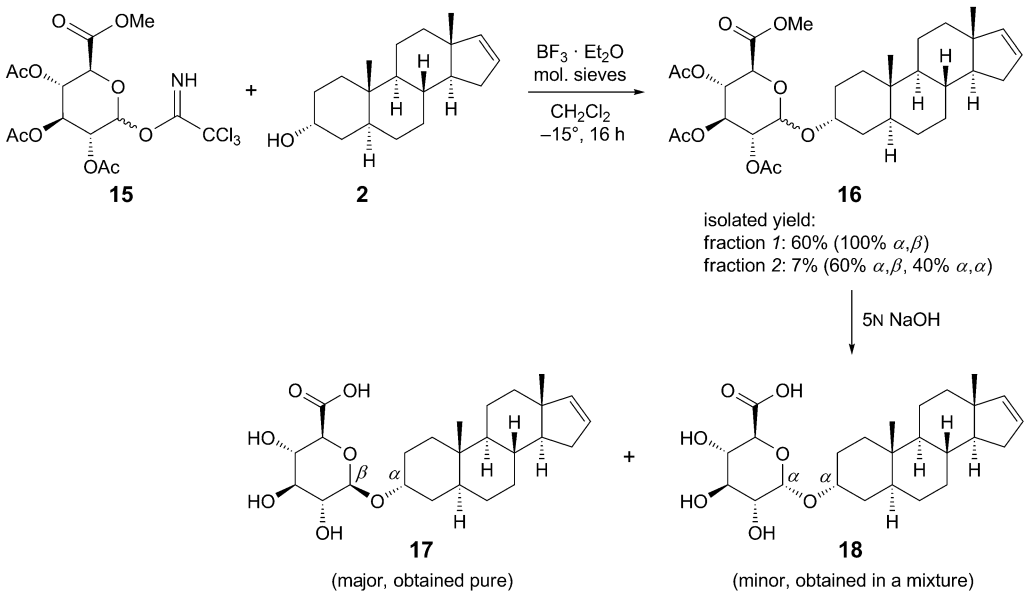


Fig. 1. *Analysis by UPLC/MS triple quadrupole in negative-ion heated electrospray ionization mode* (HESI; base peak neg.-ion ESI 130.00–500.00). *a*) Full scan in base peak of male sweat. *b*) Base peak of male sweat in parent-ion scan of 97 in negative HESI.

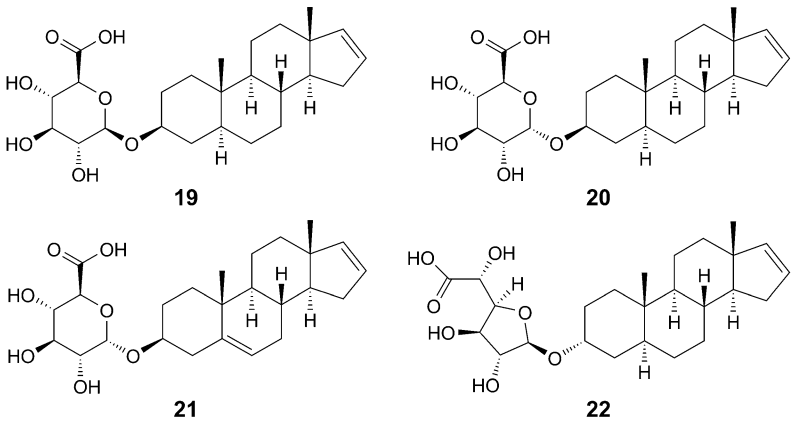
The sweat steroid sulfates were concentrated 27 times by SPE (solid phase extraction) and reinjected: no α- and β-androstenols **10** or **12**, or β-androstadienol sulfate **14**, were detected; therefore, if they were present, their concentrations should have been below our detection limit.

Preparation of Glucuronide Conjugates. The coupling of methyl acetobromo-α-D-glucuronidate to α-androstenol **2** with Ag_2CO_3 under *Koenigs–Knorr* reaction conditions [14][15] failed to give the desired product in reasonable yield. As described by *Brown et al.* [16], the imidate of the methyl 2,3,4-tri-*O*-acetyl-1-hydroxy-α,β-D-glucopyranuronate **15** was coupled to α-androstenol **2** to give **16**, which was then deprotected, in 30% yield, to α-androstenol β-glucuronide **17**, containing a small quantity of α-androstenol-α-glucuronide **18** (*Scheme 2*). The configuration of α-androstenol β-glucuronide (**17**) was confirmed by ^{1}H-NMR, based on the coupling constant (3.75 Hz) between the anomeric H-atom (δ(H) 4.90 ppm) and the H-atom at C(2) of the sugar moiety. α-Androstenol α-glucuronide (**18**) was a minor product obtained only in mixture, but this allowed us to compare its ^{1}H-NMR coupling constant

Scheme 2. *Preparation of the Steroid Glucuronides as Authentic Standards*



(δ(H) 4.33 (J = 7.80 Hz)) to that of **17**. β-Androstadienol **8**, obtained from DHEA **7**, was also coupled in the same way to prepare β-androstadienol β-glucuronide **21**. α-Androstenol β-glucuronide **17** was assumed to be in a pyranose form, but it could also be in a furanose form. To exclude this possibility, the α-androstenol β-glucuronide furanose form **22** was prepared starting from glucuronolactone. The coupling was performed in $BF_3 \cdot Et_2O$, and the lactone-ring opening was achieved in aq. NaOH solution; after purification steps, pure α-androstenol β-glucuronide, furanose form, **22** was obtained. A UPLC method was developed to separate $\alpha\beta$-, $\alpha\alpha$-, $\beta\alpha$-, and $\beta\beta$-androstenol glucuronides (*Fig. 2*, *a*–*c*).



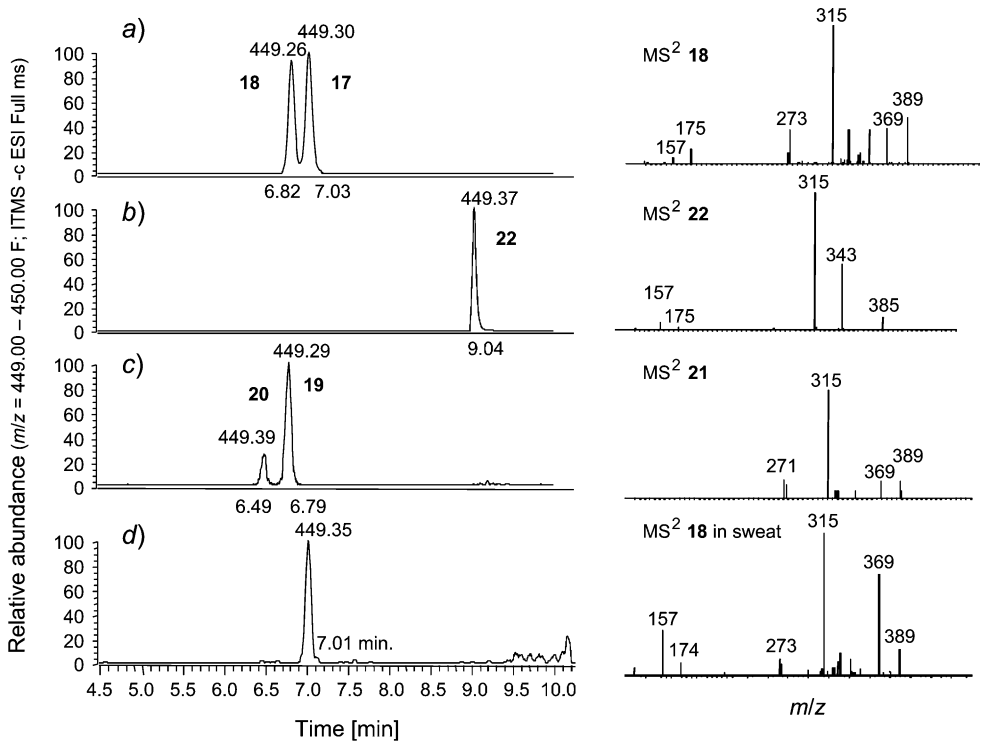


Fig. 2. *Analysis by UPLC/MS LXQ in negative-ion mode of male sweat concentrated ten times. Left:* UPLC trace of HESI full MS, monitoring in the range of *m/z* 448.75–449.75 (exact *m/z* 449.25). *Right:* MS/MS. *a*) α-Androstenol-α- and -β-glucuronides. *b*) α-Androstenol-β-glucuronide, furanose form. *c*) β-Androstenol-α- and -β-glucuronides. *d*) Male axillary sweat.

Analysis of Glucuronide Steroids in Axillary Sweat. Sterile sweat was concentrated ten times and analyzed in full MS scan. The LC/MS trace displayed in *Fig. 2,d*, corresponds to the extract ion with a peak at *m/z* 449.35. At 7.03 min, a peak with a mass spectrum corresponding to the α-androstenol β-glucuronide (**17**) was detected. No peaks with *m/z* 446.75–447.75, which could correspond to β-androstadienol-β-glucuronide **21**, could be detected. Compound **18**, with a detection limit of 0.025 μg/ml (S/N 8) was not found.

Quantitative Estimation of α-Androstenol β-Glucuronide **17** *in Sterile Sweat.* The concentration of **17** was established in an eccrine and apocrine axillary sweat pool collected from 25 women and 24 men, who used a sauna over a three-year period [17]. A 4-ml aliquot of sterile sweat was concentrated ten times and analyzed by LC/MS (HESI) in negative-ion mode. From an external calibration curve in SIM at *m/z* 448.75–449.75, we found **17** at 79 ng/ml (±2) for the female pool and at 241 ng/ml (± 70) for the male pool.

Enzymatic Release of α-Androstenol **2** *from α-Androstenol β-Glucuronide* (**17**). Synthetic **17** was incubated with *Streptococcus agalactiae*, which possesses glucuronidase activity [18], and with an isolated commercially available enzyme, β-glucuroni-

dase/arylsulfatase. In both cases, **17** disappeared. The fermentation broth was then extracted with CH_2Cl_2 and injected on GC/MS; α-androstenol **2** was detected, but not in the blank system.

A homology search (a microbial protein blastp search on www.ncbi.nlm.nih.gov) was carried out between the *S. agalactiae* ATCC 13813 β-D-glucuronidase amino acid sequence (621 amino acids, accession No. EFV97073) and the *Gram*-positive bacteria genome. Some identities were found with glucuronidases from the underarm bacterial species *Staphylococcus warneri*, *Staphylococcus haemolyticus*, and *Propionibacterium acnes* [19–21]. After incubation with α-androstenol β-glucuronide (**17**), the three species were able to generate α-androstenol (**2**). The percentage reductions of **17** after a one-day incubation at 37° were 99, 29, and 22% with *S. warneri* DSM20316, *S. haemolyticus* DSM20263, and *P. acnes* ATCC6919, respectively.

In parallel, the male sweat pool was incubated with the commercial β-glucuronidase/arylsulfatase enzyme at 37° for one night. We confirmed by LC/MS the disappearance of **17**; the formation of **2** was confirmed after extraction with CH_2Cl_2 (containing α-androstanol as internal standard). α-Androstanol was quantified by GC/MS in SIM mode (*m*/*z* 274), and the concentration of **2** (*m*/*z* 276) was determined. In sterile male sweat, **17** was found at a concentration of 241 ng/ml ± 70 (0.53 μmol/ml ± 0.15) and, after incubation, **2** was found at a concentration of 167 ng/ml ± 12 (0.61 μmol/ml ± 0.04).

Discussion. – In human underarm secretions, steroid sulfate derivatives are more abundant than glucuronides [6][22–24]. This finding could be the reason for the assumption that sulfate conjugates were the precursors of odorant steroids [4]. In a previous work, the concentration of DHEA sulfate **13** in Asian and Caucasian axillary sweat secretions was compared [13], but it is difficult to deduce a correlation between the concentration of DHEA sulfate **13** and α-androstenol β-glucuronide (**17**), implying that the quantification of **13** cannot be directly linked to body odor.

Brooksbank and *Haselwood* in 1961 claimed [25] that the precursor of α-androstenol was not a sulfate. They extracted the acid fraction of sweat, and, after solvolysis in AcOEt, they could not find α-androstenol (**2**). Sulfate is a good leaving group, and, under acidic conditions or thermolysis, the formation of odorant androstene derivatives [8][12] has been observed; therefore, we did not know whether this claim was valid [2].

The precursor of **2** is its corresponding β-glucuronide, which is not surprising, because it was already identified in urine [25], and recently we demonstrated its importance in the odor of stale urine [18]. In the literature, bacterial β-glucuronidase activities have already been detected in both facultatively and strictly anaerobic strains belonging to the family Enterobacteriaceae (*Escherichia coli*), and the genera *Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, anaerobic *Corynebacterium*, and *Bacteroides*. Genes encoding B-glucuronidase (GUS) have been characterized from *E. coli*, *L. gasseri*, *Staphylococcus* spp., *Clostridium perfringens*, *S. aureus*, *Thermotoga maritima*, and *Ruminococcus gnavus* [26–29]. In moist areas such as the human axilla, *Corynebacterium* spp. and *Staphylococcus* spp. dominate the resident flora, which was demonstrated earlier through culture-based approaches [19–21]. Here, we have shown that at least three bacteria present in the underarm, *S. warneri*

and *S. haemolyticus*, and to a minor extent *P. acnes*, were able to cleave the β-glucuronide conjugate to generate α-androstenol (**2**).

Furthermore, we noticed that β-androstenol (**4**) and β-androstadienol (**8**) are enzymatically oxidized to ketones, which is also in agreement with the literature [23][24]. This could explain the MS fragment ion corresponding to the peak at *m*/*z* 271 (*Fig. 2*,*c*) for **19**, instead of the expected fragment ion with the peak at *m*/*z* 273 observed for **17**. Even if we cannot clearly confirm this hypothesis now, we strongly believe that androstenone is bacterially generated from α-androstenol (**2**), and probably to a lesser extent from β-androstenol (**4**), which is in agreement with the literature [23][24]. Androstenone may, therefore, build up over time to enhance human sweat odor after the primary liberation of conjugated steroid alcohols.

We are grateful to Dr. *Eric Ohleyer* to guide us in the synthesis of glucuronide derivatives, Mrs. *Sabine Beccucci* for providing the sterile sweat and bacterial solutions, Dr. *Eric Frerot* for LC/MS guidance and suggestions, and Dr. *Monica Bandera* and *Laurent Wünsche* for reviewing the manuscript.

Experimental Part

Materials. All chemical and reagents were purchased from *Fluka–Sigma–Aldrich* (CH-Buchs), *Acros* (BE-Geel), and *TCI Europe N.V.* (BE-Zwjindrecht). All solvents, including Et_2O *p.a.* and CH_2Cl_2 (*Atrasol*), were purchased from *Carlo Erba Reactifs SDS* (FR-Val de Reuil Cedex), H_2O for ultra-performance liquid chromatography (UPLC) was purchased from *Biosolve* (NL-Valkenswaard), and MeCN for UPLC was purchased from *Merck* (DE-Darmstadt). $HCOONH_4$ *puriss p.a.* for the UPLC solvent was from *Fluka–Sigma–Aldrich.* Na_2SO_4 (99%) was obtained from *Sigma–Aldrich* (DE-Steinheim). Silica gel (SiO_2, *60A*; 32–63 μm) for flash chromatography (FC) was purchased from *Brunschwig* (CH-Basel). β-Glucuronidase/arylsulfatase (*Helix pomatia*) was purchased from *Roche* (CH-Rotkreuz). Samples were filtered prior to injection on UPLC with *UptiDisc* 13-mm syringe filters (0.45 μm, *Acrodisc*) and regenerated cellulose membrane from *Interchim* (FR-Montluçon). The solid phase extraction (SPE) cartridges were *Supelclean ENVI-18 SPE* tubes of 6 or 3 ml from *Supelco* (CH-Buchs). Previously urine-isolated *Streptococcus agalactiae* (100% similarity with *Streptococcus agalactiae* ATCC 13813 16S rDNA sequence) were grown in liquid tryptic soya broth (*Beckton Dickinson*, FR-Pont de Claix) for 24 h at 37°. The culture was then washed two times with 0.9% NaCl (saline soln.) and concentrated in saline soln. to 10^{10} cfu/ml. Sterile sweat samples (human apocrine and eccrine secretions) were collected from the axillae of 49 Caucasian volunteers, who used a sauna over three winter seasons, and the samples were pooled by sex [17]. *Staphylococcus warneri* DSM 20316, *Staphylococcus haemolyticus* DSM 20263, and *Propionibacterium acnes* ATCC 6919 were purchased from DSMZ (*German Collection of Microorganisms and Cell Cultures GmbH*, DE-Braunschweig) and cultivated as described for *S. agalactiae*, except for *P. acnes*, which was cultivated on *Schaedler* agar containing 5% sheep blood (*BioMérieux*, CH-Geneva) and *Wilkins–Chalgren* liquid broth (*Oxoid*, CH-Basel) at 37° for 48 h under anaerobic conditions.

^{1}H- and ^{13}C-NMR Spectra. The NMR spectra were recorded in $CDCl_3$ or MeOD on a *Bruker Avance-500* spectrometer (CH-Zurich) at 500.13 and 125.76 MHz (^{1}H and ^{13}C), resp. Chemical shifts δ in ppm were recorded rel. to Me_4Si as internal standard. The assignments were completed by 2D spectroscopy, correlated spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond coherence (HMBC) experiments with standard *Bruker* software (Topspin).

UPLC/MS. Analyses were performed on a *Waters Acquity* system (*Waters*, CH-Baden-Dättwil) coupled to a mass spectrometer. The separations and quantifications of sulfates were performed on an *Acquity BEH-C18* column (2.1 mm i.d. × 100 mm, 1.7 μm i.d.). One μl was injected; the elution solvents were H_2O containing 5 mM $HCOONH_4$ (solvent *A*), and a mixture of 90% MeCN and 10% H_2O containing 5 mM $HCOONH_4$ (solvent *B*). The gradient profile started at 20% *B* and was then increased

to 98% *B* in 6.0 min. The flow rate was 0.3 ml/min. The separation of glucuronides and their quantification were performed on an *Acquity BEH* shield *RP-18* column (2.1 mm i.d. × 100 mm, 1.7 μm i.d.). The gradient started at 35% *B* and was increased to 45% *B* in 8.0 min, and then to 98% in 0.1 min and held for 1 min. The flow rate was 0.3 ml/min.

The mass spectrometer was a *Thermo Finnigan LXQ* (*Thermo Fisher Scientific*, CH-Basel) with heated electrospray ionization (HESI) operated in negative-ion mode. The spray voltage was 4.0 kV, the vaporizer temp. was 250°, and the cap. temp. 350°. The sheath gas was N_2 at a flow rate of 50 (*Finnigan* arbitrary units). The auxiliary gas was also N_2 at a flow rate of 20 (*Finnigan* arbitrary units). The events were either in full-scan mode [80–800], which could be linked with a data-dependent MS/MS of the 1st most intense ion from scan event 1; collision energy, 25 V; or in single-ion monitoring (SIM) mode based on the mass that we were interested in ($[M-1]$).

When specified, the mass spectrometer for parent-ion scanning experiments was a *Thermo Finnigan TSQ Quantum Ultra* (*Thermo Fisher Scientific*) with HESI operated in negative-mode. The spray voltage was 4.0 kV, the vaporizer temp. was 400°, and the cap. temp. 370°. The sheath gas was N_2 at a flow rate of 50 (*Finnigan* arbitrary units). The auxiliary gas was also N_2 at a flow rate of 15 (*Finnigan* arbitrary units). The injections were made in two scan events: first, full Q3MS and, second, full parent-ion scan; product mass, 96,900; collision energy, 35 V.

Gas Chromatography (*GC*)/*MS.* An *Agilent-GC-6890* system connected to an *Agilent-MSD-5973* quadrupole mass spectrometer (*Santa Clara*, CA, USA) was operated at 70 eV. He was the carrier gas set at a constant flow rate of 0.7 ml/min. Separations were performed on fused-silica cap. columns, coated with *SPB-1* (*Supelco*, 30 m × 0.25 mm i.d., 0.25 μm). The temp. program: 50°, increased to 250° at 10°/min, and held for 15 min. MS Interpretation was based on the *Wiley* MS database.

Synthesis of Steroids. 5α-Androst-16-en-3α-ol (=*α-androstenol*; **2**) [12]. *3α-Hydroxy-5α-androstan-17-one* (=*androsterone*; **1**; 11 g, 37.9 mmol) and $TsNHNH_2$ (7.2 g, 37.9 mmol) were heated in dry EtOH (185 ml) at reflux for 2 h and then concentrated under vacuum to dryness. Et_2O (185 ml) was added to the residue, followed by a 1.6M soln. of MeLi (118 ml, 189 mmol), added dropwise for 1.5 h at 22°. After 15 h, the mixture was poured onto ice-cold aq. NH_4Cl, and the org. phase was washed twice with brine, and then dried (Na_2SO_4), filtered, and concentrated. The product was purified by FC on SiO_2 (CH_2Cl_2/Et_2O 9:1) to afford odoriferous **2** (6.0 g, 58%). $[\alpha]_D^{20} = +15.1$ ($c=1$, $CHCl_3$) [12]. ^{13}C-NMR (125 MHz, $CDCl_3$): 11.2 (*q*); 17.1 (*q*); 20.8 (*t*); 28.6 (*t*); 29.0 (*t*); 32.0 (*t*); 32.0 (*t*); 32.1 (*t*); 34.1 (*d*); 36.0 (*t*); 36.4 (*s*); 39.4 (*d*); 45.6 (*s*); 55.2 (*d*); 56.2 (*d*); 66.6 (*d*); 129.3 (*d*); 144.0 (*d*).

5α-Androst-16-en-3β-ol (=*β-Androstenol*; **4**). The same procedure used for **2** with *3β-hydroxy-5α-androstan-17-one* (=*epiandrosterone*; **3**; 3 g, 10.33 mmol) afforded **4** (820 mg, 29%). $[\alpha]_D^{20} = +13.8$ ($c=1$, $CHCl_3$) [12]. ^{13}C-NMR (100 MHz, $CDCl_3$): 12.3 (*q*); 17.1 (*q*); 21.2 (*t*); 28.7 (*t*); 31.5 (*t*); 32.0 (*t*); 32.1 (*t*); 34.1 (*d*); 35.8 (*s*); 36.0 (*t*); 36.8 (*t*); 38.2 (*t*); 45.1 (*d*); 45.6 (*s*); 55.2 (*d*); 56.1 (*d*); 71.3 (*d*); 129.3 (*d*); 144.0 (*d*).

Androsta-5,16-dien-3α-ol (=*α-Androstadienol*; **6**). Prepared by reduction of **5** as described in [12]. ^{13}C-NMR (125 MHz, $CDCl_3$): 16.9 (*q*); 18.6 (*q*); 20.6 (*t*); 28.9 (*t*); 30.4 (*d*); 31.8 (*t*); 32.1 (*t*); 33.2 (*t*); 35.9 (*t*); 37.7 (*s*); 39.9 (*t*); 45.4 (*s*); 51.2 (*d*); 56.2 (*d*); 67.1 (*d*); 123.8 (*d*); 129.4 (*d*); 138.9 (*s*); 143.8 (*d*).

Androsta-5,16-dien-3β-ol (=*β-Androstadienol*; **8**). The same procedure used for **2** with *3β-hydroxyandrost-5-en-17-one* (DHEA; **7**; 2 g, 6.93 mmol) furnished **8** (956 mg, 51%). $[\alpha]_D^{20} = -67.5$ ($c=1$, $CHCl_3$) [12]. ^{13}C-NMR (90 MHz, $CDCl_3$): 16.9 (*q*); 19.4 (*q*); 20.9 (*t*); 30.5 (*d*); 31.7 (*t*); 31.8 (*t*); 32.1 (*t*); 35.9 (*t*); 36.8 (*s*); 37.2 (*t*); 42.4 (*t*); 45.4 (*s*); 51.0 (*d*); 56.2 (*d*); 71.8 (*d*); 121.6 (*d*); 129.4 (*d*); 141.1 (*s*); 143.8 (*d*).

Synthesis and Analysis of Steroid Sulfates. General Procedure [8]. $ClSO_3H$ (2 equiv.) was added dropwise to a soln. of the steroid alcohol (1 equiv.) in pyridine (34 equiv.). The mixture was stirred for 2 h at 22°. The pyridine was removed by washing three times with pentane (5 × the volume of pyridine). The residue was concentrated, and H_2O (2 ml) was added. The pH must be <2; if not, a few drops of 2N H_2SO_4 was added. The soln. was loaded on an *SPE ENVI-18* cartridge (3 ml for 10–50 mg of product, or 6 ml up to 1 g of product). The cartridge was rinsed with sat. aq. $NaHCO_3$ soln., followed by ^{t}BuOMe and then MeOH. The MeOH fraction was concentrated and then lyophilized to give the corresponding steroid sulfate in powder form.

3α-Hydroxy-5α-androstan-17-one (*Androsterone*) *Sodium Sulfate* (**9**). ^{13}C-NMR (125 MHz, MeOD): 11.9 (*q*); 14.2 (*q*); 21.2 (*t*); 22.7 (*t*); 27.9 (*t*); 29.3 (*t*); 32.1 (*t*); 32.9 (*t*); 33.9 (*t*); 34.6 (*t*); 36.4 (*d*); 36.8 (*t*); 36.9 (*s*); 40.8 (*d*); 49.2 (*s*); 52.8 (*d*); 55.9 (*d*); 76.3 (*d*); 224.2 (*s*).

5α-Androst-16-en-3α-ol (*α-Androstenol*) *Sodium Sulfate* (**10**). ^{13}C-NMR (125 MHz, MeOD): 11.9 (*q*); 17.5 (*q*); 21.9 (*t*); 27.9 (*t*); 29.6 (*t*); 32.9 (*t*); 33.4 (*t*); 33.8 (*t*); 34.7 (*t*); 35.5 (*d*); 37.0 (*s*); 37.2 (*t*); 41.0 (*d*); 46.7 (*s*); 56.8 (*d*); 57.6 (*d*); 76.4 (*d*); 130.2 (*d*); 145.0 (*d*).

3β-Hydroxy-5α-androstan-17-one (*Epiandrosterone*) *Sodium Sulfate* (**11**). ^{13}C-NMR (125 MHz, MeOD): 14.0 (*q*); 19.8 (*q*); 21.5 (*t*); 22.7 (*t*); 30.0 (*t*); 31.9 (*t*); 32.7 (*t*); 32.8 (*d*); 36.7 (*t*); 37.8 (*s*); 38.4 (*t*); 40.4 (*t*); 49.2 (*s*); 51.8 (*d*); 53.0 (*d*); 79.7 (*d*); 122.8 (*d*); 141.8 (*s*); 223.9 (*s*).

5α-Androst-16-en-3β-ol (*β-Androstenol*) *Sodium Sulfate* (**12**). ^{13}C-NMR (125 MHz, MeOD): 12.7 (*q*); 17.5 (*q*); 22.3 (*t*); 29.8 (*t*); 29.9 (*t*); 33.0 (*t*); 33.4 (*t*); 35.5 (*d*); 36.4 (*t*); 36.8 (*s*); 37.2 (*t*); 38.1 (*t*); 46.6 (*d*); 46.7 (*s*); 56.7 (*d*); 57.6 (*d*); 79.7 (*d*); 130.2 (*d*); 145.0 (*d*).

3β-Hydroxyandrost-5-en-17-one (*DHEA*) *Sodium Sulfate* (**13**). ^{13}C-NMR (125 MHz, MeOD): 14.0 (*q*); 19.8 (*q*); 21.5 (*t*); 22.7 (*t*); 30.0 (*t*); 31.9 (*t*); 32.7 (*t*); 32.8 (*d*); 36.7 (*t*); 37.8 (*s*); 38.4 (*t*); 40.4 (*t*); 49.2 (*s*); 51.8 (*d*); 53.0 (*d*); 79.7 (*d*); 122.8 (*d*); 141.8 (*s*); 223.9 (*s*).

Androsta-5,16-dien-3β-ol (*β-Androstadienol*) *Sodium Sulfate* (**14**). ^{13}C-NMR (125 MHz, MeOD): 17.3 (*q*); 19.7 (*q*); 22.0 (*t*); 30.0 (*t*); 31.8 (*d*); 32.9 (*t*); 33.0 (*t*); 37.1 (*t*); 38.0 (*s*); 38.5 (*t*); 40.5 (*t*); 46.5 (*s*); 52.6 (*d*); 57.7 (*d*); 79.8 (*d*); 123.2 (*d*); 130.3 (*d*); 142.0 (*s*); 144.9 (*d*).

Analysis of Steroid Sulfates in Sterile Sweat. Four solns. of **13** in MeOH at different concentrations (2.7, 18.9, 40.7, and 81.3 μg/ml) were injected in LC/MS (*LXQ*) in SIM mode (*m*/*z* 366.56–367.56) for the external calibration curve (RSQ: 0.953). Two sterile sweat samples from men (the same pool as described in [17]) were injected twice; the result was an average of the two values. The detection limits were determined by injecting decreasing concentrations of the reference compounds in MeOH in SIM mode: **10**: SIM *m*/*z* 352.8–353.8, detection limit (signal-to-noise ratio (S/N) 3 (=0.05 μg/ml)); **12**: SIM *m*/*z* 352.8–353.8; detection limit S/N 10 (=0.0125 μg/ml); **14**: SIM *m*/*z* 350.8–351.8; detection limit S/N 4 (= 0.025 μg/ml)). The analysis of steroid sulfates in sterile sweat was performed after pre-concentrating the sweat (27 g) and loading it on a 6-ml *SPE ENVI-18* cartridge, previously washed with MeOH (3 ml) and then conditioned with H_2O (7 ml). It was rinsed three times with 2 ml of H_2O and then desorbed with MeOH (5 ml). MeOH was concentrated to obtain dry material (1.2 mg). MeOH was then added (1.00 ml) to obtain a 27× concentrated sterile sweat soln.

*Synthesis of Steroid Glucuronides. α-Androstenol β-*D*-glucopyranosiduronic Acid* (=*α*-(*Androstenol β-glucuronide*; **17**). Imidate **15** was prepared as described in [16]. The saponification was slightly modified: *α-androstenyl methyl 2,3,4-tri-O-acetyl-β-*D*-glucopyranosiduronate* (**16**; 63 mg, 0.107 mmol) in MeOH (1.6 ml) and 5N aq. NaOH (107 μl) were stirred for 1 h. The MeOH was concentrated under vacuum. The residue was loaded on an SPE cartridge *ENVI-18* (6 ml; conditioned as described above). The SPE was then washed with H_2O (2 ml), and the glucuronide was desorbed with MeOH. MeOH was then evaporated to afford **17** (18 mg), 37%). ^{13}C-NMR (125 MHz, MeOD): 11.9 (*q*); 17.5 (*q*); 21.9 (*t*); 25.9 (*t*); 29.7 (*t*); 33.0 (*t*); 33.4 (*t*); 33.5 (*t*); 35.6 (*d*); 35.6 (*t*); 37.2 (*s*); 37.3 (*t*); 40.8 (*d*); 46.7 (*s*); 56.5 (*d*); 57.8 (*d*); 73.9 (*d*); 74.2 (*d*); 75.1 (*d*); 76.3 (*d*); 78.1 (*d*); 102.0 (*d*); 130.2 (*d*); 145.1 (*d*); 177.1 (*s*).

α-Androstenol α-Glucuronide (**18**). A mixture of *α*-androstenyl methyl 2,3,4-tri-*O*-acetyl-*α*- and -*β*-D-glucopyranosiduronate **16** (35 mg, 0.059 mmol) in MeOH (0.9 ml) and 5N aq. NaOH (60 μl) was stirred for 1 h. The same purification as above afforded a 7:3 mixture of *α*-androstenol *β*-glucuronide (**17**) and *α*-androstenol-*α*-glucuronide (**18**) (9 mg) in 34% yield. ^{13}C-NMR (125 MHz, MeOD): 11.9 (*q*); 17.5 (*q*); 21.9 (*t*); 28.7 (*t*); 29.8 (*t*); 33.0 (*t*); 33.4 (*t*); 33.5 (*t*); 34.2 (*t*); 35.6 (*d*); 37.2 (*t*); 37.3 (*s*); 40.9 (*d*); 46.7 (*s*); 56.6 (*d*); 57.7 (*d*); 72.1 (*d*); 73.4 (*d*); 74.3 (*d*); 74.6 (*d*); 74.9 (*d*); 98.9 (*d*); 130.2 (*d*); 145.1 (*d*); 178.1 (*s*).

β-Androstenol β-Glucuronide (**19**) *and β-Androstenol α-Glucuronide* (**20**). Prepared as described for **17**. Unfortunately, because of the small quantities of starting materials and the poor yield, LC/MS t_R and MS data were obtained for **19**/**20**.

β-Androstadienol β-Glucuronide (**21**). *β-Androstadienyl methyl 2,3,4-tri-O-acetyl-β-*D*-glucopyranosiduronate* (**16**; 90 mg, 0.153 mmol) in MeOH (2.3 ml) and 5N aq. NaOH (153 μl) were stirred for 1 h. The workup as described above afforded **21** (13 mg, 19%). ^{13}C-NMR (125 MHz, MeOD): 17.3 (*q*); 19.8 (*q*);

22.0 (*t*); 30.6 (*t*); 31.9 (*d*); 33.0 (*t*); 33.1 (*t*); 37.1 (*t*); 38.2 (*s*); 38.6 (*t*); 39.7 (*t*); 46.5 (*s*); 52.7 (*d*); 57.7 (*d*); 73.8 (*d*); 75.0 (*d*); 76.3 (*d*); 78.0 (*d*); 79.4 (*d*); 102.2 (*d*); 122.6 (*d*); 130.3 (*d*); 142.4 (*s*); 144.9 (*d*); 177.0 (*s*).

*α-Androstenol β-*D*-Xylohexofuranosiduronic Acid* (*=α-Androstenol β-Glucuronide Furanose Form*; **22**). $BF_3 \cdot Et_2O$ (10.97 mg, 0.077 mmol) was added at r.t. (22.2°) to a mixture of (+)-D-glucuronic acid γ-lactone (136 mg, 0.77 mmol) and **2** (212 mg, 0.77 mmol) in dioxane (2 ml). The mixture was stirred for 6 h at 35° and then concentrated under vacuum. The residue was extracted with AcOEt/EtOH 4 :1 and washed twice with brine. The org. phase was then dried (Na_2SO_4), concentrated under vacuum, and purified by FC (SiO_2; pentane/AcOEt 5 :5) to give α-androstenol β-D-glucurono-3,6-lactone (117 mg, 35%; white solid). The lactone was dissolved in acetone (2.3 ml) and the soln. was cooled in an ice bath. NaOH (0.5M; 1 ml) was added dropwise, and the soln. was stirred for 3 h at 22° before concentration under vacuum. H_2O (3 ml) was added, and purification was performed on an *SPE ENVI-18* 6 ml cartridge (as described above) to afford **22** (44 mg, 36%). ^{13}C-NMR (125 MHz, MeOD): 12.0 (*q*); 17.5 (*q*); 21.9 (*t*); 25.4 (*t*); 29.6 (*t*); 33.0 (*t*); 33.4 (*t*); 33.7 (*t*); 35.3 (*t*); 35.6 (*d*); 37.2 (*s*); 37.3 (*t*); 41.0 (*d*); 46.7 (*s*); 56.8 (*d*); 57.7 (*d*); 72.8 (*d*); 73.5 (*d*); 78.2 (*d*); 82.7 (*d*); 84.2 (*d*); 106.8 (*d*); 130.2 (*d*); 145.0 (*d*); 177.7 (*s*).

Analysis of **17**. To quantify **17** in sweat, four solns. of **17**, prepared in MeOH (0.51, 2.55, 5.1, and 7.65 μg/ml), were injected in to LC/MS (HESI$^-$) in SIM mode (*m*/*z* 448.75–448.75) for the calibration curve (RSQ: 0.9959). The sweat samples were injected in duplicate after concentrating them by ten times, as described above.

Analysis of **21**. The detection limit of **21** was estimated by injecting decreasing concentrations of **21** in MeOH and injecting them in to LC/MS (HESI$^-$) in SIM mode (*m*/*z* 446.75–447.75).

GC/MS Quantification of **2**. This was performed by using α-androstanol as the internal standard (I.S.). The response factor between **2** (274 in SIM mode) and the I.S. (276 in SIM mode) was measured in CH_2Cl_2 solns., containing 0.1 μg of I.S. and 0.051, 0.102, and 0.205 μg of **2**. Prior to injection, the volumes were reduced to *ca.* 20 μl under a gentle Ar flow. Each soln. was injected twice in GC/MS (1 μl; in SIM mode at *m*/*z* 274 and 276) and the following equation was applied for the response factor (rf):

$$\text{rf} = \text{Area I.S.} \times \frac{\text{Mol. wt. of } \mathbf{2}}{\text{Area of } \mathbf{2} \times \text{Mol. wt. of I.S.}} = 0.3845 \pm 0.03.$$

α-Androstenol Formation after Incubation of Sterile Sweat. The I.S. soln. (500 μl, 0.1 μg) was added to the sterile sweat (500 μl) incubated for one night at 37° with the enzyme β-glucuronidase/arylsulfatase (from *Helix pomatia*). The incubated samples were centrifuged at 3260*g* for 3 min. The lower phase was removed with a pipette, and the soln. was washed two times with CH_2Cl_2 (100 μl). The org. layers were combined, dried (Na_2SO_4), filtered on cotton, and concentrated under Ar to *ca.* 20 μl; 1 μl was injected into the GC/MS in SIM mode at *m*/*z* 274 and 276. The concentration was determined from the GC/MS peak areas corrected by the rf value. The result is the average of three samples from the same pool. In parallel, human sterile sweat (or *α-androstenol* conjugates) was incubated with a bacterial soln. to end up with a final concentration of 10^8 cfu/ml.

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