Thesis

The biosynthetic pathway of sulfur-containing molecules in Human Axillary Malodor: from precursors to odorous volatiles

TROCCAZ, Myriam

Abstract

A côté des stéroïdes et des acides gras, une troisième classe de molécules ayant un seuil de perception très faible, les sulfanylakanoles, contribuent à l'odeur caractéristique de la transpiration humaine. Ce travail de thèse a permis l'identification de la bactérie S. haemolyticus, comme capable de générer le (R) et le (S)-3-methyle-3-sulfanylhexan-1-ole (forme majoritaire, ayant une odeur d'oignon) après incubation avec un conjugué de la cystéine et de la glycine présent dans les secrétions axillaires. Les enzymes de S. haemolyticus issues du gene metC tel que la cytathionine β-lyase ont peu d'implication dans cette transformation. Etonnamment, le ratio entre la concentration des précurseurs acides (conjugés de la glutamine) et des précurseurs soufrés est trois fois plus important chez l'homme que chez la femme. Des analyses sensorielles ont confirmé le caractère essentiel de la composition de la sueur sur le développement des odeurs corporelles qui sont ensuite modulées par la flore cutanée.

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THESE

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The biosynthetic pathway of sulfur-containing molecules in Human Axillary Malodor: from precursors to odorous volatiles

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Au bout de tout savoir et de tout accroissement de notre savoir, il n'y a pas un point final, mais un point d'interrogation.

Herman Hesse (1877-1962)

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List of Abbreviations

m_{sw}: sweat rate

3M2HA: 3-Methyl-2-Hexenoic Acid

Apo D: Apolipoprotein D

ASOB1: Apocrine Odor-Binding protein 1

ASOB2: Apocrine Odor-Binding protein 2

ATCC: American Type Culture Collection

C. jeikeium: Corynebacterium jeikeium

Cauxin: Carboxylesterase-like urinary excreted protein

CBL: Cystathionine β-Lyase

CFU: Colony Forming Unit

CGL: Cystathionine γ-Lyase

CGS: Cystathionine γ-Synthase

C-S lyase: Carbon-Sulfur lyase

Cys-*S*-conjugate: Cysteine-*S*-conjugate; [1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteine

Cys/Met metabolism enzyme: Enzyme involved in cysteine and methionine metabolism

Cys-Gly-*S*-conjugate: Cysteine-Glycine-*S*-conjugate; [1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine

DHT: Dihydrotestosterone

DSMZ: Deutsche Sammlung von Mikroorganism und Zellkulturen

DTNB: 5,5'-Dithiobis(2-Nitrobenzoic Acid)

E. coli: Escherichia coli

E3M2HA: (E)-3-Methyl-2-Hexenoic Acid

EDTA: EthyleneDiamineTetraAcetic acid

FMO3: Flavin-containing MonoOxygenase

FSH: Follicle Stimulating Hormone

GC/MS: Gas Chromatography-Mass Spectrometry

GC-AED: Gas Chromatography coupled to an Atomic Emission Detector

HAM: Human Axillary Malodor

HMHA: 3-Hydroxy-3-MethylHexanoic Acid

HSD: 3-HydroxySteroid Dehydrogenase

IPTG: Isopropyl-beta-thio-galactoside

kDa: kiloDalton (1 Da = 1,67 × 10^{-24} g)

LH: Luteinizing Hormone

MD-GC: Chiral Multidimensional Gas Chromatography

MeSH: Methanethiol

MGL: Methionine γ-Lyase

MHC: Major Histocompatibility Complex

NCBI: National Center for Biotechnology Information

Nr: Number of repetitions

OAH/OAS sulphydrylase: *O*-Acetylserine/*O*-Acetylhomoserine Sulphydrylase

ODT: Odor Detection Threshold

PAGE: Polyacrylamide gel electrophoresis

pCBL: Putative Cystathionine β-Lyase

PLP: Pyridoxal-5'-Phosphate

HMHA: 3-Hydroxy-3-Methyl-2-Hexanoic Acid

MSH: 3-Methyl-3-SulfanylHexan-1-ol

SD: Standard Deviation

SDS: Sodium Dodecyl Sulphate

SEM: Standard Error of the Mean; SEM = SD/√Nr-1

S. epidermidis: Staphylococcus epidermidis

S. haemolyticus: Staphylococcus haemolyticus

Tc: hypothalamic core temperature

Ts: average skin temperature

UV-HPLC: High-performance liquid chromatography-UV absorption spectrometry

VFA: Volatile Fatty Acids

VNO: VomeroNasal Organ

VSC: Volatile Sulfur Compound

$Tcset$: set temperature of the blood

$Tsset$: set temperature of the skin

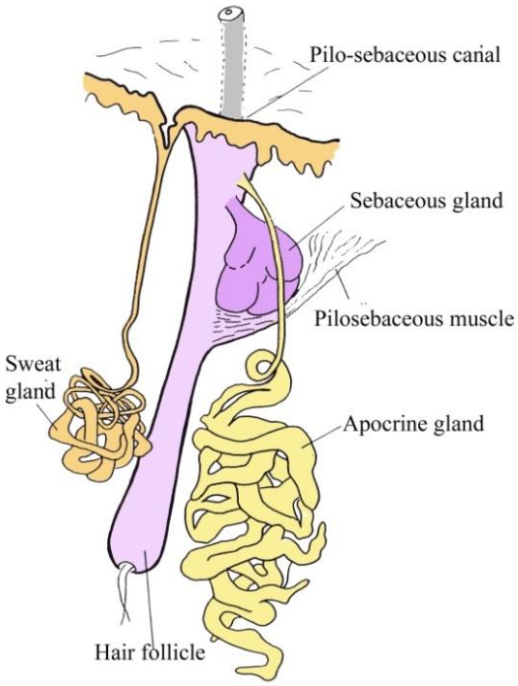
Note: the numbering of compounds, Tables and Figures is not consecutive throughout the thesis, but is consecutive in each of the major chapters.

The biosynthetic pathway of sulfur-containing molecules in Human Axillary Malodor: from precursors to odorous volatiles

Summary of Thesis

Human apocrine sweat gland secretions are known to produce a highly individual scent upon the action of skin bacteria (mainly staphylococci and corynebacteria) that are present in high concentrations in the armpit (see *Scheme 1*). Volatile steroids, aliphatic, branched and unbranched fatty acids have all been reported as major contributors to Human Axillary Malodor (HAM). The odorous (*R/S*)-3-hydroxy-3-methyl-2-hexanoic acid (*R/S*-HMHA) was found to originate from a glutamine conjugate present in axillary secretions and released by the action of a Zn^{2+} dependent aminoacylase from corynebacteria. However, few studies concerning the Volatile Sulfur Compounds (VSCs) have been forthcoming, principally due to the difficulties in studying high impact, low threshold molecules.

Scheme 1: Diagram of a human hair follicle and associated glandular structure.



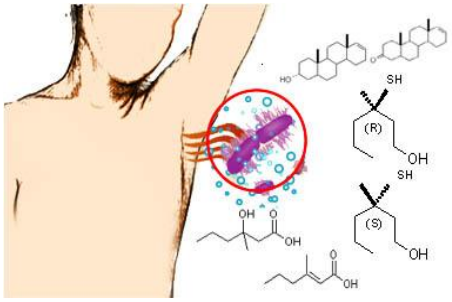
This thesis sets out to redress the lack of knowledge in this area. In 2004 an exercise/sauna facility was built in order to collect underarm sweat secretions from 30 volunteers. Surprisingly, after extensive olfactive evaluation, the axillary isolated Gram (+) strain of *Staphylococcus haemolyticus* produced the most sulfury sweat character after incubation with odorless sweat secretions. From this experiment, (R)- or (S)-3-methyl-3-sulfanylhexan-1-ol (*R*/*S*-MSH) was identified as the major descriptor for the human axilla-sweat odor profile by GC/MS, MD-GC, and GC AED (atomic emission detector) (see *Scheme 2*). (S)-3-methyl-3-sulfanylhexan-1-ol (*S*-MSH) was described as sweat and onion-like; its opposite enantiomer, (*R*)-3-methyl-3-sulfanylhexan-1-ol (*R*-MSH), was described as fruity and grapefruit-like. The (S)-form was found to be the major enantiomer. The odorous sulfur volatile, (*R*/*S*)-MSH was perceived at a concentration 100x lower than the (*R*/*S*)-HMHA and may explain its important contribution in human malodor even when present at low concentrations. Since then, three groups have independently discovered, at the same time, that MSH is a major descriptor for the human axilla-sweat odor.

The biosynthetic pathway of sulfur-containing molecules in Human Axillary Malodor: from precursors to odorous volatiles

Sulphanylalkanols are particularly known in Sauvignon white wine and passion fruit aroma and derived from odorless Cysteine-*S*-conjugates. In this study, the non-volatile precursor of VSCs was investigated. The precursor of MSH was identified as a Cysteine-Glycine-*S*-conjugate **([1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine)**. The *(S)*-enantiomer of this precursor was found to be the most abundant (ratio *(S)*/(*R*) was 4:1). It appears that the *S. haemolyticus* enzyme does not affect the stereochemistry of the volatile organic thiol.

Whereas the axillary malodor is attributable to the microbial biotransformation of odorless, natural secretions into volatile odorous products, gender may influence the sweat odor profile. In the present work, analyses of the acid and thiol precursor concentrations in fresh axillary secretions from 49 males and females was carried out over a period of three years. Surprisingly, the ratio between the acid precursor, a glutamine conjugate, and the sulfur precursor, Cysteine-Glycine-*S*-conjugate, was three times higher in men than in women. Indeed, women have the potential to liberate significantly more volatile sulfur compounds such as *R*/*S*-MSH, with a tropical fruit and onion-like odor than *R*/*S*-HMHA (possibly transformed into *E*/*Z*-3-methyl-2-hexenoic acid), which has a cheesy, rancid odor. Sensory analysis on sweat incubated with isolated skin bacteria confirmed that intrinsic composition of sweat is important for the development of body odors and may be modulated by gender differences in bacterial compositions. Sweat samples having the highest "sulfur" intensity were also found to be the most intense and the most unpleasant.

Scheme 2: Human axillary volatiles.



Although of considerable interest to the cosmetic industry, the identification of metabolic mechanisms involved in the generation of VSCs in axillary malodour has proved difficult. A primary reason is a lack of knowledge regarding the enzymes involved. Cysteine-Glycine-*S*-conjugates are key intermediates in the glutathione biodetoxification pathway, however, neither glutathione-*S*-conjugate nor Cysteine-*S*-conjugate was detected in sterile sweat samples. In addition, incubation of glutathione-*S*-conjugate never led to the formation of thiol under the experimental conditions employed. During the study of the bio-generation of MSH, it was possible to show that enzymatic extracts from *S. haemolyticus* were more active on synthetic Cysteine-

Glycine-*S*-conjugates with respect to synthetic Cysteine-*S*-conjugates, whereas it is the reverse after incubation with *C. jeikeium* bacterial strain.

The last part of this thesis was to study *S. haemolyticus met*C enzymes in the generation of volatile axillary sulfur compounds in the underarm region. A pyridoxal-5'-phosphate (PLP)-dependent *met*C-cystathionine *β*-lyase gene (CBL; EC 4.4.1.8) from the axillary isolated strain *S. haemolyticus* Ax3 has been cloned and characterized. From this work, we conclude that *Staphylococci metC* CBL, whose function within the growing cell is primarily related to amino acid catabolism, appears unlikely to be involved in sulfur compound generation in sweat. Recently, a MalY-type Carbon-Sulfur *β*-lyase from *C. jeikeium* and *C. striatum* similar to bacterial aminotransferase has been reported to be involved in the generation of thiols from cysteine-*S*-conjugate. Thus, future work involving gene knockout or the expression of *S. haemolyticus* MalY-type enzymes with *β*-lyase activity may confirm whether a single enzyme or a multiple enzyme mechanism is responsible for the release of sulphanylalkanols by *S. haemolyticus* in body odors.

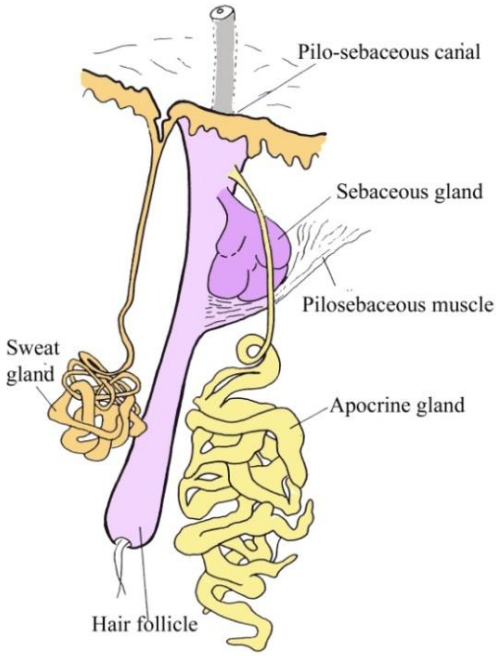
To conclude, this thesis has contributed to the advance in the formation of human perspiration odor by skin bacteria. Besides steroids and volatile fatty acids, a third class of volatiles, the odorous sulfanylalkanols, has now been synthesized to recreate body odors in cosmetic perfume performance evaluation. Even though huge variations were found between individuals, sensory analysis confirmed that intrinsic composition of sweat is important for the development of body odors and may be modulated by gender differences in bacterial compositions. Interestingly, we found that an axillary isolated strain of *S. haemolyticus* Ax3 generated the most intense underarm odor after incubation with a cysteine-glycine-*S*-precursor present in odorless sweat secretions. These results open a new perspective in volatile thiol formation. Advances in the field include bacterial strain identification, new analytical chemistry, sensorial analysis and discovery of genes which are involved in the release of volatile thiols. Finally, this work could form the basis of a new strategy for the counteraction of male and female body odor.

The biosynthetic pathway of sulfur-containing molecules in Human Axillary Malodor: from precursors to odorous volatiles

Résumé de Thèse

Les secrétions humaines des glandes sudorales apocrines sont reconnues pour exprimer une odeur propre à chaque être sous l'action de la flore cutanée (principalement des staphylocoques et des corynébactéries) présente sous les aisselles (voir *Schéma* 1). Les stéroïdes volatiles ainsi que les acides gras aliphatiques, branchés ou non, ont été reportés comme contributeurs majeurs dans l'odeur désagréable de transpiration. L'odeur du (*R/S*)-3-hydroxy-3-methyle-2-hexanoique acide (*R/S*-HMHA) a pour origine l'action d'une aminocylase zinc-dépendante de corynébactéries sur un conjugué de la glutamine présent dans les secrétions axillaires. Cependant peu d'études ont montré l'implication des composés volatiles soufrés dans l'odeur de sueur, notamment à cause des difficultés pour étudier des molécules à impact olfactif fort avec des seuils de détection très faibles.

Schéma 1: Structure d'un follicule de poil humain et des glandes axillaires associées.

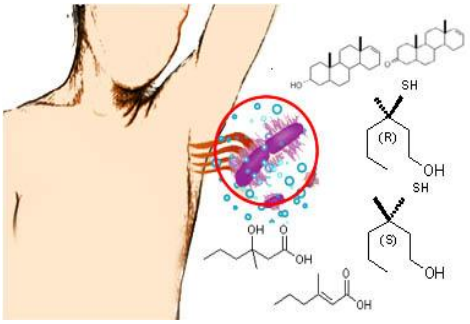


Ainsi, ce travail de thèse vise à combler le manque de connaissance dans ce domaine. En 2004, une salle d'exercice avec sauna a permis la collecte de sécrétions axillaires à partir d'une trentaine de volontaires. Des évaluations olfactives ont démontré le rôle important de *Staphylococcus haemolyticus* (bactérie Gram positive isolée des aisselles) dans la formation des odeurs soufrées. Suite à ces travaux, le (R) ou le (S)-3-methyle-3-sulfanylhexan-1-ole (*R*/*S*-MSH) a été décrit comme un des descripteurs majeurs de l'odeur de transpiration humaine par l'analyse GC/MS, MD-GC et GC-AED (détecteur à émission atomique) (voir *schéma 2*). Le (S)-3-methyle-3-sulfanylhexan-1-ole (*S*-MSH) a une odeur de sueur et d'oignon alors que la forme (R) a une odeur plus fruitée comparable au pamplemousse. L'énantiomère majeur présent dans la sueur est la forme (S). Le racémate a un seuil de perception 100 fois plus faible que l'hydroxy-acide HMHA ce qui peut expliquer sa forte contribution dans l'odeur de transpiration même lorsqu'il est présent en faible quantité.

Depuis cette découverte, trois groupes ont isolé indépendamment le MSH dans des prélèvements de transpiration humaine.

Les sulfanylalkanoles sont particulièrement connus dans le Sauvignon blanc et les fruits de la passion. Ils sont généralement dérivés de conjugués de la cystéine présents dans le mou ou le fruit. Dans cette étude, la recherche des précurseurs soufrés de l'odeur de transpiration humaine a permis d'isoler un conjugué de la cystéine et de la glycine. La forme énantiomérique (S) de ce précurseur est la plus abondante (avec un ratio S/R de 4:1). Il semble donc que l'action de l'enzyme de *S. haemolyticus* ne change pas les rapports énantiomériques.

Schéma 2: Molécules odorantes présentes dans l'odeur de transpiration.



Alors que l'odeur désagréable de sueur relève d'une transformation bactérienne, d'autres facteurs tels que le sexe peuvent influencer l'odeur générale. Pour élucider cette question, l'analyse des précurseurs des acides et des soufrés a été conduite à partir de secrétions non-odorantes issues de 49 hommes et femmes sur une période de trois ans. Étonnamment, le ratio entre la concentration des précurseurs acides (conjugués de la glutamine) et des précurseurs soufrés (conjugués de la cystéine et de la glycine) est trois fois plus important chez l'homme que chez la femme. Ainsi, nous pouvons en déduire que les femmes produisent potentiellement plus de composés soufrés volatiles tels que le MSH, qui a une odeur fruitée et oignon que de l'hydroxy-acide (éventuellement transformé en 3-methyle-2-hexenoique acide) qui a une odeur plus proche du rance ou du fromage. En parallèle, des études sensorielles à partir de bactéries humaines ont confirmé le rôle important de la composition de la sueur sur la formation des odeurs, qui peuvent être modulées par les différences de compositions de la flore cutanées entre les deux sexes. Il a été démontré que les échantillons de transpiration ayant une odeur soufrée plus intense étaient aussi jugés comme les plus désagréables pour le nez humain.

Bien que cela suscite un grand intérêt pour l'industrie cosmétique, l'identification des mécanismes impliqués dans la formation des odeurs soufrées de transpiration se révèle difficile. Une des premières interrogations concerne les enzymes impliquées. Les conjugués de la cystéine et de la glycine sont des intermédiaires clés bien connus dans le mécanisme de détoxification du glutathion. Cependant, ni le conjugué de la glutathione ni le conjugué de la

cystéine n'ont été détectés dans les échantillons de sueur. De plus, l'incubation des conjugués de la glutathionne n'a jamais permis la production de thiols volatiles dans les conditions d'expérience utilisées. Nous avons démontré que l'addition de *S. haemolyticus* était plus actif sur les conjugués soufrés de la cystéine et de la glycine que sur les conjugués de la cystéine alors que cette tendance est inversée après incubation avec *C. jeikeium*.

La dernière partie de cette thèse avait pour objectif d'étudier les enzymes de *S. haemolyticus* issues du gene *met*C. La cytathionine *β*-lyase de *S. haemolyticus* (une enzyme nécessitant le pyridoxal phosphate comme cofacteur) (CBL, EC 4.4.1.8) a été clonée et exprimée chez *E. coli*. De ce travail, nous pouvons conclure que cette enzyme, qui trouve son importance dans la formation des acides aminés, n'est pas responsable de la formation des odeurs soufrées de la transpiration. Récemment, des enzymes *β*-lyases de la liaison carbone-soufre de *C. jeikeium* et *C. striatum* de type MalY ont été décrites. Des travaux futurs impliquant la suppression ou l'expression de ce type de gènes présents chez *S. haemolyticus* permettront de confirmer si un tel mécanisme existe chez *S. haemolyticus* et si une ou plusieurs enzymes sont impliquées.

Pour conclure, ce travail de thèse a contribué à l'avancement majeur dans la recherche des molécules présentes dans les odeurs de transpiration humaine. A côté des stéroïdes et des acides gras volatiles, une troisième classe de molécules, les sulfanylakanoles, sont aujourd'hui synthétisés pour recréer des modèles d'odeurs corporelles pour l'évaluation de parfums destinés à des applications tels que les déodorants. Même si d'importantes variations ont été trouvées entre les individus, des analyses sensorielles ont confirmé le caractère essentiel de la composition de la sueur sur le développement des odeurs corporelles qui sont ensuite modulées par les bactéries présentes sur la peau. Dans ce cadre là, la bactérie *S. haemolytis* Ax3 isolées des aisselles, est capable de générer une odeur soufrée caractéristique de la transpiration humaine après incubation avec un conjugué de la cystéine et de la glycine présent dans les secrétions axillaires. Ces résultats permettent d'ouvrir de nouvelles persceptives sur la formation des composés volatiles soufrés. Les avancées dans ce domaine impliquent l'identification bactérienne, de nouvelles méthodes analytiques et d'analyses sensorielles, ainsi que la découverte de nouveaux gènes impliqués dans la formation des volatiles soufrées. Finalement, ce travail ouvre de nouvelles stratégies pour contrecarrer la formation des odeurs corporelles désagréables indépendamment chez l'homme et la femme.

1. INTRODUCTION

1.1. General picture of human body odors

1.1.1. Human skin: structure and function

The skin is a highly complex and organized structure comprising two main layers: the outer **epidermis** (0.05-1.50 mm thickness) and the inner **dermis** (3.0-5.0 mm thickness**)**, separated by the basement membrane. The deeper **hypodermi**s (subcutaneous or fat layer) is situated below the dermis (*Fig. 1*). 90% of the epidermis cells are keratinocytes, the remaining cells are melanocytes, producing melanin as skin color and UV protection, and Langerhans cells (known as dendritic cells), involved in the epidermal immune system. The dermis comprises a dense matrix of connective tissue (90% of the dermis is collagen) and various cells including fibroblasts, macrophages, mast cells and lymphocytes, together with nerves, blood vessels, and lympatics. The dermis also contains a variety of **epidermal appendages** such as **hair follicles**, **eccrine and apocrine sweat glands,** and **sebaceous glands**. The skin and its appendages together are called the **integumentary system** [1][2].

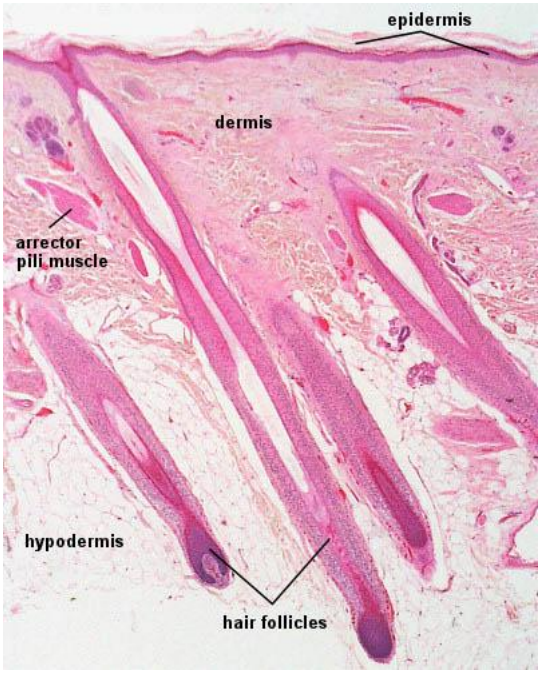


Fig. 1. Microscopic view of a histologic specimen of human skin stained with hematoxylin and eosin[1]

The skin is the body's largest organ. Ten percent of the average adult's weight is skin, and it covers a surface area of nearly 2 square meters. It is a vital part of immune system; 5% of the body's macrophages, the Langerhans cells, reside beneath the skin, and a sensory organ that transmits sensations such as pressure, touch, warmth, cold, and pain. The skin provides an interface between the external environment and the internal media of the living organism. As such, its major role is to protect and maintain the internal composition of the body, by limiting the entry of unwanted agents, including bacteria and xenobiotics, whilst preventing the escape of vital constituents such as nutrients and water. The epidermal skin layer is also implicated in the production of Vitamin D_3 (cholecalciferol) when exposed to the sun's rays, specifically ultraviolet B radiations [3].

[1]http://www.lab.anhb.uwa.edu.au/mb140/CorePages/integumentary/integum.htm *(University of Western Australia)*

1.1.2. Sweat glands present on skin

a) *Sudoriferous glands in humans and histology*

Sweating is the elaboration of a fluid secretion on the body surface produced by two sweat (sudoriferous) glands, named for the first time in 1917, **the eccrine** and **the apocrine sweat glands** [4]. These glands are developed only in mammals, in which they evolved, along with **sebaceous glands**, mainly for defence at the skin surface against microbial invasion and for thermoregulation function. In some mammalian species, *e.g.* the hippopotamus, the elephant, or members of the Cetacea, sweat glands are absent; in others, the glands either do not respond to heat, as in the pig or rodent, and cat footpads, or play an insignificant role in the control of body temperature, as in sheep and goats [5-7]. Humans have more eccrine glands than appocrine glands on body surfaces, whereas gibbons and orang-utans have more apocrine glands than eccrine glands in their hairy skin [8]. In contrary to the eccrine glands, sebaceous and apocrine glands are under the control of hormones and start to be active at puberty, suggesting that their activity might be related to sexual reproduction. In humans there are two other sudoriferous glands, ceruminous glands (producing ear wax) and mammary glands (producing milk), which will not be discussed here.

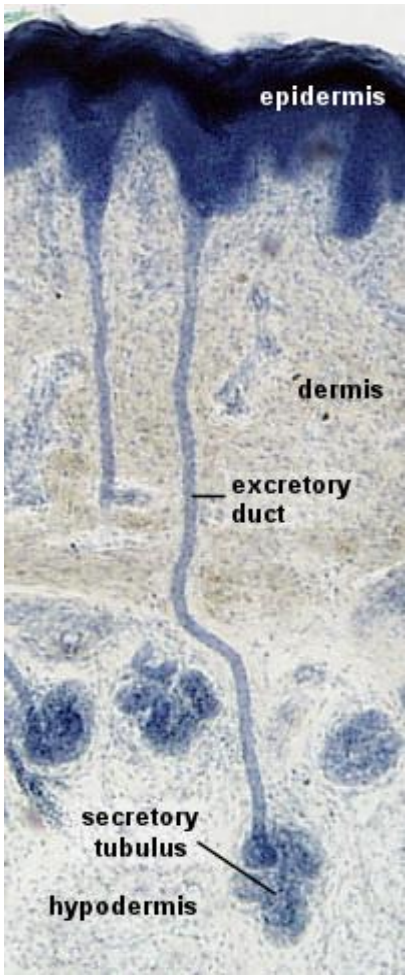


Fig. 2. Light micrograph section through an eccrine gland[1].

Eccrine sweat glands (merocrine glands) are of critical importance for the regulation of body temperature. They are stimulated by sympathetic cholinergic nerves, which are controlled by a center in the hypothalamus. Indeed, lesions of hypothalamus regions generally results in anhidrosis [7].

Eccrine sweat glands are simple tubular glands *(Fig. 2).* The excretory ducts empty directly onto the surface of the skin. *It* is estimated that between 2 and 4 million eccrine sweat glands are distributed over almost the entire body surface of humans, which are capable of secreting about 1.3 litres of fluid per hour (if we consider that 50% of the glands are active and one individual gland produces 4-28 nl/min of sweat) [7][9]. The highest densities of eccrine glands (up to 700/cm^2) are found on the back, chest, and forehead. In 1987, the presence of an anomalous sweat gland, the **apoeccrine gland**, with a duct similar to eccrine

gland, was described [10]. These glands apparently develop after adolescence in both sexes and are able to produce nearly sevenfold larger volumes of secretion than the eccrine sweat gland. However, the existence of the apoeccrine gland remains controversial. No evidence of apoeccrine glands was found either by histology or by immunofluorescence [11].

Apocrine glands are larger sweat glands, restricted to the hirsute areas, predominantly in the axillae, perineal region, mammary areolae, scalp, and abdomen. They do not contribute to thermoregulation, but rather serve as scent glands [9]. The excretory duct of apocrine sweat glands does not open directly onto the surface of the skin. Instead, the excretory duct empties the sweat into the upper part of the hair follicle *(Fig. 1)*. Apocrine sweat glands are therefore part of the pilosebaceous unit comprising the follicle hair and sebaceous glands. Apocrine sweat produced by an individual gland in response to stimulation is approximately 0.001 ml with important race and gender variations (see *part 1.1.3*) [12][13]. Apocrine glands are stimulated by emotional stress (pain and fear) and sexual excitement. The injection of sympathomimetic drugs, such as epinephrine (adrenaline), stimulates them, whereas they may be blocked by injection of a local anesthetic drug such as procaine (also named novocaine or 2-(diethylamino)ethyl 4-aminobenzoate) into the skin [7][14].

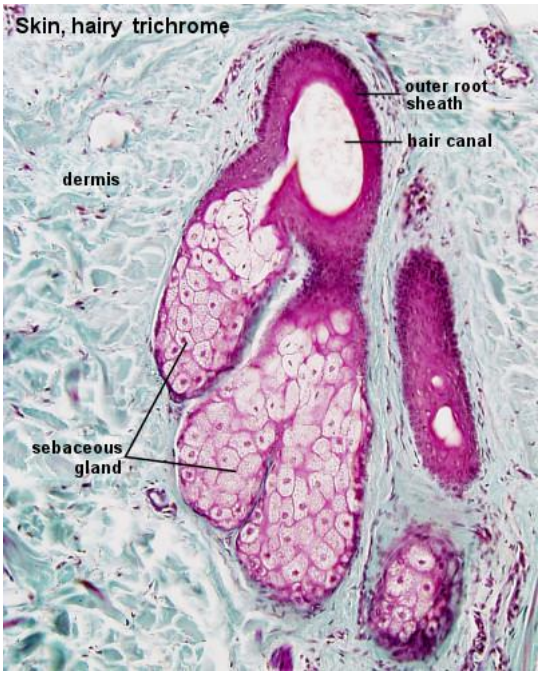


Fig. 3. Light micrograph section through a sebaceous gland[1].

Sebaceous glands (holocrine glands) empty their secretory product into the upper parts of the hair follicles (*Fig. 3*). They are widely dispersed all over the body and are specially found in parts of the skin where hair is present. However, sebaceous glands are also found in some of the areas where no hair is present, for example, lips and external genitalia [9]. Cell size increases with the accumula-tion of sebum as the cells are gradually displaced towards the opening of the gland into the hair follicle. Androgen is the dominant hormonal influence on sebaceous gland secretion and hair growth [15][16]. Clinically, the sebaceous glands are important in that they are liable to infections (acne) [15].

b) *Chemical composition of human sweat secretions*

Sweat has a pH range from 4.0 to 6.5 and is hypoosmotic relative to plasma [17]. It contains 99% water and varying concentrations of minerals, such as sodium (< 40 mM), potassium (4-20 mM), chloride (< 20 mM), carbonates (HCO_3^-) (15-20 mM), ammonia (0.5-8 mM) but also lactates (10-15 mM), urea (0.15-0.25 mg/ml), glucose (2-5ug/ml) and proteins (0.15-0.25 mg/ml). Trace elements of zinc (0.4 mg/l), copper (0.3-0.8 mg/l), iron (1 mg/l), chromium (0.1 mg/l), nickel (0.05 mg/l) and lead (0.05 mg/l), but probably many other less abundant trace minerals are excreted in sweat [18][19]. Substances such as urocanic acid have been identified in sweat as offering a protection for the skin against ultraviolet radiation [20]. Major proteins are albumin and α-1-antitrypsin, but enzymes (α-amylase, esterases), glycoproteins, renin-like substances and antibodies (IgA, IgE, and IgC) are also present (reviewed in [7]). A number of defence peptides against infectious agents, such as dermcidin, cathelicidins and defensins (produced by skin cells), have been identified [21]. Free amino-acids such as serine, glycine, and alanine occur in higher concentrations than proteins (see C*hapter 5; Appendix 7.3,* [7]).

The **apocrine sweat** is particularly rich in lipids (20 µg/µl, around 2% of apocrine sweat) and proteins (around 10% of apocrine sweat). 76.2% of the lipids are cholesterol, 19.2% are glycerides and fatty acids. Excised apocrine glands contain a number of enzymes such as *β*-glucuronidase, 3-hydroxysteroid dehydrogenase, 4-ene-5α-reducase, esterase, as well as lysozyme [22].

Human sebaceous cells produce a thick oily secretion which is unpigmented. The chemical composition of sebum changes as it passes along the ducts. Lipophilic enzymes present in the duct break down triglycerides into diglycerides, monoglycerides and free glycerol, which serve as a reservoir for the release of (odorous) free fatty acids, representing a quarter of surface lipids; 60% of the sebum is triglycerides, 23% wax esters, 12% squalene and less than 1% sterol esters [9]. Squalene is used as a fixative in fragrances and may be important in prolonging axillary odor.

The composition of gland secretions varies according to several factors: the particular stress source (exercise, sauna, etc.), the acclimatation to heat, the duration of sweating, and the composition of minerals in the body. In addition, difficulties in defining the precise composition of sweat are due to the contamination of excreted secretions by contact with substances on or in the skin surface. Care is also needed to ensure that sweat does not become concentrated by evaporation. Therefore, the given concentrations are only indicative and may vary greatly between individuals.

1.1.3. Factors influencing human body odors

a) *Skin bacteria*

The generation of odor on various sites on the human body, *e.g.* foot, mouth, or axilla, is mainly caused by microbial transformation of odorless natural skin secretions into volatile odorous molecules. The addition of antimicrobial substances in deodorants has therefore considerably reduced body odor formation. However, it is commonly known that gender, ethnicity, along with emotional, physiological and environmental factors also influence the composition and quantity of one's sweat (*Fig. 4*).

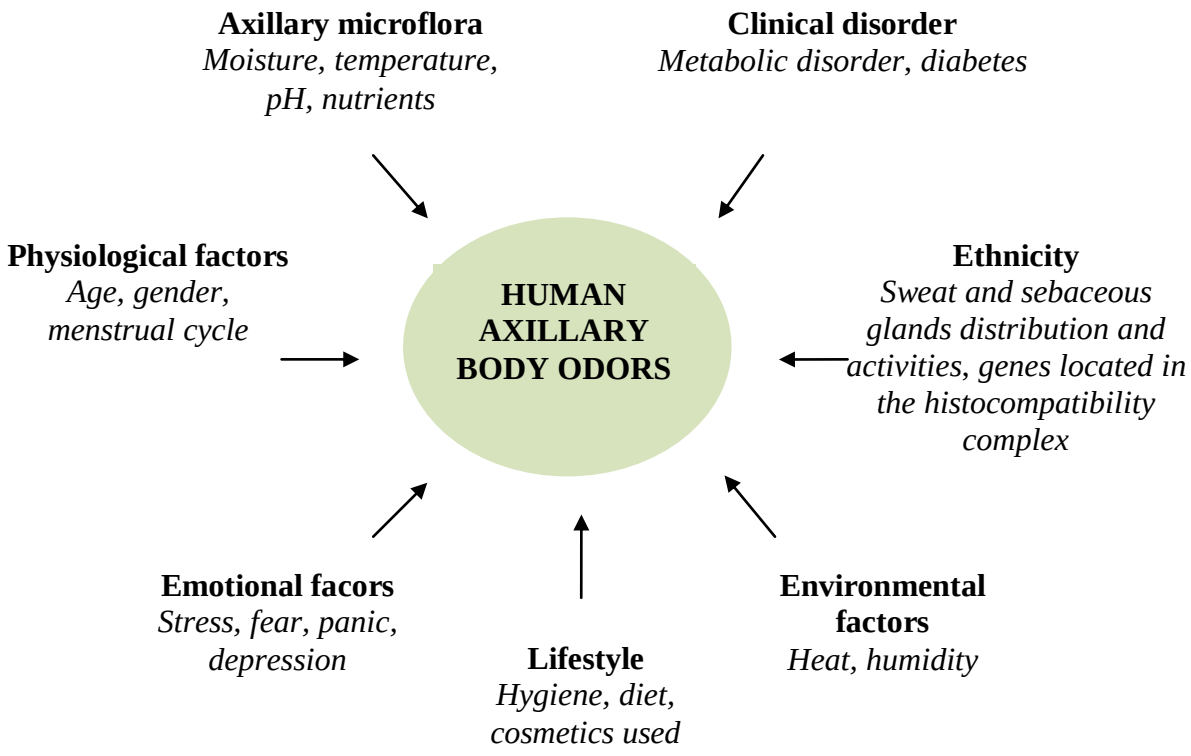


Fig. 4. Factors influencing differences in human sweat secretions and axillary body odor.

In humans, the strong and generally unpleasant odor emanating from the armpit is correlated to the high number of bacteria (10^6 cell/cm^2) present in this humid body area (*see part 1.2)* [22][23]. The axillary sweat was found to be richer in volatiles and semi-volatiles than saliva or urine [24]. Sweat glands deliver water to the surface of the axilla and provide the

critical moisture required for bacterial proliferation. In addition, sweat contains amino-acids and minerals such as copper, iron, magnesium and zinc which are important for bacterial growth. The majority of skin bacteria are Gram positive, belonging to the following groups: micrococcaceae, mainly *Staphylococcus* species; aerobic coryneforms, primarily *Corynebacterium* species, and anaerobic/microaerophilic *Propionibacterium* species (*Table 1*, [22][23][25]). Of the staphylococci isolates, 60% are *S. epidermidis*, 12% *S. haemolyticus*, 6% *S. hominis*, 3% *S. capitis* and 3% *S. saprophyticus*, respectively. Of the propionibacterial isolates, 46.9% are *P. acnes*, 40.6% are *P. avidum* and *P. granulosum* was less prevalent at 12.5% [22][23][25].

Table 1. The percent carriage and population density of microbial groups in the axillae of 34 male subjects [22][23][25].

	Mean of both axillae	
	% of people carrying each type of bacteria	Density (log CFU/cm^2)†
Total bacteria	-	5.85 ± 0.16
Aerobic *Coryneform*s	97.1	5.79 ± 0.30
Total *Micrococcacea*e	100	4.76 ± 0.37
Staphylococcus	85.3	4.38 ± 0.51
Micrococcus	52.9	1.90 ± 0.52
Propionibacterium	100	3.66 ± 0.35
Gram-negative bacteria	5.9	*
Yeasts	2.9	*

*density not calculated due to low number of carriers; †mean log CFU/cm^2 ± SEM. CFU: Colony Forming Unit.

The great stability of the skin flora has been globally described as “host resistance”, and has been attributed to such factors as physiological skin pH, relative skin humidity, skin lipid composition, desquamation of the stratum corneum, skin temperature and the interaction between resident and transient members of the skin flora. In the skin flora ecosystem, there are complex interdependences, feedbacks and multiple interactions among all these factors, which would be extremely difficult to simulate *in vitro*. Cutaneous microorganisms have been shown to produce bacteriocins with inhibitory effects on the growth of other bacteria and such inhibition may occur between species residing on the skin [26][27]. Additionally, there may be an interdependence between bacterial groups residing on human skin [28].

During the summer season, the bacterial numbers recovered from both axillae are reported to be higher, with aerobic corynebacteria exhibiting the greatest variations in numbers with a range of more than four orders of magnitude [29]. However, the magnitude of the variation in the total number of bacteria in each axilla of one individual is less than that for each individual group, which may indicate a limit to the total biomass that can be sustained by the axilla [29][30]. Reasons for the large variations in bacterial numbers between individuals are not

known, but may well reflect changes in local environment, *e.g.*, extent of gland secretions on axillary skin or humidity levels [31]. Under conditions of high nutrient supply and humidity, the growth of coryneforms is favoured, and it is possible that they are also able to suppress the growth of cocci [32][33]. Jackman has also confirmed the presence of two distinct types of axillary flora, either a coryneform or coccal dominated flora, with the coryneforms being more prevalent in males and contributing the more pronounced body odor [32][33]. Apocrine sweat components, sebum and the breakdown products of keratin, but also other cell debris, can serve as substrates for bacterial degradation, and may contribute to the overall odor. Lipophilic and large colony corynebacteria are generally described as generating typical pungent axillary odors and micrococci a sweaty, isovaleric acid type odor when incubated with apocrine sweat [33]. However, some diphteroids (corynebacteria) after repeated subcultivation failed to generate strong odors on incubation with apocrine secretion [33].

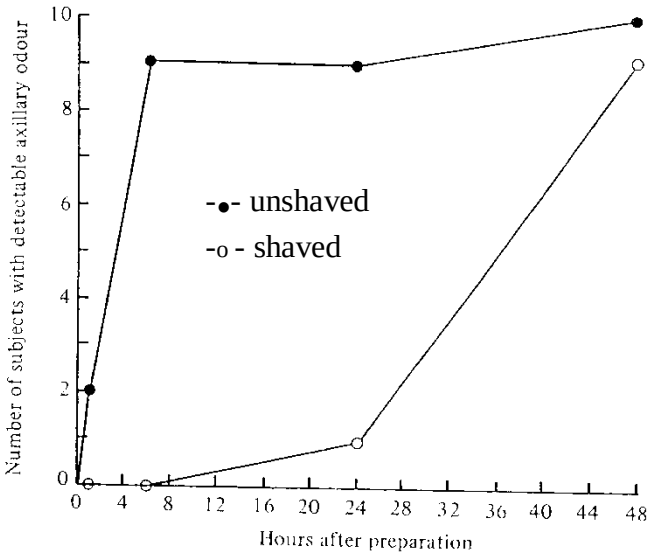


Fig. 5. Effect of shaving the axillae on axillary odor [9].

b) *Lifestyle: diet, hygiene, and cosmetic use*

Though secretions are considered inodorous, the diet such as garlic and curry absorbtion may influence the character of these secretions. Tsuda and co-workers have found that thirty minutes after the intake, caffeine was secreted with sweat, and its detection continued for more than 4 h [34]. A protein-rich diet may also influence sweat composition. It was reported that eating habits such as a non-meat diet, induce a less intense body odor, without a clear explanation of the mechanism involved [35]. Similarly, people with a fish diet reportedly exude a fishy, trimethylamine-like scent [9].

Hygiene habits such as shaving axillae or use of cosmetics may change odor profile by increasing odor substantivity or changing bacterial profile, sweat volumes or metabolites. Axillary hair provides a very large surface that may lodge bacteria but also increases odor substantivity (*Fig. 5*) [9][36]. Data on male sweat are generally more documented in

comparison to female sweat as women extensively use antiperspirants which may reduce apocrine sweat gland response and modify skin microflora or microflora metabolism [37][38]. For instance, the skin bacterium *C. jeikeium* was shown to respond with differential gene expression under the influence of the cosmetic ingredient 4-hydroxy-3-methoxybenzyl alcohol [39]. Antiperspirants may reduce or obliterate the apocrine and sebum sweat gland response, *e.g.* reduce substrates for bacterial growth and transformation [13][37][40]. Some ingredients may also directly provide nutrients for the resident microflora, *e.g.* glycerine, amino-acids, hydrolysed collagen, or they may contain antimicrobials that increase the presence of resistant strains on skin and/or decrease or change the bacterial profile [40].

c) *Environmental factors: heat and humidity*

Sweating is our most effective means of dissipating heat in hot environments. As mentioned previously (*part 1.1.2*), sweating is controlled from a center in the preoptic and anterior regions of the hypothalamus where thermosensitive neurons are located. In a hot environments, there is an increased sweat rate (m_{sw}, [41][42]), increased sweat gland sensitivity relative to the hypothalamic ('core') temperature (T*c*), and a decreased hypothalamic set point (T*cset*) for sweating [43]. The heat regulatory function of the hypothalamus is also affected by inputs from temperature receptors in the skin: high skin temperature (T*s*) reduces T*cset* and increases the gain of the hypothalamic feedback system in response to variations in Tc.

The following mathematical equation summarises the major influence on the local sweat rate [44]:

$$\frac{dm_{sw}}{dt} = a(Tc - Tcset) + b(Ts - Tsset)$$

Where $\frac{dm_{sw}}{dt}$ is the local sweating rate, Tc is the core temperature, Ts is the average skin temperature, $Tcset$ and $Tsset$ are the set temperature of the blood and the skin, respectively.

The process of sweating decreases core temperature T*c*, whereas the process of evaporation of sweat from the skin surface decreases surface temperature (cooling effect). Hence, in hot weather, or when the individual's muscles heat up due to exercise, more sweat is produced. In humid environments, in which the evaporation process is less effective, more sweat will be produced to counteract this effect [45].

d) *Physiological factors: age, gender, and menstrual cycle*

It was reported that apocrine glands are non active in children, their activity is initiated at puberty and they become "non-functional" after menopause in females (no information is available in males) [13][46]. Sweat electrolytes content increases with age. In adults, sweat electrolytes were found to be twice the level of children, whereas sebum production decreases, which may be related to changes in hormonal levels [47]. In both sexes, skin lipid production is highest between the ages of 20 to 39 years [9].

The ratio between cocci and coryneform bacteria in the armpit seems to be inverted according to the gender (more coryneforms in male axillae) [32]. Sweat composition, e.g bacterial substrates, may also vary according to gender. Women have 75 % more apocrine glands in their armpits than men, but male apocrine glands are larger and may be more active [9]. The skin pH of men is slightly less acid (pH 5.8) as compared to women (pH 5.5) [48][49].

Female body odor is more variable due to the menstrual cycle. Androgen hormones are known to increase during the menstrual cycle. For instance, it has been shown that production of these hormones peak around the time of ovulation [50][51]. Sebum glands are androgen-dependent in both sexes; this could explain why they are larger in men than in women. Flushes of acne, a common affliction of the sebaceous ducts, in the days immediately preceding menstruation suggest that progesterone might also be involved, whereas oestrogens appear to inhibit sebaceous secretions [46].

e) *Genetics and ethnicity*

Even if day-to-day variation exists, individuals are thought to have their own distinctive scent, analogous to a signature that may be genetically determined [24][52]. Recently, Kuhn and Natsch have shown that common pattern of odorant carboxylic acids may be found in monozygotic human twins [53]. Those results are in agreement with previous studies in which odors of identical twins (but not dyzygotic twins) were matched by the human nose at rates higher than chance, even when the twins are living apart [54]. It was found that the sweat gland distribution and activity and the human body odor may be due to genes located in the Major Histocompatibility Complex (MHC) [55][56]. In those studies, an association between body odor types and genes in the human leucocyte antigen (HLA) locus appears to exist.

A number of workers have pointed out that substantial racial differences exist in axillary organ size, especially in apocrine glands [57]. The term 'race' is preferred to 'ethnic' as it defines one specific population in terms of genetic similarities (*e.g.* Japanese) whereas 'ethnic' usually defines groups of populations with common culture and/or language [9][56][58][59]. In

African Negroes and Europids the axillary organs are large and highly active, whereas Mongolids and Koreans have no or only few apocrine glands [9][12]. Only two to three percent of Chinese have any axillary odor and when they do, it is said to be musky in character (steroid-like odors) [9]. A recent review on the differences between ethnic skin types reveals increased pore size, increased sebum secretions and skin surface microflora in black subjects, but low stratum corneum lipids and lower desquamatory enzyme levels [58]. Concentrations of chloride in sweat were found to be lower in white Americans compared to black ones. Asian skin may be more sensitive to exogenous chemicals, probably due to a thinner stratum corneum and a higher eccrine gland density [58].

f) *Emotional factors*

Both apocrine and sebum secretions have been reported to increase due to certain emotional factors. Higher levels of apocrine sweating have been demonstrated in response to stress such as pain and fear [13]. Similarly, stress, fear, panic, and depression may increase sebum secretion through increased androgen hormone levels and the subsequent release of acetylcholine [60].

g) *Clinical disorders*

Spinal chord injuries may induce hyperhydrosis and more intense axillary odor as a consequence [61]. The presence of ammonia in sweat odors may be due to a renal insufficience. It has also been shown that hospitalized adults may increase skin colonization with antibiotic resistant bacterial strains [62].

Diabetes and metabolic disorders may also influence body odors. For instance, trimethylaminuria (or fish odor syndrome) includes a transient or mild malodor caused by the production of an excessive amount of malodorous trimethylamine. The causal factor has been shown to be either reduced trimethylamine oxidase enzyme capacity, or may be due to substrate overload. Both of which could also be the result of an inherited deficiency hormonal modulation, or liver damage [63]. For trimethylaminuria, at least 40 genetic polymorphisms of the flavin-containing monooxygenase (FMO3) gene have been reported [64][65]. Menstruation can also be an additional factor causing transient trimethylaminuria, even in healthy women harbouring functionally active FMO3 [66].

1.1.4. Odorous axillary volatiles

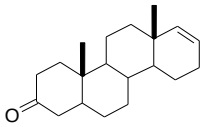
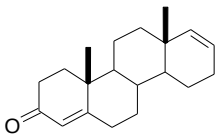
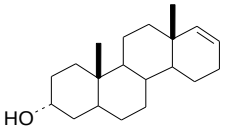
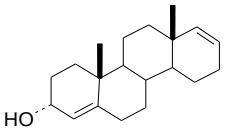
A wide range of volatile malodorous substances has been implicated in axillary odor and is thought to be microbiologically produced from precursors contained in the apocrine secretions [67]. The dominant notes are: sweat, musky, goat-like, urinous, onion-like odors, imparted by three major classes of compounds: **steroids**, **fatty acids** and **sulfur compounds**. Other volatiles such as aldehydes, esters, ketones, and alcohols may act as odor modifiers. In this paper we do not refer to artificial contamiants that may occur in soaps, cosmetics, fragrances, shampoos, detergents and tobacco that may be found in human axillary samples but have no bacterial or metabolic origin [24].

a) *Volatile steroids*

Many steroids have been detected in axillary secretions. Of particular interest are cholesterol, unsaturated steroids of the androstane (C19) family (androsterone and dehydroepiandrosterone), and 16-androstenes, including the odoriferous **5α-androst-16-en-3-one** (androstenone), **androsta-4,16-dien-3-one** (androstadienone), and their respective alcohols, **5α-androst-16-en-3α-ol** (androstenol) and **androsta-4,16-dien-3α-ol** (androstadienol) (*Table 2*) [68-70]. Ohloff *et al.* established that many 3α-sterols have intense odors, whereas the corresponding 3*β*-sterols usually have little or no detectable odor [70]. Gower was the first to detect androstenone in the axilla [71], androstenol was then detected on axillary pads in 1974 [72]. Studies by Monel Institute found a significant change in androstenol levels throughout the menstrual cycle [73]. The level of the odorous steroid 5α-androst-16-en-3-one, was found to be significantly higher in men than in women (five times more) and the levels are linearly related to the axillary cholesterol concentrations, but not to those of squalene (present in sebum) [68][71]. 16-androstenes are known to be present in large quantities in boar and pigs saliva as sexual attractants (see *Part 1.1.4.d*, [68]). They have musky and urine-like odors [72][74][75]. However, human olfactory perception differs enormously between individuals. Androstenone (5α-androst-16-en-3-one) is variously perceived as offensive ("sweaty, urinous"), pleasant ("sweet", "floral"), or odorless [76][77]. Compared to males, the perception of females to androstenone is more unpleasant. One explanation may be that it is present in male sweat in greater quantities that in female sweat. Indeed, males have a lower sense of its presence [77]. The mean olfactory threshold is as low as 0.18 parts per billion in gas phase (and 6.2 ppb for its respective alcohol) [78]. Recently, Keller *et al* reported that genetic variation in a human odorant receptor, OR7D4, may be responsible for the variation in

perception of the androstenone and androstadienone steroidal odors [79]. At a concentration of one-tenth of detection threshold, androstenone has been shown to enhance the intensity of unpleasantness of short-chain fatty acids [77].

Table 2. Major volatile odoriferous steroids present in axillary body odors.

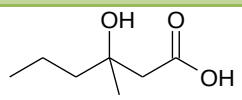
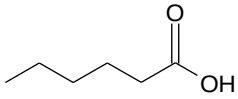
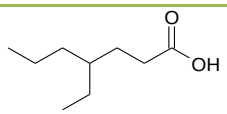
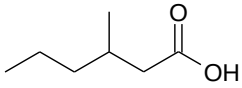
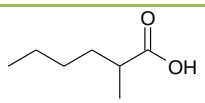
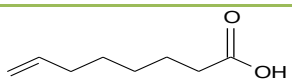
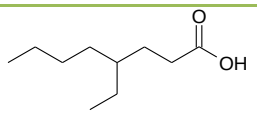
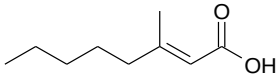
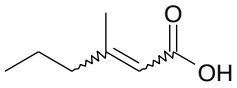
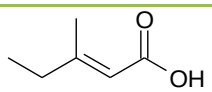
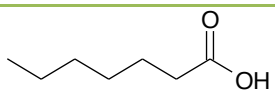
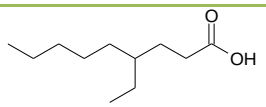
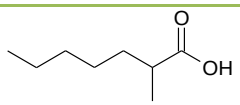
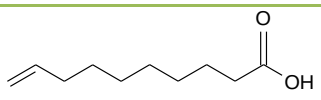
Name	*Structure*	*Odors*	*Bibliography*
5α-Androst-16-en-3-one*		Urinous, perspiration	[71][78][80]
Androsta-4,16-dien-3-one		Urinous, perspiration	[80][81]
5α-Androst-16-en-3α-ol*		Musky, strong	[72][78][82]
Androsta-4,16-dien-3α-ol		Musky, strong	[83]

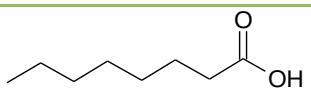
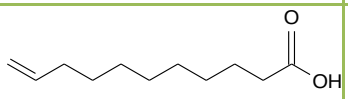
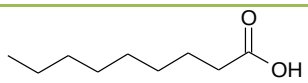
*key impact body malodorants.

b) *Volatile fatty acids*

Initial studies on sweat samples have identified the presence of odorous *low molecular weight and short chain branched volatile fatty acids C2-C5* such as butyric acids and isovaleric acid (3-methylbutyric acid) [81]. In subsequent studies, Zeng *et al.* identified *long chain volatile fatty acids C6-C11* in male axillary secretion extracts [84]. Important odor contributors were terminally unsaturated acids, 2-methyl C6-C10 acids and 4-ethyl C5-C11 acids, such as 4-ethylheptanoic acid (odor detection threshold: 1.6 ppb [23]). However, the major contributor appeared to be **(E)-3-methyl-2-hexenoic acid (E3M2H)** (*Table 3*). This acid was also identified in asiatic populations [85]. The analysis of volatile organic compounds extracted from patches reveals that the E3M2H is twice more concentrated in male than in female sweat samples. The E/Z ratio ((*E*)-3-methyl-2-hexenoic acid / (*Z*)-3-methyl-2-hexenoic acid) was 16:1 for females and 10:1 for males [86]. E3M2H and androstenone were found at concentrations of 357 ng/µl and 0.5 ng/µl axillary extract, respectively. The presence of specific anosmia for both diastereoisomers was also detected; however, the olfactory threshold for the E-diastereoisomer is within an order of magnitude of that for androstenone [87][88].

Table 3. Major volatile fatty acids present in axillary body odors.

Name	*Structure*	*Odors*	*Bibilography*
(RS)-3-Hydroxy-3-methylhexanoic acid**		Acid, spicy	[89][90]
n-Hexanoic acid			[84][90]
4-Ethyl-heptanoic acid*		Hircine, goat-like	[84][79]
3-Methyl-hexanoic acid			[84]
2-Methylhexanoic acid		Fruity, cheese, oily fatty, lard	[91]
7-Octenoic acid*			[84][90]
4-Ethyl-octanoic acid*		Goat acid	[84]
E-3-Methyl-2-octenoic acid			[91]
E/Z-3-Methyl-2-hexenoic acid*			[84][90]
E-3-Methyl-2-pentenoic acid			[91]
n-Heptanoic acid			[84]
4-Ethyl-nonanoic acid			[84]
2-Methyl-heptanoic acid		Waxy, green, cheese, sweaty, butter, milk	[84]
9-Decenoic acid		Waxy, green, fruity, fatty, soapy	[84]

n-Octanoic acid			[84][90]
10-Undecenoic acid			[84]
n-Nonanoic acid		Rancid	[84][90]

*Major contributors of axillary body odors; **recently identified as key impact body malodorants.

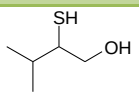
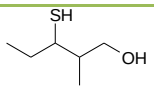
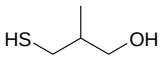
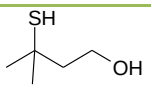
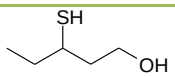
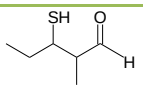
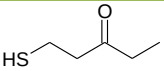
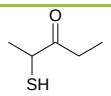
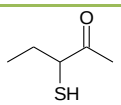
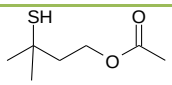
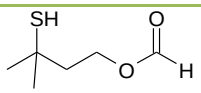
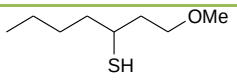
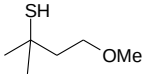
Recently, the hydrated analogue of **3M2H**, **(R/S)-3-hydroxy-3-methylhexanoic acid (HMHA)**, which has a very pungent axillary odor, was confirmed to be the most-abundant odorant in axilla secretions with an extremely low odor threshold value of 4.4 ng/µl (*Table 3*, [89][90]. This hydroxyl acid, but also other odorous hydroxy-acids such as 3-hydroxy-4-methylhexanoic acid, 3-hydroxy-4-methylheptanoic acid, 3-hydroxy-4-methyloctanoic acid, and 3-hydroxyoctanoic acid may be released from N_{α}-acyl-glutamine-conjugates, present in sweat secretions, by skin bacterial enzymes (see *Part 1.2.2*, [92]).

c) *Volatile sulfur compounds*

Sulfur-containing molecules, and especially thiols, are probably the most famous key flavor ingredients in many foods and beverages. Although only present in trace quantities, these VSCs contribute to the overall aroma, due to their extraordinarily low odor threshold values in the range of 1 pg/l, and their high volatilities. Furthermore, two studies have demonstrated the additive effects of volatile thiols in sensory tests, thus confirming their olfactory impact on the overall aroma of wine [93][94]. In some cases they are described as having highly desirable fruity notes, such as 4-mercapto-4-methyl-2-pentanone in white wine, but they may also exhibit onion, sweat, pungent, and leek character [95].

In 1979, Labows *et al.* have created synthetic versions of human axilla-sweat odors [96][97]. These mixtures of fatty acids and steroids are organoleptically similar to the real odor, as determined by a sensorial panel, but clearly lack important impact molecules. These reconstitutions only lack Volatile Sulfur Compounds (VSCs), for which no structures have been reported [98] due to their difficult identification. Van de Waal *et al.* successfully attempted to substitute the missing VSCs with 1-methoxyhexane-3-thiol from clary sage (*Salvia sclarea* L.) [99]. Some thiols isolated from food were described as having an onion or sweat-like odor (*Table 4*, [100]) but up to now have not been identified in sweat samples.

Table 4. Structures of volatile sulfur-containing molecules isolated from different food matrices characterised by their onion, sweaty and pungent odor (reviewed in [100]).

Name	*Matrix occurrence*	*Structure*
2-Mercapto-3-methyl-1-butanol	Beer	
3-Mercapto-2-methyl-1-pentanol	Onion	
3-Mercapto-2-methyl-1-propanol	Wine	
3-Mercapto-3-methyl-1-butanol	Coffee	
3-Mercapto-1-hexanol	Wine, grapefruit, passion fruit	
3-Mercapto-2-methylpentanoal	Onion	
1-Mercapto-3-pentanone	Beef liver	
2-Mercapto-3-pentanone	Wine, lamb, poultry chicken, yeast extract	
3-Mercapto-2-pentanone	Wine, cheese, beef, ham, lamb, pork, chicken yeast extract	
3-Methyl-3-mercaptobutyl-acetate	Passion fruit	
3-Methyl-3-mercaptobutyl-formate	Beer, coffee	
1-Methoxyheptane-3-thiol	Rue	
4-Methoxy-2-methylbutane-2-thiol	Tomato, sage, rue	

d) *Other volatiles present in axillary samples*

Ammonia, amines, indoles, hydrogen sulphide, phosphine (phosphorus trihydride, PH_3), esters, ketones, aldehydes and alcohols were also isolated from axillary extracts. Some of these additionally malodorous volatile components are probably derived from the autooxidation of lipids in sebum and apocrine sweat. Aldehydes (decanal, hexanal, heptanal, nonanal, octanal, undecanal, (E)-2-nonenal and benzaldehyde) originate from the oxidative degradation of

unsaturated fatty acids present in sebum (*i.e.* similar to scalp malodor) [101]. For instance, 2-nonenal, present in the odor of individuals, whose average age was 40 years is produced by oxidative degradation of monosaturated fatty acids, such as palmitoleic acid and vaccenic acid [101]. Sweat also contains alkanes, such as dodecane, heptadecane, nonadecane, undecane, and alcohols such as benzyl alcohol, phenol, undecanol, dodecanol, tridecanol, 2-methylphenol (*o*-cresol), 1-octen-3-ol and 4-methylphenol (*p*-cresol). 1-Octen-3-ol and 4-methylphenol also present in human sweat, were described as being mosquito attractants [102]. Finally, ketones such as 2-piperidone and γ lactones (recognized for their stale urine smell) were also identified in trace amounts [86][90][101].

e) *Pheromones*

Pheromones are referred to as ecto-hormones: chemical messengers that are transported outside the body and have the potential to evoke certain responses, such as physiological or behavioral changes in a conspecific (that is in another individual belonging to the same species) [103]. In most mammals, hormone levels are endogenously regulated in response to pheromones (for a review, see [104][105]). Although human behavioral phenomena, such as menstrual synchrony, resemble to pheromonal effects in rodents, whether or not human pheromones exist remains a controversial issue [106-108].

Juette showed that an aqueous mixture of five ovulatory fatty acids evoked increased saliva testosterone levels in men, and resulted in female attractiveness [109][110]. The steroid androstadienone (4,16-androstadien-3-one), a derivative of testosterone, present in human male secretions such as sweat, saliva, and semen, has been implicated as a putative human pheromone [111]. Androstadienone influences context-dependent mood, physiological arousal, and brain activity, in both a sex-specific [111-119], and a sexual orientation-specific manner [120][121]. Wyart *et al*. have recently shown that androstadienone can influence the hormonal balance by maintaining higher levels of the hormone cortisol (implicated in alarm reaction) in female saliva [122]. It has been shown that sulfur-containing volatile compounds such as 3-mercapto-3-methylbutan-1-ol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthiobutan-1-ol, and 3-methyl-3-(2-methyldisulfanyl)-butan-1-ol, can function as pheromones used for conspecific recognition and reproductive purposes in mature cats [123]. However, the pheromonal effect of sulfur-containing volatiles in humans has not been studied.

Some recent scientific evidence has shown that molecules in the sweat from apocrine glands may be involved in how a woman selects a potential mate, by guiding them towards

those with genetically different MHC profiles, and those who are potentially better for healthy offspring [55][124]. Unfortunately, no chemical structures have been reported. In addition, there is no clear mechanism explaining how these molecules might act on the human brain. In animals, pheromones are usually detected by the vomeronasal organ (VNO), present inside the nose which detect airborne pheromones and relay messages directly to the brain. In humans, there are no neurons linking the VNO to the brain, and the human genes no longer code for functional pheromone receptor proteins [79]. A recent study found that mice detect alarm pheromones by the Grueneberg ganglion organ which is also present in humans [79][125] and may open new research perspectives to prove or disprove hypothetic mechanisms related to pheromones.

The ontogenetic link between olfaction and hormones becomes evident in patients suffering from X-linked Kallmann's syndrome. They show underdeveloped gonads, completely lacking secondary sexual characteristics, and both male and female patients are anosmic [103][125][126]. However, to prove that human pheromones are active, they have to be clearly identified, synthesised and critically tested (in a relevant number of subjects) to see whether they trigger a real behavioural change. In addition, it was reported that female emotional reactions towards putative male pheromones such as androstenone, androstenol, and endrostadienone may change at the time of ovulation [122][127][128], which may increase the difficulties of such experiments. Similarly, body odor attractiveness may change during the human menstrual cycle [35]. In this respect, there are still many questions that remain unanswered to prove a pheromonal effect in humans.

1.2. Bacterial transformations of human axillary secretions

1.2.1. Volatile steroids

Early findings concluded that aerobic coryneforms are responsible for generating androstenol and androstenone from unidentified odorless substrates in apocrine secretion. However, axillary bacteria were unable to generate 16-androstenes from testosterone and pregnenolone, as previously shown in pigs and porcines [69][129][130]. Testosterone was converted to dihydrotestosterone (DHT), androstenedione and androstanedione by *Corynebacterium* spp. isolates, indicating 4-ene reductase and 3-hydroxysteroid dehydrogenase (HSD) activities [131]. Micrococci could only produce low levels of androstenedione, while staphylococci and propionibacteria were incapable of transforming either of the precursors. Some studies demonstrated that the axillary *Corynebacterium* spp. could only generate odorous 16-androstenes from precursors that already contain the C(16) double bonds, such as 5,16-androstadien-3-ol **3** and 4,16-androstadien-3-one **6** (*Fig. 6*, [132][133]). However, no single isolate was shown to carry out a full complement of the observed biotransformations, and most incubations were for periods exceeding 72h. A key observation was that very low prevalence of microorganisms capable of biotransforming 16-androstenes were present on skin [133].

The formation of steroidal malodors appears more complex than previously anticipated, *e.g.* odors resulting from bacterial enzymatic hydrolysis of glucuronide- and sulfate-conjugates, such as dehydroepiandrosterone sulfate or androsterone sulfate [134][135]. The steroidal biotransformations begin by the formation of androstadienol and its subsequent modification to androstadienone, androstenone, and then further reduction to α- or *β*-androstenol. Decreau *et al*. have proposed a G41 *Coryneform* pathway in which the biological origin of the non-odorous sterol **1** in axilla remains undetermined and sterol **2** was shown to afford testosterone after 24h (*Fig. 6*, [83]). Further research is required both to assess the contribution level of 16-androstenes to underarm odor (as high levels of specific anosmia are reported for the 16-androstenes), and to further elucidate the pathways and the odor molecules formed by corynebacteria.

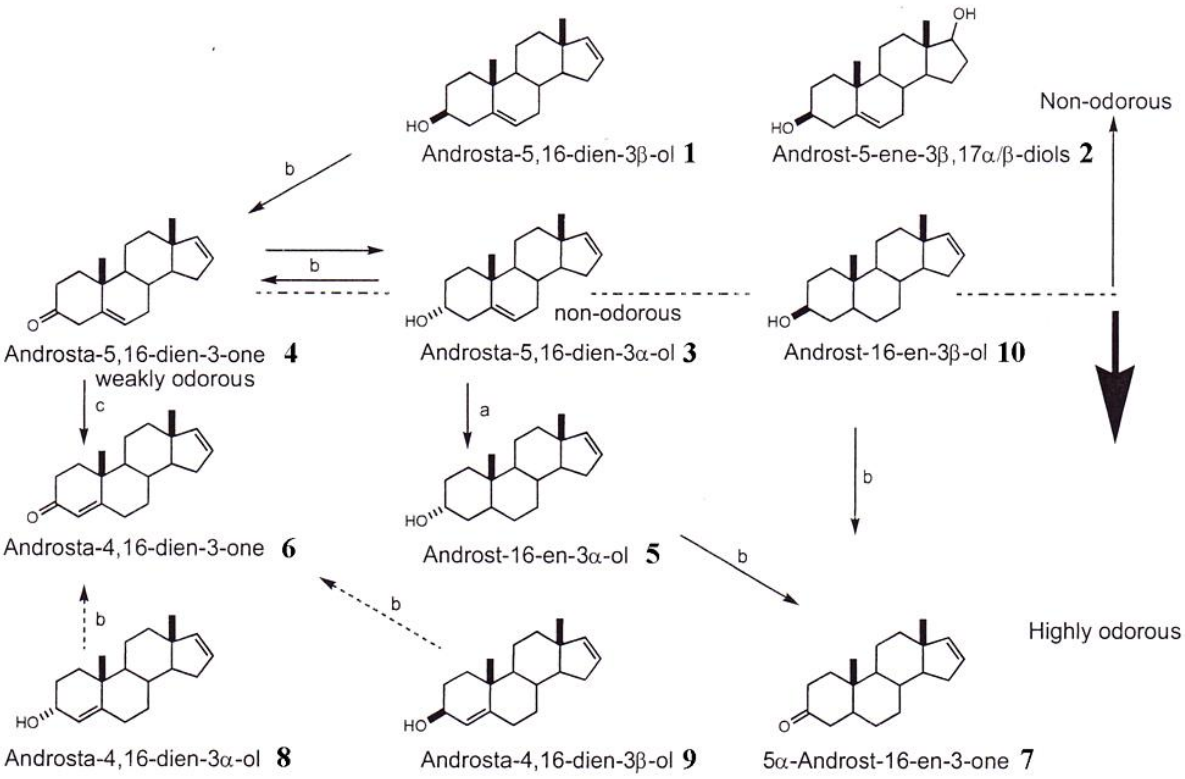


Fig. 6. Proposed biotransformations effected by G41 *Coryneform* bacteria in the production of steroidal malodor in human axillae [89]. Enzymes: (a) 4,5- or 5α-reductase; (b) 3α(β)-sterol dehydrogenase; (c) steroid 4,5-isomerase.

1.2.2. Volatile fatty acids

The production of short chain (C2-C5) fatty acids (VFAs) by skin bacteria is a consequence of a particular substrate found under physiological conditions by organisms in human apocrine sweat. In rare cases it may be due to the metabolism of skin bacteria, *e.g.* production of propionic acid by *Corynebacterium* spp. [136]. Propionibacteria and staphylococci metabolise glycerol and lactic acid to form large amounts of short chain VFAs, acetic, and propionic acids. Lactic acid is present in eccrine sweat and in the pilosebaceous follicle, and its level increases during hard work and exercise. Furthermore, staphylococci have been shown to be capable of converting branched aliphatic amino acids, such as leucine, to methyl-branched VFAs, such as isovaleric acid [137]. Propionibacteria, staphylococci and corynebacteria possess lipase activity that may be involved in the hydrolysis of triglyceride components present in sebaceous and apocrine lipids that may be subsequently metabolised by other skin bacteria [138].

Zeng *et al.* were the first to investigate the chemical nature of the precursors of the malodorant acid (E)/(Z)-3-methylhexenoic acid **13**. They showed that this acid can be liberated from odorless water soluble components of apocrine secretion by either saponification or bacteriolysis by corynebacteria [139]. This was the first indication that acids must be covalently bound to a water-soluble carrier moiety. A few years later, the same research group reported that a strong interaction may be present between the acid and two glycoproteins present in apocrine glands and secretions, the odor-binding protein 1 and 2 (ASOB1 and ASOB2), with apparent molecular masses of 26 and 45 kDa, respectively [140][141]. ASOB2 is identical in its polypeptide sequence to apolipoprotein D (Apo D), a member of a carrier protein superfamily known as lipocalins. Therefore an apoliprotein-acid complex was suggested as the malodorant acid precursor present in axillary sweat [142]. Firm analytical evidence for the acid precursor has only been recently found. (E/Z)-3M2H **13** and HMHA **14** are covalently linked to a glutamine residue (**11** and **12**, respectively) present in fresh axillary secretions and released by a Zn^{2+} dependent aminoacylase (*Fig. 7*) [89]. The aminoacylase enzyme was purified from *Corynebacterium striatum* AX20 and cloned in *Escherichia coli* Top10 for characterisation [89]. Several odorous acids and hydroxy acids, such as (Z) and (E)-4-methyloct-3-enoic acid and 3-hydroxy-4-methylheptanoic acid, together with (Z) and (E)-3-methyl-2-hexenoic acid and 3-hydroxy-3-methylhexanoic acid, were identified after incubation of sweat with the recombinant enzymes [92].

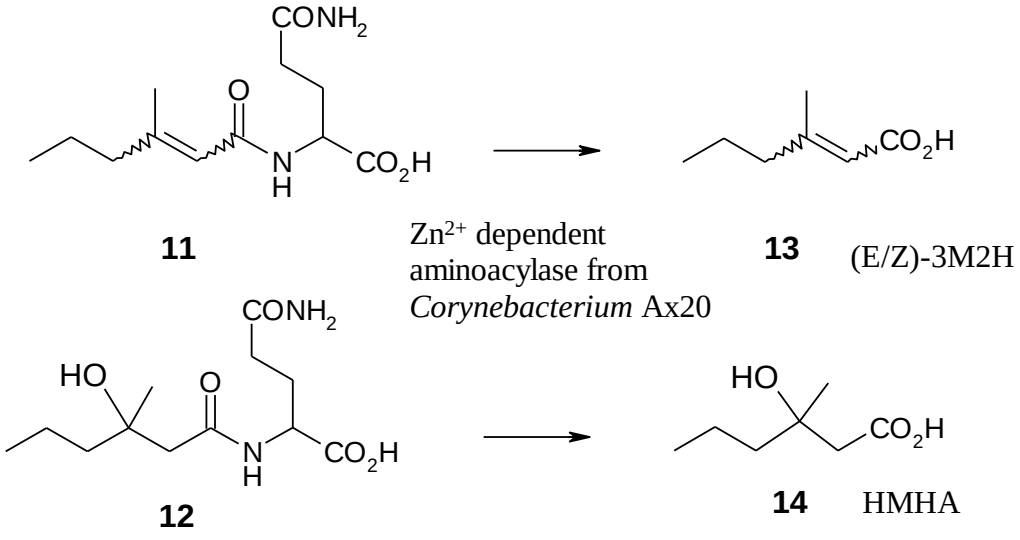


Fig. 7. Release of malodorants carboxylic acids from glutamine conjugate [143].

1.2.3. Volatile sulfur compounds[1]

a) *C-S lyases in the formation of volatile sulfur compounds*

Besides having a central role in catalysing reactions of amino-acid metabolism and H_2S production, **C-S lyases** such as alliin lyase (EC 4.4.1.4), cystathionine *β*-lyase (cystine lyase, E.C 4.4.1.8), alkylcysteine *β*-lyase (EC 4.4.1.6), and cysteine conjugate *β*-lyase (EC 4.4.1.13) contribute to the formation of important sulfurcontaining volatiles from cysteine-*S*-conjugate precursors. They catalyse *β*-elimination reactions of S-substituted cysteines to yield pyruvate, ammonia, and the corresponding thiols. Enzymes of this type have been isolated from rats, humans, zebra fish, bacteria, yeast, and plants. They are generally encoded by *met*C, *mal*Y, *pat*B or *aec*D genes in bacteria [144-147] and they require pyridoxal 5,-phosphate (PLP) as co-factor. In most cases, cysteine conjugates of aromatic compounds serve as substrates for these enzymes, while the enzymes extracted from bacteria have rather broad substrate specificities. They may catalyze the *β*-elimination reaction of several cysteine derivatives, such as *S*-alkyl-, *S*-aryl—cysteines [148-151]. The activity is generally inhibited by hydroxylamine and potassium cyanide. Other mammalian PLP-containing enzymes which have been shown to catalyse a cysteine *S*-conjugate *β*-lyase reaction are alanine aminotransferase and aspartate aminotransferase [152-155].

In plant and mammalian cells, cysteine-S-conjugates originate from a glutathione-S-conjugate precursor in the detoxification pathway (*Fig. 8*). Glutathione **15** is an endogenous tripeptide, which plays a key role in cell protection *via* two enzymatic mechanisms: glutathione peroxidase (EC 1.11.1.9), which removes hydroperoxides by reduction, and the **glutathione-*S*-transferase** (EC 2.5.1.18), which removes xenobiotics or potentially toxic compounds from the cell by conjugation [156-159]. Glutathione-S-tranferases are present in animal tissues where they catalyze the nucleophilic attack of the glutathione thiolate on an electrophilic centre, generally on a hydrophobic substrate (*Fig. 8A*). The resulting thioether **16** is the substrate for **γ-glutamyl transpeptidases**, which remove the glutamate residue to give **17** (*Fig. 8D*). Then several enzymes, including **aminopeptidases**, **dipeptidases** or **carboxypeptidases**, may cleave the peptide bond of **17** to release glycine (*Fig. 8E*) [160][161]. Finally, the Cysteine *S*-conjugate **18** can be cleaved by **cysteine S-conjugate *β*-lyases,** eliminating the sulfur-containing fragment **19,** or excreted as their mercapturate form **20** (which is less toxic and more water-soluble than the

[1] Partially published in Flavour and Fragrance Journal. Starkenmann C., Troccaz M., Howell K. The Role of Cysteine and Cysteine-*S*-Conjugates as odor precursors in the flavor and fragrance industry. *Flavour Fragr. J.* 2008, 23,369-381.

parent compounds, and thus readily excreted) [161]. In higher plants, the glutathione-S-conjugates **16** are rapidly metabolized by either preferred route B through to C, or route D to E (*Fig. 8*). In animals, glutamate is removed first, and therefore route D to E (*Fig. 8D*) is preferred [157].

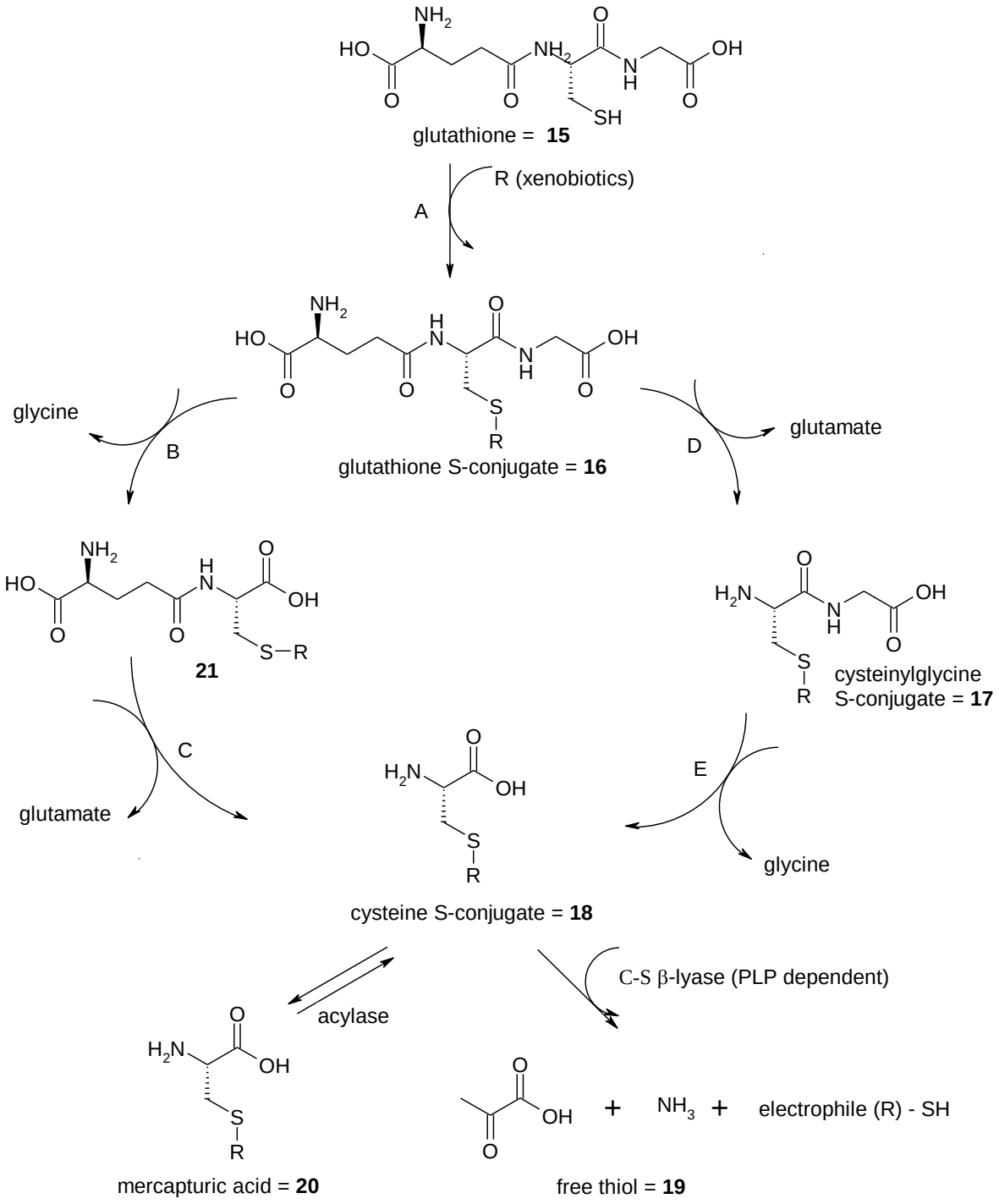


Fig. 8. General detoxification pathway using glutathione as scavenger.

The mechanisms of PLP-dependent enzymes are well documented. The PLP is stabilized by electrostatic interactions with an aldimine (comprising the ε-amino group of a lysine residue present on the active site of the enzyme). Entry of a substrate amino-acid into the active site results in transimination to form a new substrate-PLP complex (Schiff base) [162]. The free thiol is eliminated from the substrate-PLP Schiff base and the PLP is regenerated. This mechanism is outlined for the S-cysteine conjugate as a substrate in *Fig.* 9.

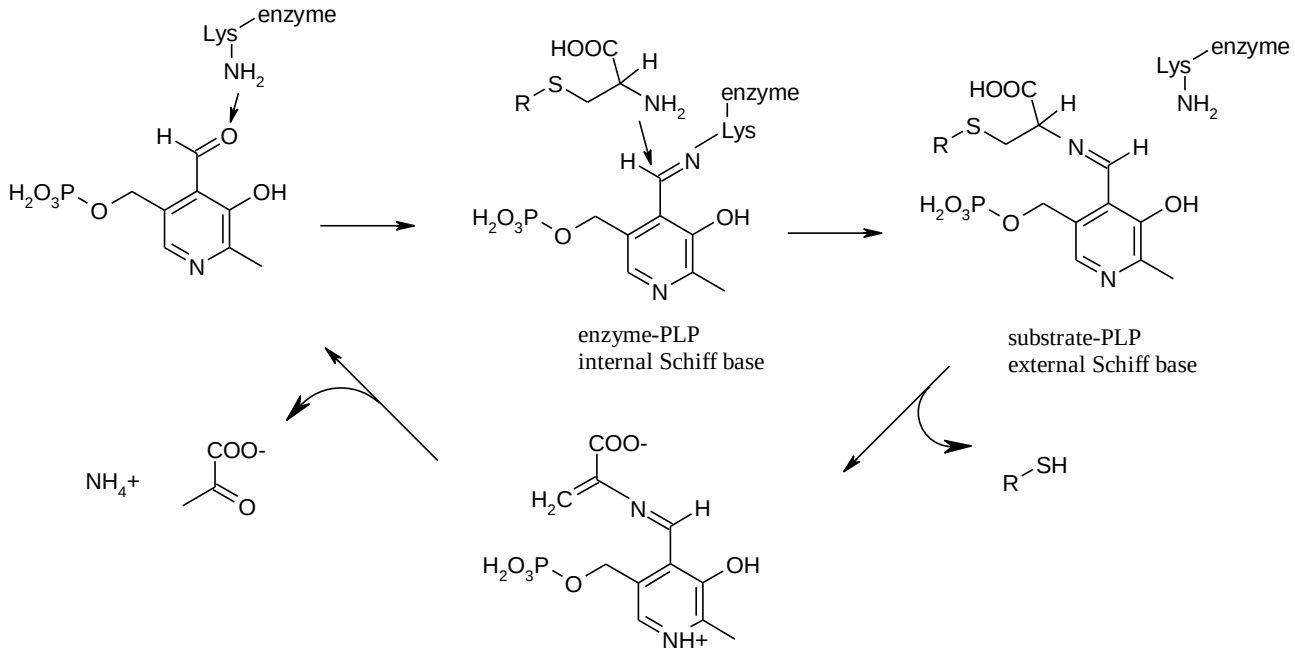


Fig. 9. Mechanism of the reaction of the active site of the enzyme with PLP and substrate, and *β*-elimination of the substrate-PLP Schiff base (modified from [176]).

b) *Examples of natural scents generated from cysteinylated precursors*

▪ **Wine and Passion fruit:** Organosulfur compounds are important for the characteristic aromas of wine and tropical fruit. When Dubourdieu and co-workers treated wine with copper sulfate the "boxtree" and "tropical fruit" odor disappeared, suggesting that these compounds contain sulfur. They were later shown to be 3-sulfanylhexan-1-ol **24**, 4-methyl-4-sulfanylpentan-2-one**,** and 4-methyl-4-sulfanylpentan-2-ol [94][163]. When sulfur compounds are observed as impact odorants in fruit and vegetables, it appears likely that there is a corresponding cysteine-S-conjugate. For instance, cysteinylated precursors have been identified in asparagus, Trifolate orange (*Poncirus trifoliate)* and bell pepper (*Capsicum annum*) [164]. Recently, C-S lyases have attracted new attention, because S-cysteine conjugates **22** have been described as a new type of non-volatile flavor precursor in Sauvignon white must (*Vitis vinifera)* and passion fruit

(*Passiflora edulis*), and cysteine *β*-lyase has been shown to release volatile thiols from these conjugates (*Fig. 10*, [165-169]).

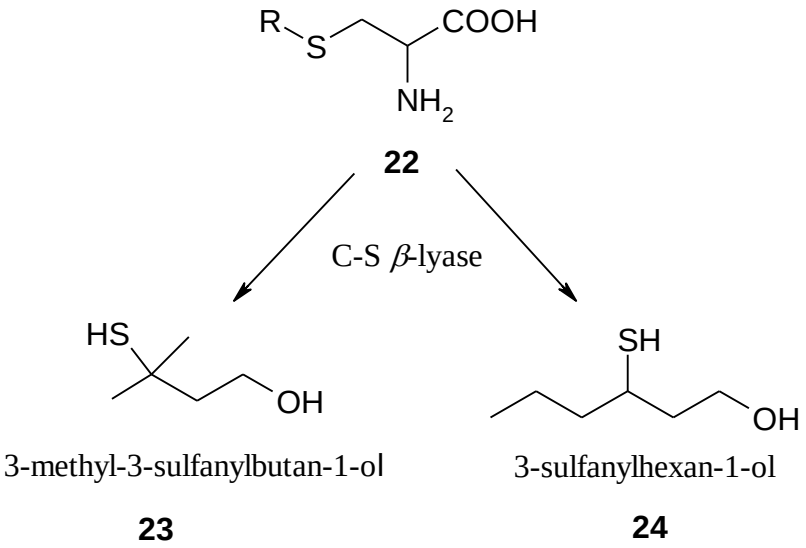


Fig. 10. Sulfur-containing volatiles formed by *β*-lyase-catalysed cleavage of non-volatile precursors in passion fruit [166].

The presence of cysteine-S-conjugates in Sauvignon must was confirmed by the addition of a tryptophanase from *Escherichia coli* (EC 4.1.99.1) or a cell-free extract of *Eubacterium limosum*, which has a cysteine conjugate *β*-lyase activity (EC 4.4.1.13) that released the flavor active thiols [167][170]. During wine fermentation, *Saccharomyces cerevisiae* was reported to cleave non-volatile cysteinylated precursor present in grape juice liberating the volatile thiol 4-sulfanyl-4-methylpentan-2-one. Those results led to the identification of four genes encoding carbon-sulfur lyases that influence the release of the volatile thiol (see *Table 5*) [171][172].

Table 5. Yeast genes identified as having potential carbon-sulfur lyase activity on a cysteinylated precursor in wine [173].

Systematic name		*Gene function*	*EC*	*Comment*
YJL060w	BNA3	Biosynthesis of nicotinic acid		Similar to human/rat cysteine conjugate lyases
YML004c	GLO1	Glyoaxylate 1, lactoylglutathione lyase	4.4.4.5	Yeast C-S lyase
YFR055C	METC	Cystathionine *β*-lyase	4.4.1.8	Yeast C-S lyase, 26% similar to YFR055c, 26% similar to YLR303w
YAL012w	CYS3	Cystathionine *γ*-lyase	4.4.1.1	Yeast C-S lyase

In further experiments, it appears that glutathione-S-conjugates may be involved in the production of cysteine-S-conjugates in wine aroma. Experiments using an immobilized *γ*-glutamyltranspeptidase column increased the amount of *S*-3-(hexan-1-ol)-*L*-cysteine, the precursor of 3-sulfanylhexan-1-ol in Sauvignon white grape must [174][175]. Dubourdieu and co-workers suggested that *S*-3-(1-hexanol)-*L*-cysteine adducts might be released *via* the catabolism of *S*-3-(1-hexanol)-glutathione by two consecutive enzymes: **γ-glutamyltranspeptidase**, eliminates glutamine and **carboxypeptidase**, eliminates glycine [168][176]. Quantification of such precursors in grapes or musts may allow the assessment of the aromatic potential of Sauvignon white grape must and other *V. vinifera* cultivars, as quality control [177][178].

▪ **Allium species:** The *Allium* plants belong to the *Liliaceae* family. They are characterized by their strong sulfury odor. The odorants are enzymatically produced by damage of the plant tissues. Alliin, (+)-*S*-allyl-*L*-cysteine sulfoxide **25** was the first non volatile precursor of an odorant identified in a natural product, isolated from *Allium* species [179]. *S*-(+)-Alk(en)yl-*L*-cysteine sulfoxide itself does not have any specific odor, but it can be converted into volatile flavors by alliin lyase when the plant is cut or crushed. *S*-alk[en]yl-*L*-cysteine sulfoxide precursors exist in the cytosol of mainly mesophyll cells, while the enzyme, alliin lyase (EC 4.4.1.4 *S*-alk[en]yl-*L*-cysteine sulfoxide lyase), exists in the vacuoles of the vascular bundle sheagth cells around the xylem or phloem [180]. Investigation using radio-labelled carbon and sulfur sources showed that cysteine is incorporated from the glutathione cycle (*Fig. 8*) [181]. The next step is the addition of glutathione **15** to methacrylic acid, and the resulting *S*-(2-

carboxypropyl) glutathione **26** can be detected in both onion and garlic bulb tissues [181][171]. The glycine residue is cleaved early in the process to give *γ*-*L*-glutamyl-*S*-(2-carboxy-propyl)-*L*-cysteine **27** *via* route B to C (*Fig. 8*, [182]). The next step is decarboxylation to give *γ*-L-glutamyl-*S*-prop-1-enyl)-*L*-cysteine **28** and later *γ*-*L*-glutamyl-*S*-prop-1-enyl)-*L*-cysteine sulfoxide **29**. As long as the *α*-amino group of cysteine is conjugated to the glutamic acid residue, alliin lyase or other C-*S*-lyases cannot liberate the volatile organic sulfur compounds. When the cysteine-*S*-conjugates are liberated from the glutamyl residue, upon enzymatic action of the alliin lyase, the C-*S* bond is cleaved to generate *S*-alk(en)yl sulfoxide. Different aroma's are produced depending on the various flavor precursors found in the cultivated species [183][183][184].

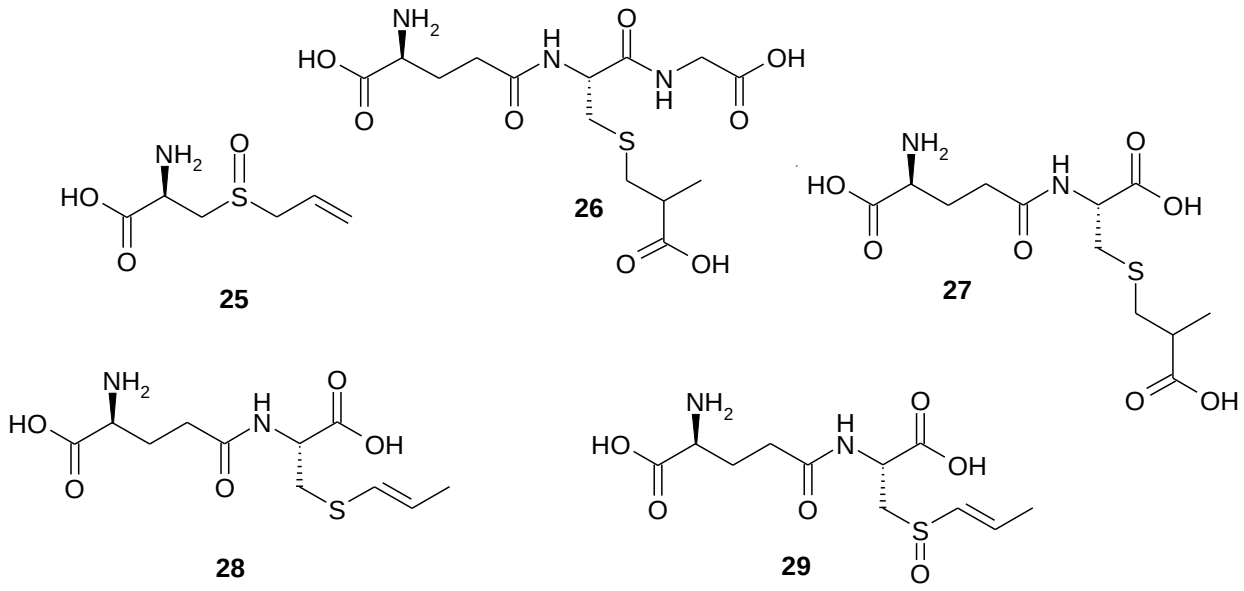


Fig. 12. Principal S-(+)-alk(en)yl-L-cysteine sulphoxides in *Allium* plants.

▪ **Cat urine:** To understand the frequently used association between the "catty" odor and "blackcurrant" aroma, Joulain *et al.* analysed the urine of *Felis catus* and discovered compound **33** and several other related sulfur-containing compounds [185]. Early work by Westall *et al.* in 1952, elucidated the structure of an unusual amino-acid in the urine of domestic cats (*Felis domesticus*), as the 2-methyl-3-hydroxy-butyl-*S*-cysteine conjugate named *felinine* **32 (***Fig. 13)* [186]. This compound was also identified in bobcat (*Lynx rufus*) [187][188]. It is believed to be the precursor of a putative cat pheromone, 3-methyl-3-sulfanyl-butan-1-ol **33**, a key odoriferous component of cat urine [123]. Miyasaki *et al.* discovered that, as well as felinine, there were also

high concentrations of a carboxylesterase called cauxin (carboxylesterase-like urinary excreted protein) in the urine of male cats [189]. It appears that cauxin is present in the urine of other big cats, such as the Bengal tiger, as a semiochemical-releasing enzyme (*Fig. 13*, [190])

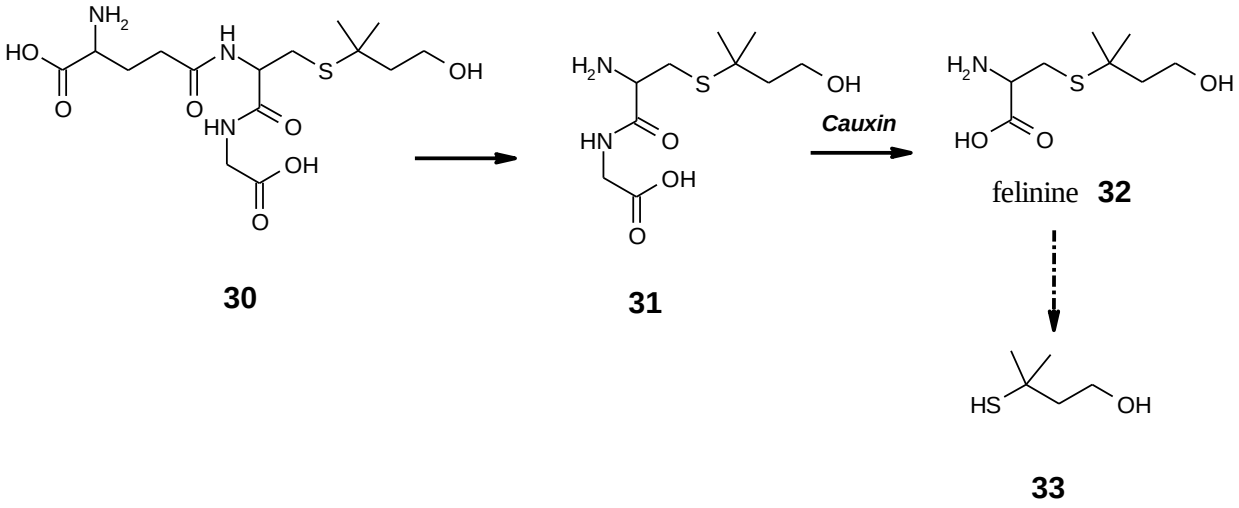


Fig. 13. Chemical structures of felinine-related molecules and cauxin activity. **30**: 3-methylbutanol-glutathione. **31**: 3-methylbutanol-cysteinylglycine; **32**: 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid (felinine); **33:** 3-methyl-3-sulfanyl-butan-1-ol (modified from [123]).

Cauxin is excreted in a species-, sex-, and age-dependent manner [191]. Its excretion is higher in male cats older than about 3 months, and its excretion increased with age. This enzyme contains both a serine hydrolase active site (with a GXSXG motif) and a carboxylesterase-specific sequence. The enzyme is produced and excreted from the kidneys of the domestic cat [189]. Cauxin cleaves 3-methylbutanol-cysteinylglycine **31**, formed from **30** by an enzyme (probably a γ-glutamyltransferase), into felinine and glycine [123][192][193]. It remains unknown how felinine breaks down into the volatile sulfur compound in cat urine. Enzymatic and non-enzymatic reactions may be involved.

1.3. Aim of the thesis

Volatile steroids, such as 5-α-androst-16-en-3-one and 5-α-androst-16-en-3-α-ol and volatile aliphatic, branched and unbranched fatty acids ((*E)*-3-methyl-2-hexenoic acid (3M2HA) and (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid) have been reported to be major contributors to Human Axillary Malodors (HAM). However, reconstitutions of human axilla-sweat odors composed of fatty acids and steroids, although organoleptically similar to the real odor, as determined by sensorial panel, clearly lacks important impact molecules. One reason may be the presence of specific anosmia for both androstenone and 3M2HA [194][195]. It was discoverd that these reconstitutions only lack Volatile Sulfur Compounds (VSCs). No structures have been reported [98], principally due to the difficulties in identifying high impact, low threshold volatiles in the concentration range of 1pg/l. Van de Waal *et al.* successfully replaced the missing VSCs with 1-methoxyhexane-3-thiol, a natural product found in clary sage (*Salvia sclarea* L.) [99]. VSCs are known to be key flavour ingredients in many foods and beverages; in some cases their odors are described as pleasant and fruity, but can also be onion-like, with an unpleasant sweaty, pungent character.

The first aim of the thesis was to identify the presence of VSCs in axillae. We thus first followed a pragmatic approach by building an exercise/sauna facility, in order to collect enough underarm sweat from volunteers after excessive sweating, and then incubate it with skin bacterial isolates as described in Chapter 2 (see *Chapter 2*: *Major sulfur volatiles in axillary malodor*). The level of contribution of VSCs to underarm odor was confirmed by odor detection threshold measurements of the isolated thiols.

Another interest, particularly in the cosmetic industry, is the identification of metabolic mechanisms involved in the generation of VSCs in axillary malodor. Further research was then required; both to isolate and identify the precursors involved, and then to further elucidate the pathways and the bacterial enzymes responsible for the bioconversion. The second aim of this thesis was then to understand axillary malodor formation and more precisely, to rationalise the formation of VSCs in typical European sweat malodor with respect to the precursors and the bacteria involved. (see *Chapter 3*: *Identification of sulfur odor precursors*). The odorless sterile sweat was therefore fractionated by liquid chromatography. An aliquot of each fraction was then treated with axillary bacteria to identify which fraction could generate sulfur odors. In analogy to what occurs in mammals in the glutathione detoxification pathway, or in wine fermentation, we expected to find cysteinyl-S conjugates as odorless precursors present in sweat, and focussed our research accordingly.

In parallel we were curious to evaluate the gender influence on the formation of VSCs in the axillary region. In previous studies, it was found that 3M2HA, which has an acid, rancid, animal-like odor, was twice as concentrated in male as compared to female sweat samples, and in far greater quantities than steroids [84]. Are those differences to be attributed to the high proportion of coryneform bacteria in the male armpit? No information is available regarding the content of potential precursors in either male or female odorless secretions and how they may influence the sweat odor profile (see *Chapter 2*, [196]). The third aim of this thesis was thus to collect sterile sweat in sufficient quantities from either males or females, in order to address this question (see *Chapter 4: Gender and body odor: it is all in the chemistry*).

Once the VSCs precursors and bacteria present in the armpit had been elucidated, the focus of the final part of the thesis was to study the enzymatic systems required for the biosynthesis of axillary volatile thiols in HAM (see *Chapter 5: Enzymes involved in sulfur compound biosynthesis*). Such transformations may well involve bacterial transaminases and *β*-lyases such as *met*C gene products endowed with C-S lyase activity. For this purpose, relevant enzymes from isolated bacteria, known to convert odorless precursors to odorous sweat (see *Chapter 2*, [197]), were cloned and characterized. The understanding of such mechanisms could well be used to generate a new strategy for malodor counteraction in the personal care and cosmetic industries.

Bibliography

[1] A. C. Williams. *Transdermal and Topical Drug Delivery*, Pharmaceutical Press, London, UK, **2003**.

[2] J. A. McGrath, R. A. J. F. Eady, F. M. Pope. Anatomy and Oragnization of Human Skin, In: *Textbook of Dermatology*, Burns, T., Breathnach, S., Cox, N.,Griffiths C., **2004**.

[3] A. W. Norman, *Am. J. Clin. Nutr.* **1998**, *67*, 1108.

[4] P. Schiefferdecker, *Biologisches Zentralblatt.* **1917**, *37*, 534.

[5] L. Giacometti, *Anat. Rec.* **1967**, *159*, 69.

[6] F. Smith, *J. Anat.* **1890**, *24*, 493.

[7] P. M. Quinton, H. Y. Elder, D. McEwan Jenkinson, D. L. Bovell. Structure and function of human sweat glands, In: (Ed.: K. Laden), *Antipersiprants and deodorants*, **1999**, p. 15.

[8] W. Montagna, *J. Hum. Evol.* **1985**, *14*, 13.

[9] D. M. Stoddart. *The Scented Ape: The Biology and Culture of Human Odour.*, (Ed.: New York: Cambridge University Press.), **1990**.

[10] K. Sato, R. Leidal, F. Sato, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **1987**, *252*, R166-R180.

[11] D. L. Bovell, A. D. Corbett, S. Holmes, A. MacDonald, M. Harker, *Br. J. Dermatol.* **2007**, *156*, 1278.

[12] H. J. Hurley, W. B. Shelley. *The Human Apocrine Sweat Gland in Health and Disease*, (Ed.: Charles C), Thomas, Springfield, Illinois, **1960**.

[13] W. B. Shelley, H. J. Hurley, *J. Invest. Dermatol.* **1953**, *20*, 285.

[14] W. C. Lobitz, C. J. Campbell, *Arch Dermatol Syphil.* **1953**, *67*, 575.

[15] P. E. Pochi, J. S. Strauss, *J. Invest. Dermatol.* **1974**, *62*, 191.

[16] F. J. G. Ebling. The hormonal control of hair growth, In: (Eds.: C. E. Orfanos and R. Happle), *Hair and Hair Diseases*, Springer-Verlag, Berlin, **1990**, p. 267.

[17] L. S. Constanzo. *BRS Physiology*, Lippincott Williams and Wilkins, **2006**, p. 155.

[18] J. R. Cohn, E. A. Emmett, *Ann. Clin. Lab. Sci.* **1978**, *8*, 270.

[19] R. Saraymen, E. Kilic, Y. Suleyman, *Inonu Universitesi Tipe Fakultesi Dergisi.* **2004**, *11*, 7.

[20] M. Kent. *The Oxford Dictionary of Sports Science & Medicine*, Oxford University Press, Oxford, **2006**.

[21] B. Schittek, R. Hipfel, B. Sauer, J. Bauer, H. Kalbacher, S. Stevanovic, M. Schirle, K. Schroeder, N. Blin, F. Meier, G. assner, C. arbe, *Nat. Immunol.* **2001**, *2*, 1133.

[22] D. B. Gower, A. Nixon, A. I. Mallet. The significance of odorous steroids in axillary odour, In: *Perfumery: The Psychology and Biology of Fragrance*, Chapman and Hall, London, **1988**, p. 47.

[23] J. N. Labows, J. T. Reilly, J. J. Leyden, G. Preti. Axillary odor determination, formation, and control, In: (Ed.: K. Laden), *Antiperspirants and deodorants*, **1999**, pp. 59-80.

[24] D. J. Penn, E. Oberzaucher, K. Grammer, G. Fischer, H. A. Soini, D. Wielser, M. V. Novotny, S. J. Dixon, Y. Xu, R. G. Brereton, *J. R. Soc. Interface.* **2007**, *4*, 331.

[25] P. J. Rennie, D. B. Gower, K. T. Holland, *Br. J. Dermatol.* **1991**, *124*, 596.

[26] E. A. Eady, K. T. Holland, W. J. Cunliffe, *J. Appl. Bacteriol.* **1983**, *55*, 461.

[27] H. L. Ko, G. Pulverer, J. Jeljaszewicz, *Zentralbl. Bakteriol.* **1978**, *241*, 325.

[28] D. J. Bibel, R. J. Smiljanic, D. J. Lovell, *Applied Env. Microbiol.* **1978**, *35*, 1136.

[29] D. Hopwood, M. D. Farrar, R. A. Bojar, K. T. Holland, *Acta. Derm. Venereol.* **2004**, *85*, 363.

[30] D. Taylor, A. Daulby, S. Grimshaw, G. James, J. Mercer, S. Vaziri, *Int. J. Cos. Science.* **2003**, *25*, 137.

[31] J. J. Leyden, K. Nordstrom, K. J. McGinley. Cutaneous microbiology, In: (Ed.: L.A.Goldsmith), *Physiology, biochemistry, and Molecular Biology of the skin,* Oxford University Press, New-York, **1991**, pp. 1403-1424.

[32] P. J. Jackman, W. C. Noble, *Clinical and Experimental Dermatol.* **1983**, *8*, 259.

[33] J. J. Leyden, K. J. McGinley, K. Heolzle, J. N. Labows, A. M. Kligman, *J. Invest. Dermatol.* **1981**, *77*, 413.

[34] T. Tsuda, S. K. S. M. T. Noda, *Biochemical chromatography.* **22000**, *14*, 505.

[35] J. Havlicek, L. Bartos, R. Dvorakova, J. Flegr, *Ethology.* **2006**, *112*, 81.

[36] W. B. Shelley, H. J. Hurley, A. C. Nicholas, *Arch. Dermatol. Syph.* **1953**, *68*, 430.

[37] M. D. Sulzberger, F. G. Zak, F. Hermann, *Arch. Dermat. Syph.* **1949**, *60*, 404.

[38] K. T. Holland, A. B. Richard, *Am. J. Clin. Dermatol.* **2002**, *3*, 445.

[39] I. Brune, A. Becker, Paarmann D, Albersmeier A, Kalinowski J, Puhler A, Tauch A, *J. Biotech.* **2006**, *127*, 21.

[40] K. T. Holland, R. A. Bojar, *Am. J. Clin. Dermatol.* **2002**, *3*, 445.

[41] J. P. Libert, V. Candas, J. J. Vogt, *Eur. J. Appl. Physiol.* **1983**, *50*, 935.

[42] K. Sato, F. Sato, *Am. J. Physiol.* **1983**, *245*, 203.

[43] E. R. Nadel, K. B. Pandolf, M. F. Roberts, J. A. J. Stolwijk, *J. Appl. Physiol.* **1974**, *37*, 515.

[44] E. R. Nadel, R. W. Bullard, J. A. Stolwijk, *J. Appl. Physiol.* **1971**, *31*, 80.

[45] P. Agache, V. Candas. Appareil sudoral eccrine, In: (Ed.: P. Agache), *physiologie de la peau et explorations fonctionnelles cutanees,* **2000**, pp. 300-457.

[46] W. Montagna, P. F. Parakkal. Apocrine glands, In: *the structure and function of skin,* London, New York: Academic Press., **1974**, pp. 332-365.

[47] R. Choudhry, M. B. Hodgins, T. H. Van der Kwast, A. O. Brinkmann, W. J. A. Boersma, *J. Endocrinol.* **1992**, *133*, 467.

[48] B. Brody, *Psychiatry.* **1975**, *38*, 278.

[49] R. L. Doty, M. M. Orndorff, J. Leyden, A. Kligman, *Behav. Biol.* **1978**, *23*, 373.

[50] J. Havlícek, R. Dvoráková, L. Bartoš, J. Flegr, *Ethology.* **2006**, ***112***, 81.

[51] R. Thornill, S. W. Ganestad, R. Miller, G. Scheyd, J. K. McCollough, M. Franklin, *Behav Ecol.* **2003**, *14*, 668.

[52] A. H. Porter, J. M. Cernoch, R. D. Balogh, *Physiol Behav.* **1984**, *34*, 445.

[53] F. Kuhnl, A. Natsch, *J. R. Soc. Interface.* **2009**, *6*, 377.

[54] S. C. Roberts, L. M. Gosling, T. D. Spector, P. Miller, D. J. Penn, M. Petrie, *Chem. Senses.* **2005**, *30*, 651.

[55] C. Wedekind, S. Furi, *Proc. Biol. Sci.* **1997**, *264*, 1471.

[56] C. Wedekind, T. Seebeck, F. Bettens, A. J. Paepke, *Proceedings of the Royal Society B: Biological Sciences.* **1995**, *260*, 245.

[57] S. Ikeda, J. Arata, T. Nishikawa, M. Takigawa. *Standard Dermaology.*, (Ed.: T. Igakushoin), **2009**.

[58] A. V. Rawlings, *Int. J. Cos. Sc.* **2006**, *28*, 79.

[59] E. H. Starling. *Principles of human physiology,* (Eds.: H. Davson and M. G. Eggleton), Philadelphia : Lea & Febiger, **1968**.

[60] H. E. Knaggs, E. J. Wood, R. L. Rizer, O. H. Mills, *Int. J. Cos. Science.* **2004**, *26*, 129.

[61] L. S. Andersen, F. Biering-Sorensen, P. G. Muller, I. L. Jensen, B. Aggerbeck, *Paraplegia.* **1992**, *30*, 184.

[62] E. L. Larson, K. J. McGinley, A. R. Foglia, G. H. Talbot, J. J. Leyden, *J. Clin. Microbiol.* **1986**, *23*, 604.

[63] S. C. Mitchell, *Lancet.* **1999**, *354*, 2164.

[64] D. Hernandez, S. Addou, D. Lee, C. Orengo, E. A. Shephard, I. R. Phillips, *Hum. Mutat.* **2003**, *22*, 209.

[65] H. Yamaki, H. Fujita, T. Gunji, Z. Zhang, T. Kamataki, J. Cashman, M. Shimizu, *Mol. Genet. Metab.* **2007**, *90*, 58.

[66] M. Shimizu, J. Cashman, H. Yamazaki, *BMC Medical Genetics.* **2007**, *8*, 2.

[67] P. J. Rennie, D. B. Gower, K. T. Holland, A. I. Mallet, W. J. Watkins , *Int. J. Cosmet. Sci.* . **2007**, *12*, 197.

[68] D. B. Gower, B. A. Ruparelia, *J. Endocrinol.* **1993**, *137*, 167.

[69] D. B. Gower. Biosynthesis of the androgens and other C19 steroids, In: (Ed.: H. J. Makin), *Biosynthesis of the Androgens and Other C19 Steroids*, **1984**, pp. 170-229.

[70] G. Ohloff, B. Maurer, B. Winter, W. Giersch, *Helv. Chim. Acta.* **1983**, *66*, 192.

[71] D. B. Gower, *J. Steroid. Biochem.* **1972**, *3*, 45.

[72] B. W. L. Brooksbank, R. Brown, J. A. Gustafsson, *Experientia.* **1974**, *30*, 864.

[73] G. Preti, W. B. Cutler, C. M. Christensen, H. Lawley, G. R. HUGGINS, C. R. Garcia, *J. Chem. Ecology.* **1987**, *13*, 717.

[74] R. Claus, W. Alsing, *J. Endocrinol.* **1976**, *68*, 483.

[75] S. Bird, D. B. Gower, *J. Steroid Biochem.* **1981**, *14*, 213.

[76] E. A. Bremner, J. D. Mainland, R. M. Khan, N. Sobel, *Chem. Senses.* **2003**, *28*, 423.

[77] Y. Tokunaga, Y. Omoto, T. Sangu, M. Miyazaki, R. Kon, K. Takada, *Int. J. Cos. Sc.* **2005**, *27*, 333.

[78] J. E. Amoore, P. A. O. L. Pelosi, L. J. Forrester, *Chem. Senses.* **1977**, *2*, 401.

[79] A. Keller, L. B. Vosshall, *Curr. Opin. Neurobiol.* **2008**, *18*, 364.

[80] D. B. Gower, S. Bird, P. Sharma, F. R. House, *Cellular and Molecular Life Sciences (CMLS).* **1985**, *41*, 1134.

[81] J. N. Labows. Odor detection, generation, and etiology in the axilla, In: (Eds.: K. Laden and C. B. Felger), *Antiperspirants and deodorants*, **1988**, pp. 321-343.

[82] D. B. Gower, S. Bird, P. Sharma, F. R. House, *Experientia.* **1985**, *41*, 1134.

[83] R. A. Decréau, C. M. Marson, K. E. Smith, J. M. Behan, *J. Steroid Biochem. & Mol. Biol.* **2003**, *87*, 336.

[84] X. Zeng, J. Leyden, H. Lawley, K. Sawano, I. Nohara, G. Preti, *J. Chem. Ecol.* **1991**, *17*, 1469.

[85] T. Akutsu, K. Sekiguchi, T. Ohmori, K. Sakurada, *Chem. Senses.* **2006**, *31*, 557.

[86] X. N. Zeng, J. J. Leyden, A. I. Spielman, G. Preti, *J. Chem. Ecol.* **1996**, *22*, 237.

[87] A. Baydar, M. Petrizilka, M. Schott, *Perf. Flav.* **1992**, *17*, 1.

[88] C. J. Wysocki, X. N. Zeng, G. Preti, *Chem Senses.* **1993**, *18*, 652.

[89] A. Natsch, H. Gfeller, P. Gygax, J. Schmid, G. Acuna, *J. Biol. Chem.* **2003**, *278*, 5718.

[90] Y. Hasegawa, M. Yabuki, M. Matsukane, *Chem. Biodiv.* **2004**, *1*, 2042.

[91] A. I. Spielman, X. N. Zeng, J. J. Leyden, G. Preti, *Experimentia.* **1995**, *51*.

[92] A. Natsch, S. Derrer, F. Flachsmann, J. Schmid, *Chem. Biodiv.* **2006**, *3*, 1.

[93] M. Masuda, E. Okawa, K. Nishimura, H. Yunome, *Agri. Biol. Chem.***1984**, *48*, 2710.

[94] E. Sarrazin, S. Shinkaruk, T. Tominaga, B. Bennetau, E. Frérot, D. Dubourdieu, *J. Agric. Food. Chem.* **2007**, *55*, 1437.

[95] C. Vermeulen, S. Collin, *J. Agric. Food. Chem.* **2002**, *50*, 5654.

[96] J. N. Labows, G. Preti, E. Hoelzle, J. J. Leyden, A. M. Kligman, *Steroids.* **1979**, *34*, 249.

[97] J. N. Labows, J. T. Reilly, J. J. Leyden, G. Preti. Axillary odor determination, formation and control, In: (Ed.: K. Laden), *Antiperspirants And Deodorants'*, Cosmetic Science and Technology Series, Marcel Dekker Inc., New York, **1999**, p. 59.

[98] Lyon, S., O'Neal, C., Van Der Lee, H., and Rogers, B. Amino acid beta lyase enzyme inhiboitors as deodorants. Gillette. [WO91/05541]. 1991.

[99] M. van de Waal, Y. Niclass, R. L. Snowden, G. Bernardinelli, S. Escher, *Helv. Chimica Acta.* **2002**, *85*, 1246.

[100] C. Vermeulen, L. Gijs, S. Collin, *Food Reviews Int.* **2005**, *21*, 69.

[101] A. M. Curran, S. I. Rabin, P. A. Prada, K. G. Furton, *J. Chem. Ecol.* **2005**, *31*, 1607.

[102] A. Cork, K. C. Park, *Medical and Veterinary Entomology.* **2008**, *10*, 269.

[103] J. V. Kohl, M. Atzmueller, B. Fink, K. Grammer, *Neuroendocrinology Letters.* **2001**, *22*, 321.

[104] P. A. Brennan, F. Zufall, *Nature.* **2006**, *444*, 308.

[105] L. Stowers, T. F. Marton, *Neuron.* **2005**, *46*, 699.

[106] M. K. McClintock, *Nature.* **1971**, *229*, 244.

[107] K. Stern, M. K. McClintock, *Nature.* **1998**, *392*, 179.

[108] Wysocki Charles J, Preti George, *The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology.* **2004**, *281*, 1201.

[109] A. Juette, *Diplomarbeit an der naturwissenschaftlichen Fakultät der Universität Wien.* **1995**.

[110] R. P. Michael, R. W. Bonsall, M. Kutner, *Psychoneuroendocrinology.* **1975**, *1*, 153.

[111] B. I. Grosser, L. Monti-Bloch, C. Jennings-White, D. L. Berliner, *Psychoneuroendocrinology.* **2000**, *25*, 289.

[112] S. Jacob, D. J. Hayreh, M. K. McClintock, *Physiol. Behav.* **2001**, *74*, 15.

[113] J. N. Lundström, M. Goncalves, F. Esteves, M. J. Olsson, *Hormones and Behav.* **2003**, *44*, 395.

[114] J. N. Lundström, M. J. Olsson, *Biological Psychology.* **2005**, *70*, 197.

[115] J. N. Lundström, T. Hummel, *Behavioural Brain Research.* **2006**, *166*, 197.

[116] I. Savic, H. Berglund, B. Gulyas, P. Roland, *Neuron.* **2001**, *30;31*, 661.

[117] M. Bensafi, W. M. Brown, R. Khan, B. Levenson, N. Sobel, *Behavioural Brain Research.* **2004**, *152*, 11.

[118] S. Jacob, S. Garcia, D. Hayreh, M. K. McClinock, *Hormones and Behav.* **2002**, *42*, 274.

[119] C. Villemure, M. Bushnell, *Eur. J. of Pain.* **2007**, *11*, 181.

[120] H. Berglund, P. Lindstrom, I. Savic, *Proc Natl Acad Sci USA.* **2006**, *103*, 8269.

[121] I. Savic, H. Berglund, P. Lindstrom, *Proceedings of the National Academy of Sciences of the United States of America.* **2005**, *102*, 7356.

[122] C. Wyart, W. W. Webster, J. H. Chen, S. R. Wilson, A. McClary, R. M. Khan, N. Sobel, *J. neuroscience.* **2007**, *27*, 1261.

[123] M. Miyazaki, T. Yamashita, Y. Suzuki, Y. Saito, S. Soeta, H. Taira, A. Suzuki, *Chem. Biol.* **2006**, *13*, 1071.

[124] R. Chaix, C. Cao, P. Donnelly, *PLoS Genet.* **2008**, *4*, e1000184.

[125] J. Brechbühl, M. Klaey, M. C. Broillet, *Science.* **2008**, *321*, 1092.

[126] M. Schwanzel-Fukuda, D. Blick, D. W. Pfaff, *Mol. Brain. Res.* **1989**, *6*, 311.

[127] T. Hummel, R. Gollisch, L. Wildt, G. Kobal, *Experientia.* **1991**, *47*, 712.

[128] K. Grammer, *Ethol. Sociobiol.* **1993**, *14*, 201.

[129] A. Nixon, P. Jackman, A. Mallet, D. B. Gower, *Biochem. Soc. Trans.* **1984**, *12*, 1115.

[130] T. K. Kwan, N. F. Taylor, D. Watson, D. B. Gpwer, *Biochem. J.* **1985**, *227*, 909.

[131] C. J. Tue, R. A. Bojar, K. T. Holland, *Dermatology*. **1997**, *196*, 72.

[132] D. B. Gower, K. T. Holland, A. I. Mallet, P. J. Rennie, W. J. Watkins, *J. Steroid Biochem. & Mol. Biol.* **1994**, ***48***, 409.

[133] C. Austin, J. Ellis, *J. Steroid Biochem. & Mol. Biol.* **2003**, *87*, 105.

[134] B. W. Brooksbank, *Experientia.* **1970**, *26*, 1014.

[135] C. Froebe, A. Simone, A. Charig, E. Eigen, *J. Soc. Cosm. Chem.* **1990**, *41*, 185.

[136] A. Lukacs, H. C. Korting, G. Ruckdeschel, W. Ehret, *Dermatologica.* **1991**, *182*, 32.

[137] K. Ara, M. Hama, S. Akiba, K. Koike, K. Okisaka, T. Hagura, T. Kamiya, F. Tomita, *Can. J. Microbiol.* **2006**, *52*, 357.

[138] James A.G, Hyliands D., Johnston H, *Int. J. Cos. Sc.*. **2004**, *26*, 149.

[139] X. N. Zeng, J. J. Leyden, J. Brand, A. I. Spielman, K. J. Mc Ginley, G. Preti, *J. Chem. Ecol.* **1992**, *18*, 1055.

[140] A. I. Spielman, G. Sunavala, J. A. K. Harmony, W. D. Stuart, J. J. Leyden, G. Turner, B. R. Vowels, W. C. Lam, S. Yang, G. Preti, *Arch. Dermatol.* **1998**, *134*, 813.

[141] A. I. Spielman, X. N. Zeng, J. J. Leyden, G. Preti, *Experientia.* **1995**, *51*, 47.

[142] C. Zeng, A. I. Spielman, B. R. Vowels, J. J. Leyden, K. Biemann, G. A. Preti, *Proc. Nat. Acad. Sci. USA.* **1996**, *93*, 6626.

[143] M. Gautschi, A. Natsch, F. Schröder, *Chimia.* **2007**, *61*, 27.

[144] S. Auger, M. P. Gomez, A. Danchin, I. Martin-Verstraete, *Biochimie.* **2005**, *87*, 231.

[145] J.-W. Kim, H. J. Kim, Y. Kim, M. S. Lee, H. S. Lee, *Mol. Cell.* **2001**, *11*, 220.

[146] I. Rossol, A. Puhler, *J. Bacteriol.* **1992**, *174*, 2968.

[147] E. Zdych, R. Peist, J. Reidl, W. Boos, *J. Bacteriol.* **1995**, *177*, 5035.

[148] K. Aida, T. Tokuyama, T. Uemura, *Antonie van Leeuwenhoek.* **1969**, *1969*, I15-I16.

[149] J. Soutourina, S. Blanquet, P. Plateau, *J. Biol. Chem.* **2001**, *276*, 40864.

[150] T. Tokuyama, H. Kuraishi, K. Aida, T. Uemura, *J. Gen. Appl. Microbiol.* **1973**, *19*, 439.

[151] Y. Yoshida, Y. Nakano, A. Amano, M. Yoshimura, H. Fukamachi, T. Oho, T. Koga, *Microbiology.* **2002**, *148*, 3970.

[152] A. J. L. Cooper, S. A. Bruschi, M. W. Anders, *Biochemical Pharmacology.* **2002**, *64*, 553.

[153] A. J. L. Cooper, J. T. Pinto, *Amino Acids.* **2006**, *30*, 1.

[154] H. M. Wakabayashi, W. Wakabayashi, W. Eisenreich, K. H. Engel, *J. Agric. Food Chem.* **2004**, *52*, 110.

[155] A. J. L. Cooper, S. A. Bruschi, A. Iriarte, M. and Martinez-Carrion, *Biochem. J.* **2002**, *368*, 253.

[156] D. A. Dickinson, H. J. Forman, *Ann N Y Acad Sci.* **2002**, *973*, 488.

[157] G. L. Lamoureux, D. G. Rusness. In: (Eds.: D. Dolphin, O. Avroamovic, and R. Poulson), *Glutathione. chemical, biochemical, and medical aspects. Part B.*, **1989**, pp. 153-196.

[158] K. A. Marrs, *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* **1996**, *47*, 127.

[159] A. E. Wolf, K.-J. Dietz, P. Schroder, *FEBS Letters.* **1996**, *384*, 31.

[160] T. Kasai, P. O. Larsen, *Fortschr. Chem. Org. Naturst.* **1980**, *39*.

[161] A. J. L. Cooper, *Advances in enzymology and related areas of molecular biology*. **1989**, *72*, 199.

[162] R. A. John, *Biochim Biophys Acta*. **1995**, *1248*, 81.

[163] P. Darriet, T. Tominaga, E. Demole, D. Dubourdieu, *C. R. Acad. Sci. Paris, Biologie et Pathologie végétale.* **1993**, *316*, 1332.

[164] C. Starkenmann, M. Troccaz, K. Howell, *Flavour Fragr. J.* **2008**, *23*, 369.

[165] T. Tominaga, A. Furrer, R. Henry, D. and Dubourdieu, *Flavour Fragr. J.* **1998**, *13*, 159.

[166] T. Tominaga, D. Dubourdieu, *J. Agric. Food. Chem.* **2000**, *48*, 2874.

[167] C. Peyrot des Gachons, T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2000**, *48*, 3387.

[168] C. Peyrot des Gachons, T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2002**, *50*, 4076.

[169] T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2000**, *48*, 2874.

[170] T. Tominaga, C. Peyrot des Gachons, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 5215.

[171] S. Bailly, V. Jerkovic, J. Marchand-Brynaert, S. Collin, *J. Agric. Food Chem.* **2006**, *54*, 7227.

[172] M. Subileau, R. Schneider, J. M. Salmon, E. Degryse, *FEMS Yeast Research.* **2008**, *8*, 771.

[173] K. S. Howell, M. Klein, J. H. Swiegers, Y. Hayasaka, G. M. Elsey, G. H. Fleet, P. B. Hoj, I. S. Pretorius, M. A. de Barros Lopes, *Appl. Environ. Microbiol.* **2005**, *71*, 5420.

[174] J.-L. Luisier, J. Veyrand, U. Piantini, *Chimia.* **2007**, *61*, 533.

[175] C. Peyrot des Gachons, T. Tominaga, D. Dubourdieu, *Am. J. Enology Viti.* **2002**, *53*, 144.

[176] S. Shinkaruk, C. Thibon, J.-M. Schmitter, P. Babin, T. Tominaga, M. Degueil, B. Desbat, C. Jussier, B. Bennetau, D. Dubourdieu, C. Bennetau-Pelissero, *Chem. Biodiv.* **2008**, *5*, 793.

[177] X. Chone, V. Lavigne-Cruege, T. Tominaga, C. Van Leeuwen, C. Castagnede, C. Saucier, D. Dubourdieu, *J. Int. Sci. Vigne et du Vin.* **2006**, *40*, 1.

[178] J.-L. Luisier, H. Büttner, S. Völker, T. Rausis, U. Frey, *J. Agric. Food Chem.* **2008**, *56*, 2883.

[179] A. Stoll, E. Seebeck, *Experientia.* **1947**, *3*, 114.

[180] J. Lancaster, H. Collin, *Plant Sci. Lett.* **1981**, *22*, 169.

[181] P. Rose, M. Whiteman, P. K. Moor, Y. Z. Zhu, *Nat. Prod. Rep.* **2005**, *22*, 315.

[182] A. I. Virtanen, E. J. Matikkala, *Suom. Kemistil. B.* **1961**, *34*, 53.

[183] G. R. Fenwick, A. B. Hanley, *part 3. Crit Rev Food Sci Nutr.* **1985**, *22*, 1.

[184] M. G. Jones, J. Hughes, A. Tregova, J. Milne, A. B. Tomsett, H. A. Collin, *J. Exp. Bot.* **2004**, *55*, 1903.

[185] D. Joulain, R. Laurent, *11th congress of essential oils, Fragrances and Flavours, Primlani (Ed). Oxfords, IBH, New Dehli.* **1989**, *20*.

[186] R. G. Westall, *Biochem. J.* **1953**, *55*, 244.

[187] M. J. I. Mattina, J. J. Pignatello, R. K. Swihart, *J. Chem. Ecol.* **1991**, *17*, 451.

[188] W. H. Hendriks, A. D. Woolhouse, M. F. Tarttelin, P. J. Moughan, *Bioorg. Chem.* **1995**, *23*, 89.

[189] M. Miyazaki, K. Kamile**,** S. Soeta, H. Taira, T. Yamashita, *Biochem. J.* **2003**, *370*, 101.

[190] B. Burger, M. Viviers, J. Bekker, M. le Roux, N. Fish, W. Fourie, G. Weibchen, *J. Chem. Ecol.* **2008**, *34*, 659.

[191] M. Miyazaki, T. Yamashita, M. Hosokawa, H. Taira, A. Suzuki, *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology.* **2006**, *145*, 270.

[192] M. Masao, *Aroma Res.* **2007**, *8*, 56.

[193] W. H. Hendriks, K. J. Rutherfurd-Markwick, K. Weidgraaf, C. Ugarte, Q. R. Rogers, *J. Animal Physiol. Animal Nutr.* **2008**, *92*, 53.

[194] A. Baydar, M. Petrizilka, M. Schott, *Perf. Flav.* **1992**, *17*, 1.

[195] C. Wysocki, X. Zeng, G. Preti, *Chem. Senses.* **1993**, *18*, 653.

[196] P. J. H. Jackman, W. C. Noble, *Clin. Exp. Dermatol.* **1983**, *41*, 1134.

[197] C. Starkenmann, Y. Niclass, M. Troccaz, A. J. Clark, *Chem. Biodiv.* **2005**, *2*, 705.

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2. Major sulfur volatiles in axillary malodor

3-Methyl-3-sulfanylhexan-1-ol as Major descriptor for the human axilla-sweat Odor profile

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(Supporting material in ***Appendix 7.1*****)**

Abstract

This study sets out to redress the lack of knowledge in the area of volatile sulfur compounds (VSCs) in axillary sweat malodor. Sterile odorless underarm sweat (500 ml) was collected from 30 male volunteers after excessive sweating. Five strains of bacteria, *Corynebacterium tuberculostearicum*, *Corynebacterium minutissimum*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Bacillus licheniformis*, were isolated and characterised for their ability to generate an authentic axillary odor from the sweat material collected. As expected, all of the five bacterial strains produced strong sweat odors. Surprisingly, after extensive olfactive evaluation, the strain of *Staphylococcus haemolyticus* produced the most sulfury sweat character. This strain was then chosen as the change agent for the 500 ml of odorless underarm sweat collected. After bacterial incubation, the 500-ml sample was further processed for GC-olfactometry (GC-O), GC/MS analysis. GC-O of an extract free of organic acids provided three zones of interest. The first was chicken-sulfury, the second zone was onion-like, and the third zone was sweat, clary sage-like. From the third zone, a new impact molecule, (R)-or (S)-3-methyl-3-sulfanylhexan-1-ol, was isolated and identified by GC/MS, MD-GC, and GC AED (atomic emission detector). (S)-3-methyl-3-sulfanylhexan-1-ol was sniff-evaluated upon elution from a chiral GC column and was described as sweat and onion-like; its opposite enantiomer, (R)-3-methyl-3-sulfanylhexan-1-ol, was described as fruity and grapefruit-like. The (S)-form was found to be the major enantiomer (75%).

Keywords: Sulfur**,** 3-Methyl-3-sulfanylhexan-1-ol, Sweat, *Staphylococcus haemolyticus.*

1. Introduction

The formation of underarm odor primarily arises from the combination of an increased number of apocrine sweat glands and the action of a large, mixed, stable population of microorganisms, found in the axillary region [1-3]. Clearly certain strains of bacteria have evolved to live in this niche environment and are well adapted to growing on the peculiar cocktail of odorless precursors found in apocrine sweat.

The main groups of organisms found in the human axillae are as follows: staphylococci, aerobic coryneforms, propionibacteria, micrococci and *Malassezia spp.* with hugely varying population densities from 5×10^0 to 3×10^7 colony forming units (cfu) per cm^2 [4]. It appears that skin is a relatively simple ecosystem, however a certain degree of complexity certainly arises from the individual variations in population density, in combination with which of the microbial sub-populations attains dominance [5].

The volatile metabolism that occurs under the arm is derived from a complex degradative process of human secretions. The bacterial genera best adapted to the conversion of fresh apocrine sweat to the classic male locker room smell are aerobic *Corynebacterium spp*. and some *Staphylococcus spp*. Indeed certain *Corynebacterium spp.* have been often associated with the clinical condition of plantar bromidrosis (acute offensive body odor). It comes as no surprise then, that corynebacteria and certain staphylococci possess all the enzymatic machinery required to carry out biochemical conversions of proteins, lipids and steroids that are necessary in generating body malodor. By incubating lipophilic diphtheriods (corynebacteria) or certain staphylococci with apocrine secretions generated a strong and musky odor, whereas micrococci were reported to generate a more acidic-sweat odor (isovaleric acid odor) [3]. Isolates identified as *Corynebacterium xerosis*, were capable of producing underarm odor after 6 h in an *in-vitro* model [2], from apocrine secretion collected from the human axillary vault by intradermal injection of adrenaline [3][6].

The chemical composition of male sweat odor comprises four principal components. Firstly, a steroidal fraction, containing four very odorous steroids; 5α-androst-16-ene-3-one, androsta-4,16-diene-3-one and their respective alcohols (5α-androst-16-ene-3α-ol and androsta-4,16-diene-3α-ol) [7]. However, it is only recently that evidence for the exact identity of volatile odorous steroid precursors present in apocrine sweat have been reported [8].

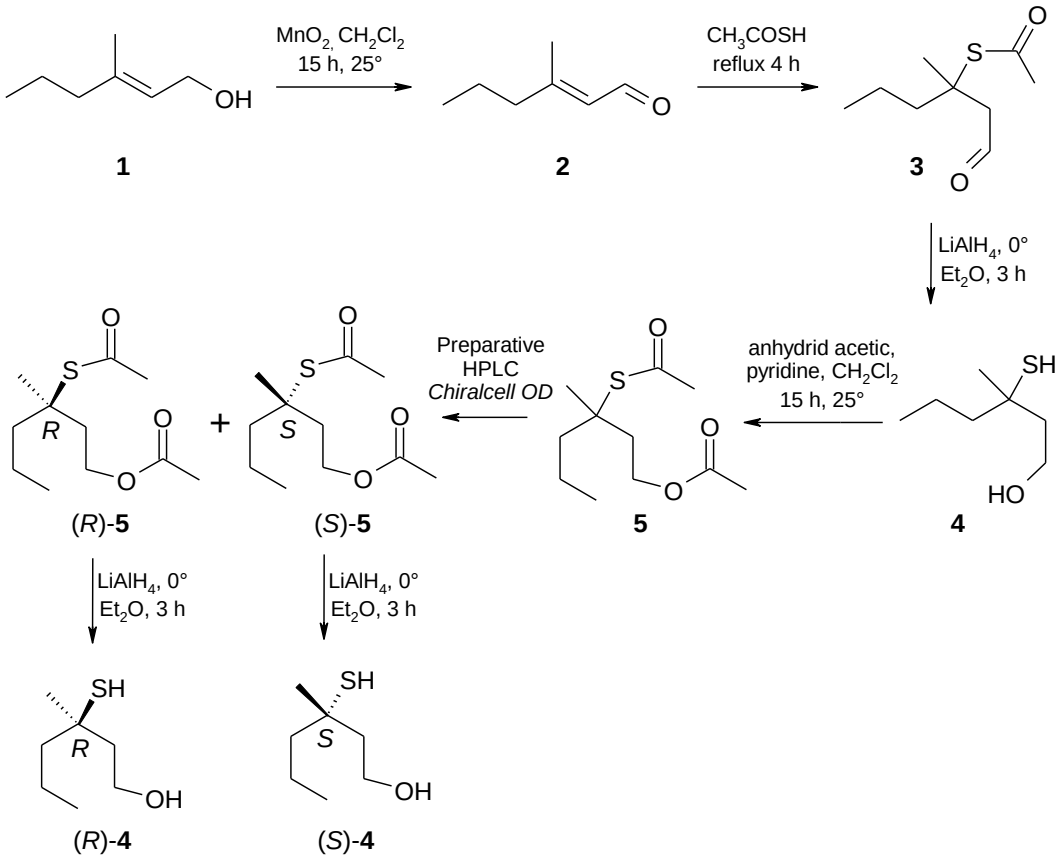
Secondly, volatile fatty acids (VFAs) can be formed by the action of certain bacteria. It is evident that leucine can act as a potential substrate for isovaleric acid generation. This conversion can be carried by *Corynebacterium spp*. Another group, the *Corynebacterium* sub-group *A* does not possess the ability to fully catabolise long chain fatty acids (LCFAs) and instead partial degradation products are formed ($C_{6–11}$ acids). This is especially the case with methyl branched fatty acids such as isostearic acid (heterogeneous cocktail of $C_{16–18}$ branched and unbranched fatty acids). With this substrate, a range of highly odorous, 2-methyl-$C_{6–14}$ fatty acids are generated as the endpoint of metabolism. VFA accumulation in apocrine sweat must depend on the relative activities of *Corynebacterium* sub-group *A* bacteria versus VFA degrading bacteria, such as *Micrococcus luteus* or *Brevibacterium epidermidis* [9].

Thirdly, the predominant olfactory contributor in axillary sweat is most likely to be (*E*)-3-methyl-2-hexenoic acid (**1**; *cf. The Scheme*) and was discovered by Preti and co-workers [10]. A comparison of the relative quantities in male sweat estimated **1** at over 700 fold higher concentration than the volatile odorous steroid androstenone [11]. The rapid release kinetics of **1** appeared inconsistent with the bacterial catabolic metabolism *e.g.* fatty acid degradation. It was subsequently demonstrated that 3M2HA is in fact bound to proteins secreted from the apocrine glands – apocrine secretion odor-binding proteins (ASOBs) [12][13]. Recently Acuna and co-workers cloned the gene for the enzyme responsible for the liberation of **1** from *Corynebacterium striatum.* They also identified that the chemically related acid 3-hydroxy-3-methylhexanoic acid represents another protein-bound form. It emerged that the ligand acids are non-covalently bound to the carrier protein in the form of glutamine conjugates. By synthesising these precursors, a Zn^{2+} dependent aminoacylase was identified, which effectively cleaved off both acids from the conjugate glutamine. Analysis of the coding region of the cloned gene revealed four conserved amino acid motifs common to a number of Zn^{2+}-dependent metalloproteases [14]. It would seem that human bacterial flora has co-evolved with the host to the extent where they present enzymes with unique substrate profiles in tune with the host secretions and chemosensory signalling.

Finally, sulfur containing amino acid degradation may also be associated with malodor generation by the formation of volatile-sulfur compounds. Mercapturates (*S*-[*N*-acetyl]-cysteine conjugates) play an important role in the detoxification of many potentially harmful xenobiotics in humans (for a review, see [15] and refs. cit. therein). Usually, a relatively stable thiol containing molecule is produced that is soluble and can be excreted. However,

certain bacteria have also been attributed with β-elimination activity for cysteine S-conjugates [16]. β-Elimination of cysteine S-conjugates can be performed by several microbial enzymes including; lipase [17], *β*-cystathionase (an enzyme involved in methionine biosynthesis), γ-cystathionase can also catalyse β-elimination [18], tryptophanase (a transaminase) and *β*-lyase from *Eubacterium limosum* [19]. It is known that sulfur containing amino acids are secreted in sweat and that pyridoxal phosphate-dependent *β*-lyase activity may be partly responsible for the generation of axillary malodor from apocrine secretions. This connection represents one of the few examples where thiols (as volatile sulfur compounds) are indicated as key components of the human axillary malodor. Although no data on structures of molecular intermediates or the purified enzyme activities were described [20].

Scheme



From the above literature Lebows and others [21] have created synthetic versions of human axilla-sweat odors. These mixtures of short chain fatty acids and steroids give a good approximation to the real odor, as determined by sensorial panel, but clearly lack important impact molecules. These re-creations are lacking only in the VSCs that has not be represented due to their problematic identification. In fact, for some twenty years researchers have looked to characterise this important subset of odorants. Whilst interesting and useful, body odor reconstitutions using mixtures of acids and steroids (including androstrenone and **1**), lacked the overall pungent nature typically found underarm. As described above, VSCs may also be major olfactive descriptors for body odor, although no structures have been reported [20]. Van de Waal *et al.*, attempted to substitute for the missing VSC component with 1-methoxyhexane-3-thiol from clary sage (*Salvia sclarea* L.) with some success [22]. However, no reports in the literature of VSC structures have been forthcoming. We wished to study the nature and generation of VSCs in human sweat malodor by following a pragmatic approach of building a exercise/sauna facility in order to collect underarm sweat from volunteers after excessive sweating. The objective was to use this odorless material to recreate an authentic axillary malodor in an *in-vitro* environment, in order to access the low threshold VSC component. Here we describe the isolation of several strains of bacteria and their expert characterisation in generating human underarm odor. Furthermore we present our initial findings and describe 3-methyl-3-sulfanylhexan-1-ol (**4**; *Scheme*) as a volatile sulfur compound generated in human sweat by microbial action.

2. Results

2.*1. Sweat collection*

The average quantities of underarm sweat collected were 3 ml/male volunteers after cycling and 10 ml after sauna. Approximately half these quantities were collected from female panelists. A total of 500 ml of non-odorous, sterile underarm sweat and 125 ml of malodorous sweat were collected from 30 volunteers over a eight-week period. Approximately 100 ml of polluted sweat (*i.e.* contaminated by fragrance or food odors) were also collected and discarded. The average protein concentration of collected underarm sweat samples was approximately 0.25 mg/ml (for both males and females). This quantity was approximately 10 times lower than the concentration reportedly found in the apocrine secretions [23].

Nevertheless, the dilution of apocrine secretions with eccrine sweat did not affect the generation of an acceptable sweat and pungent odor after bacteriolysis. As part of a control experiment we confirmed that the eccrine perspiration coming from the forehead produced no body/axillary odor when incubated with isolated bacteria (data not shown).

2.2. Bacterially generated sweat malodor

2.*2.1. Microbial diversity and its relationship to axillary malodor.* An average of 2 x 10^5 ± 5 x 10^4 cfu/cm^2 total aerobic counts were recovered from axillae, sampled 6 h after showering. As previously reported, no qualitative difference between the left and the right axilla was observed [9]. Diphtheroids were largely represented by *Corynebacterium spp.* at a concentration of 1 x 10^2 ± 2 x 10^1 cfu/cm^2. Micrococci including *Staphylococcus spp., Micrococcus spp.* and *Kocuria spp.* were the most common at a concentration of 2.5 x 10^5 ± 5 x 10^4 cfu/cm^2. A *Bacillus sp.* was recovered from just one subject at a very low concentration of 0.1 cells/cm^2. All the Gram-positive bacteria except the *Bacillus sp*. were described as microorganisms implicated in axillary malodor [2][9]. Very few Gram-negative bacteria (some *Enterobacter spp.* and *Pseudomonas spp.*) were recovered.

An olfactive screening of these microorganisms was performed with respect to their ability in biotransformation of axillary extracts. Acidic malodors were produced by the incubation of *Propionibacterium sp.,* however the signal intensity was low. Gram-negative bacteria gave nearly no contribution to the axillary malodor. By contrast corynebacteria, micrococci and the *Bacillus sp.* generated intensive and pungent malodors. Indeed five bacterial strains were selected for their ability to generate strong axillary odor and were taken on for further characterisation and descriptive sensorial analysis. The five strains were typed by 16S rDNA sequencing (*Pasteur Institute*) as: *Corynebacterium tuberculostearicum, Corynebacterium minutissimum, Staphylococcus epidermidis, Staphylococcus haemolyticus* and *Bacillus licheniformis* and were designated Ax 1–5, respectively.

2.2.2. Sensorial analyses. *Corynebacterium xerosis* DSM 20743 was chosen as a reference odor-generating strain. As expected, all six strains were able to convert sterile odorless sweat into malodorous sweat. The odor profiles after 12 h incubation with the six strains are represented in *Fig. 1*. The most intensive odors were obtained through incubation with *C. xerosis, B. licheniformis* Ax 5 and *S. haemolyticus* Ax 4, with sweat (acrid) intensity

ratings of 7.2, 7.3, and 7.9 respectively. A predominance of sweat, onion and acid notes were generated with *C. xerosis* whereas more sweat, onion and butter notes were descriptive of the *S. haemolyticus* converted sample. The latter was also responsible for the generation of a green and clary sage-like odor. *B. licheniformis* was described as sweat with a predominance of onion and chicken notes, whereas *C. minutissimum* Ax 2 was characterized with a highest floral rating (1.2). The incubation of *S. epidermidis* Ax 3, gave a chicken broth character and was evaluated as the least sweat-like.

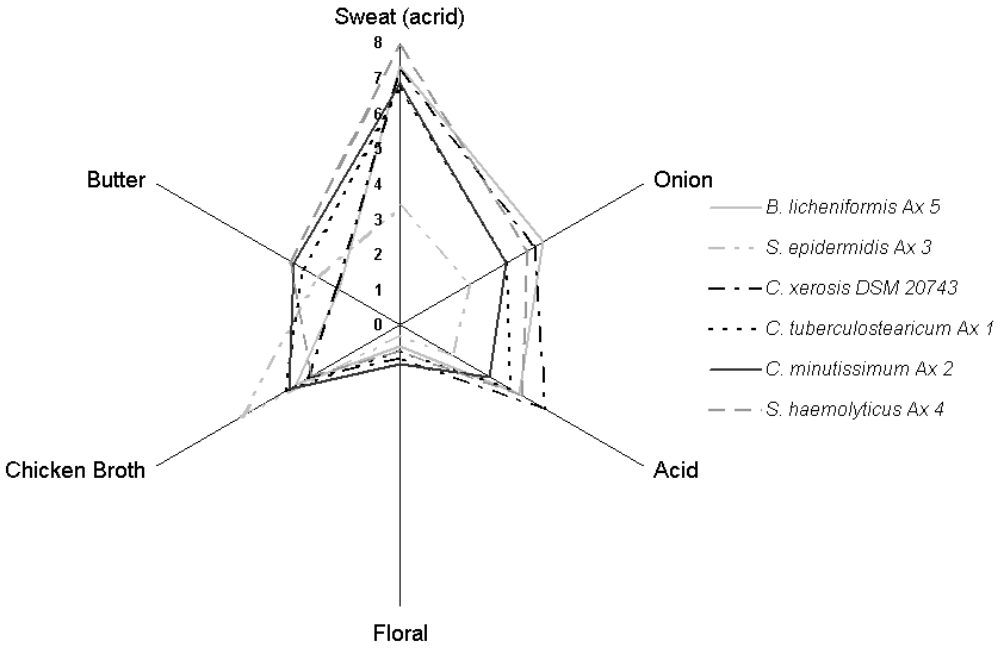


Fig. 1. Bacterially generated sweat odor profiles. Olfactive evaluation of an expert panel of assessors. Radar graphic of the average values of intensity for each of the six descriptors; acid, floral, butter, sweat, onion, and chicken broth for each of the six strains after a 12 h incubation period on sterile odorless axillary sweat.

2.2.3 Principal component analyses. The principal component analysis made from the descriptive mean scores explained 97.1% of the variance when principal component 1 (Axis 1) was plotted versus principal component 2 (Axis 2). The loading plot in *Fig. 2* shows that the six strains lie in three distinct groups with *S. epidermidis* clearly lying within a set apart. Furthermore, the attributes sweat, acid, onion and floral contribute strongly to Axis 1 and represent 88% of the results. There appears to be a positive correlation between sweat and onion and sweat and acid. A negative correlation exists between chicken and sweat and chicken and acid. A comparison of the loading plot with the score plot (*Fig. 1*) showed that the reference strain *C. xerosis* and the isolated strains, *B. licheniformis* Ax 5 and *S. haemolyticus* Ax 4 generated strong sweat, acid and onion notes rather than chicken and butter. The acidic and sweat descriptors were more characteristic of *C. xerosis*, whereas onion and sweat appear to better characterise *S. haemolyticus* Ax 4. A second group containing *C. tuberculostearicum* Ax 1 and *C. minutissimum* Ax 2 was characterized as more butter and chicken broth.

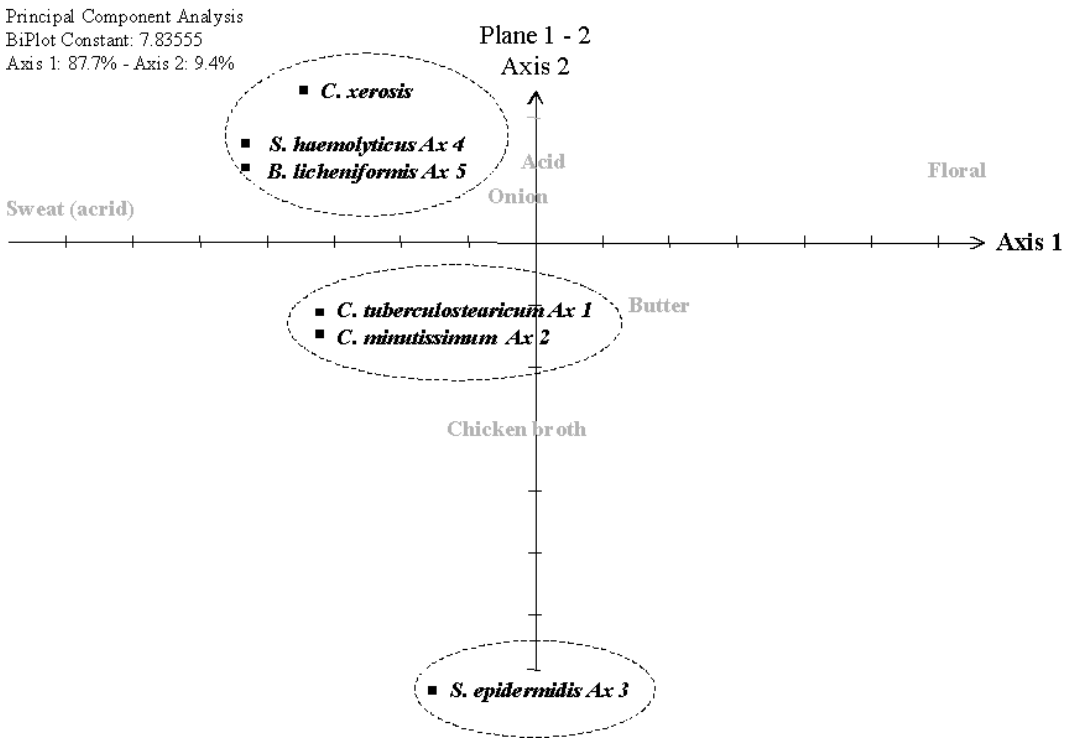


Fig. 2. Principal component analysis performed on the 5 isolated strains and C. xerosis DSM 207 43 and 6 descriptors. Axis 1 and Axis 2 represent 87.7% and 9.4% of the information respectively and were treated by descriptive analysis (FIZZ software, Biosystèmes, France).

2.3. Analysis of the key odorant molecules

The biotransformed axillary sweat was extracted with dichloromethane under acidic conditions in order to strongly protonate the carboxylic acids and thiol component. The candidate thiols were then separated from the acids by washing the organic fraction with aqueous bicarbonate. The resulting extract was injected on preparative polar *(SP-1000)* and apolar *(S-2100)* home-packed GC columns and the effluent was directed to a sniff port. A creative perfumer evaluated the effluents and his comments about the odors, at the specific times of elution, were written on the chromatogram. A solution of saturated alkanes ranging from C-5 to C-20 was used to calculate retention indices.

Three zones were identified with smells characteristic of sulfur compounds. The first zone at $I_{S\text{-}2100}$ 890 (± 20) was described as meaty and chicken broth, sulfury, the second zone at $I_{S\text{-}2100}$ 1000 (± 20) was described as green onion, sweat, floral and sulfury and the third zone at $I_{S\text{-}2100}$ 1150 (± 20) was described as typical sweat, sulfury, clary sage-like, and onion broth[2]. The sweat extract was then re-injected on GC-MS using a capillary apolar column (*Fig. 3*). In the two first zones, no relevant MS were recorded. In the last zone at $I_{SPB\text{-}1}$ 1157 (± 5) an MS spectrum having as the highest mass fragment *m/z* 148 followed by a small (M + 2) ion accounting for 5% of the signal intensity of 148, was a good indication for the presence of a molecule that contained one sulfur atom. The spectrum contained fragments at *m/z* 115 and 114 that were consistent with a loss of 33 and 34 (-SH and H_2S, respectively). The next fragment at *m/z* 97 was consistent with a loss of 17 (-OH), which suggested the presence of a hydroxyl group in the parent molecule. The sample was then injected onto a capillary apolar *SPB-1* column on a GC equipped with an AED. Sulfur-containing compounds were detected on a separate channel. Eight sulfur compounds were detected at increased levels at the bacteriolysis. The biggest signal was assigned to dimethyl sulfoxide while a large signal was present at *I SPB-1* 1153 (± 5) (*Fig. 4,b*).

[2] '*I* ' means Kovats retention index see Exp. Part for calculation.

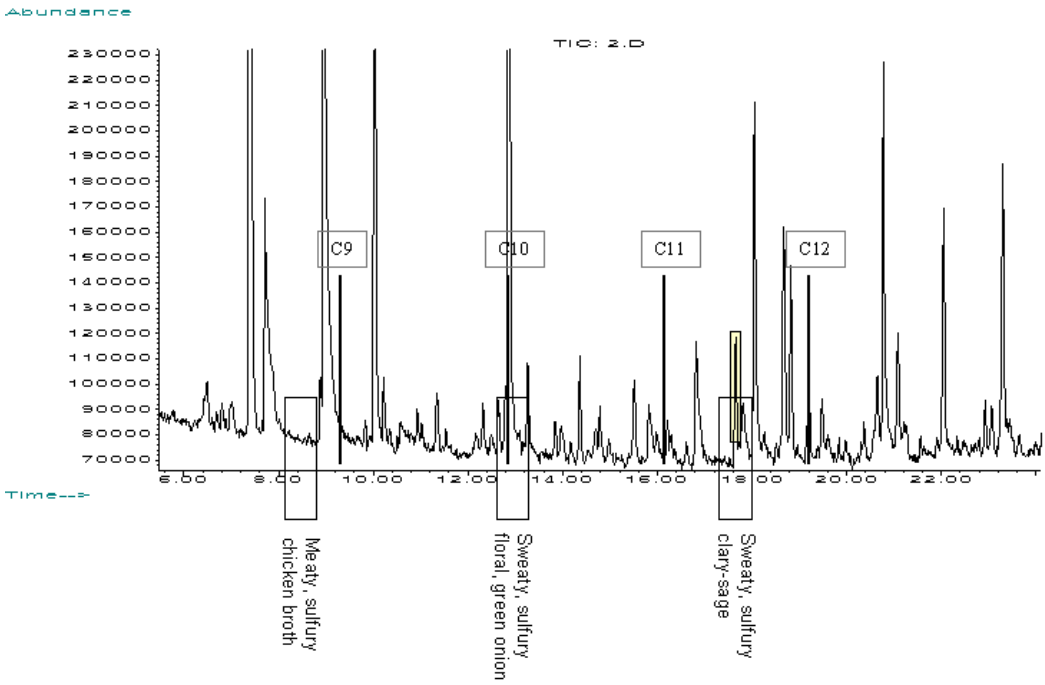


Fig. 3. GC-MS-TIC trace of S. haemolyticus biotransformed axillary sweat. The neutral fraction of a concentrated, dichloromethane-extracted sweat sample was separated on a SPB-1 column. Size markers are indicated for C_9–C_{12} alkanes/acids. The three zones of interest detected at the sniff port and their olfactive characteristics are indicated.

A similar experiment was carried out using a polar *SP-WAX* column. However, although the retention indices observed correlated poorly between the two columns, a matching odor was perceived at $I_{Sp\text{-}1000}$ 1810 (± 20) by GC-O with a preparative column. No MS having *m/z* 148 with ions 114 and 97 could be detected between retention indices 1800 to 1900, when the extract was injected on the *SP-WAX* coupled to MS. Sulfanyl compounds present in the malodorous extract were concentrated in dichloromethane, by Affigel® filtration, to 25% of the total extract volume. Injection of the Affigel® extract on a polar GC column, at $I_{SP\text{-}wax}$ 1890, resulted in a peak that gave a good mass spectrum that corresponded well to the spectrum seen with the apolar column, where the sulfur compound of interest co-eluted with benzyl alcohol. However, after Affigel® extraction the interfering benzyl alcohol was successfully removed.

To confirm that the 3-methyl-3-sulfanylhexan-1-ol was specifically generated by *S. haemolyticus* metabolism, the sweat extract (organic phase) was injected on an apolar

capillary *SPB-1* GC column coupled to an AED detector. Sulfur was specifically monitored and the resulting chromatogram was compared to the GC trace of a fresh untreated sweat extract (*Fig. 4*).

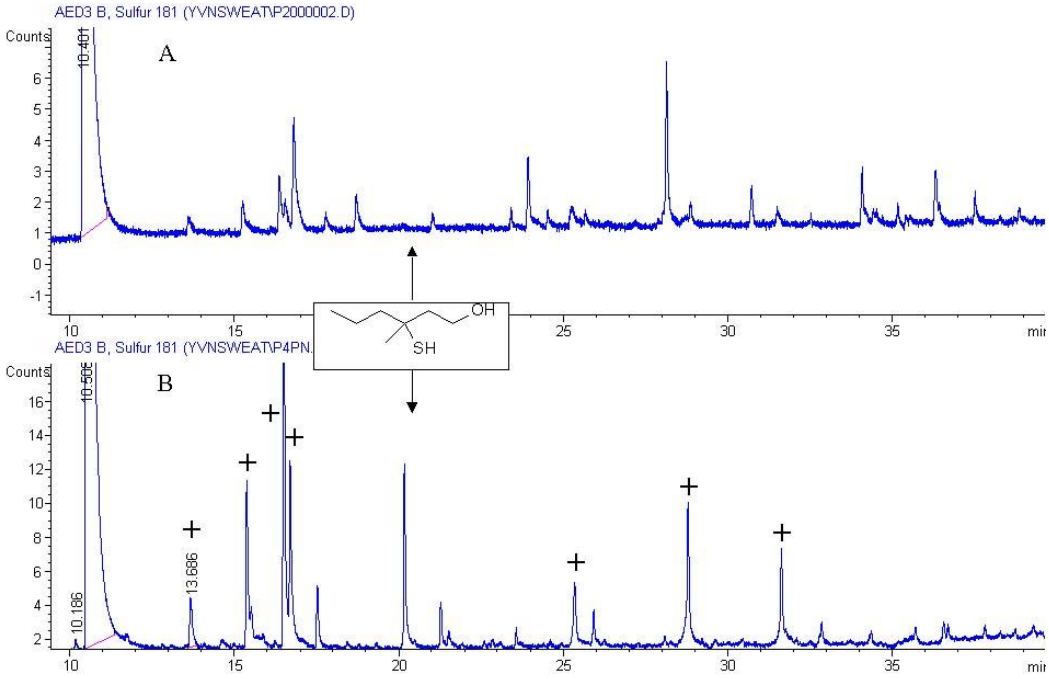


Fig. 4. GC chromatogram for the sulfur detection channel (AED detector) of the neutral fraction of a concentrated, dichloromethane-extracted untreated sweat sample (A) and after *S. haemolyticus* bacteriolysis (B). The position of 3-methyl-3-sulfanylhexan-1-ol is indicated by the boxed/arrowed structure. The positions of other sulfur compounds generated by bacteriolysis are also indicated (+).

The results of this investigation clearly showed the presence of sulfur compounds in the biotransformed sweat. At the retention index corresponding to the third zone of intense sweat odor (see above), a clear sulfur signal was detected.

*2.4. Synthesis of (*R*)-3-methyl-3-sulfanylhexan-1-ol ((R)-**4**) and (*S*)- 3-methyl-3-sulfanylhexan-1-ol ((S)-**4**)*

The synthesis of 3-methyl-3-sulfanylhexan-1-ol (**4**) was performed from the corresponding allylic alcohol **1** available in-house (*cf. Scheme*). The starting material, 3-methyl-2-hexen-1-ol (**1**), was oxidised with MnO_2 to the α,β-unsaturated aldehyde **2**, treated with thioacetic acid

and reduced with $LiAlH_4$. The synthetic 3-methyl-3-sulfanylhexan-1-ol (**4)** was injected on both polar and apolar capillary GC columns coupled to MS. We observed

good correlation of the retention indices of synthetic **4** ($I_{SP\text{-}wax}$ 1888 (± 5) and $I_{SBB\text{-}1}$ 1153 (± 5)), and the sweat compound ($I_{SP\text{-}wax}$ 1890 (± 5) and $I_{SPB\text{-}1}$ 1157 (± 5)). The mass spectrum fragmentation pattern of **4** was compared to the unknown sweat odorant molecule and was found to be identical. These clearly show that the identity of the sweat malodor compound produced by *S. haemolyticus* is 3-methyl-3-sulfanylhexan-1-ol (**4)**.

To determine the ratio of both enantiomers (*R*)-**4** and (*S*)-**4**, the synthetic racemate **4** was injected on a *Chiralsil-Dex CB*® mounted in a GC equipped with an AED detector. The separation of the synthetic enantiomers was good, but below the required 100 ppm threshold for sulfur compounds. A multidimensional GC (MD-GC) equipped with dual columns was then employed. The first column was a capillary chiral *DMePeBETACDX*, coupled to fused silica transfer line (designed by Dr. A. Chaintreau and F. Begnaud [24]). This new technology allowed the clear resolution of both enantiomers and sufficiently well as to allow good discrimination at the sniff port. The odor profile of the first eluting enantiomer (*S*)-**4** at 4.34 min was described as sweat, onion and animal, while the second eluting enantiomer (*R*)-**4** at 5.64 min appeared more fruity, grapefruit, sulfury in odor. The MD-GC configuration was changed and an apolar column was mounted before the second chiral column. With this configuration it was possible to inject the axillary malodor extract and selectively heartcut the target peak to the chiral column. From this experiment it was possible to observe a larger surface area of the first enantiomer (*S*)-**4**. Due to the minute quantity of material present in the extract, peaks areas were small and provided only a rough estimate of the (*S*):(*R*) enantiomeric ratio of 3:1. Co-injection with the synthetic 3-methyl-3-sulfanylhexan-1-ol **(4)** confirmed the identities of the two peaks. The absolute configurations of the enantiomers of **4** were determined after bis-acetylation of the 3-methyl-3-sulfanylhexan-1-ol (**4),** followed by injection on chiral preparative HPLC. The (*R*)- and (*S*)-enantiomers of 3-thioacetyl-3-methyl-1-hexylacetate (**5)** were de-protected with $LiAlH_4$ and the rotations measured. The first peak that eluted from the HPLC was assigned as the (*S*)-**4** enantiomer due to the negative rotation observed. The value corresponded to the published value [25]. We were not able to repeat the published procedure for the enantioselective preparation of the enantiomers and we were not able to find a chiral HPLC column that could separate the enantiomers of **4** directly. The use

of preparative HPLC (*Chiracel-OD*) directly on the bis-acetate **5** proved to be a successful pathway to rapidly access both enantiomers [26].

The concentration of 3-methyl-3-sulfanylhexan-1-ol (**4)** in the incubated sweat sample was estimated to be close to 4 pbb (15 nM). This estimation was performed on the basis of injections (GC-AED, *SPB-1*) of the synthetic **4** at different concentration until we obtained a comparable peak area. No internal standard was used.

3. Discussion

This study confirmed previous findings that the axillae in humans support a stable relatively simple microflora in which the major microbial microorganisms are limited to aerobic coryneforms, and members of the *Micrococcaceae*. Previously reported models, using apocrine secretions and isolated bacterial strains, succeeded in producing an acrid underarm odor. However, these studies were restricted due to the very limited amount of apocrine secretion collected [10]. It is for this reason that studies of low threshold impact molecules (such as VSCs) have been scarce. The method reported here, collecting axillary sweat from people in a sauna or whilst exercising, provides a huge advantage in allowing a continuous supply of good quality sweat for experimentation with minimum inconvenience to the volunteers involved. This method was considered suitable after expert evaluation and produced excellent and intense axillary malodor after incubation with axillary microflora (*Figs. 1* and *2*). In these experiments it was crucial to control the collected secretions for quality, *e.g.* odorless and high protein content (as a measure of the level of apocrine secretion present). In fact, in 20% of cases, the samples were rejected due to the presence of odor immediately after collection. This odorous sweat is currently under investigation to determine impact odorants for stress related sweat odor. A further 15% of polluted samples were discarded.

Interestingly, in our hands a strain of *S. haemolyticus* (Ax 4) generated the most intense underarm odor as determined by an expert panel of perfumers and trained assessors. This organism also demonstrated the mostly sulfury, onion-like and sweat notes, relative to the other four strains (*Fig. 1*). Therefore, *S. haemolyticus* Ax 4 was chosen as the change agent

for the 500 ml of sterile odorless human sweat collected. The biotransformed sweat was then taken on for GC-O, MD-GC-O, GC-AED and GC-MS analysis for the identification of VSCs. GC olfactometry identified three clear zones of interest. Under the third zone (described as

sweat, sulfur and onion) a thiol peak was detected by GC followed by atomic emission detection (GC-AED). A further 8 sulfur compounds were detected in the extract and are under current analysis (*Fig. 4*). These data appeared to confirm the importance of sulfur compounds as significant contributors to sweat malodor. In some instances, when sulfur compounds are present in very low concentrations, the use of the Affigel® technique can produce artifactual information [22]. The concentrated thiol compounds eluted from Affigel® in our experiment appeared to suffer no deleterious effects of this technique.

The GC-MS spectra of the "sulfur" peak indicated the presence of a hydroxyl group in the parent molecule. The mass spectrum suggested the unknown odorant to be 3-methyl-3-sulfanylhexan-1-ol (**4)** and this was confirmed by the synthesis of (*R*)-**4** or (*S*)-**4** and comparison of the MS fragmentation patterns. These data clearly show the identity of the sweat malodor compound and that it was generated by the metabolism action of *S. haemolyticus* on fresh, sterile, human axillary sweat. Recent work on impact odorants in Sauvignon wine and the *Michael* addition of cysteine to α/β-unsaturated aldehydes described the formation of 3-sulfanyl-alcohols such as; 3-sulfanylhexan-2-ol [27] [28]. 2-Methyl-3-sulfanylpentan-1-ol is an important odorant molecule in onion [29] and 4-methyl-2-oxo—4-sulfanylpentane is predominant in passion fruit and yuzu odors [30]. To our knowledge 3-methyl-3-sulfanylhexan-1-ol (**4**) is the first structure of a VSC described in the literature that can be assigned as a major descriptor of human underarm odor.

The (*S*)/(*R*) enantiomer ratio was determined to be *ca.* 3:1. Interestingly, the (*S*)-formhas also been described as the major enantiomer of other 3-sulfanyl compounds described in the literature.

The (*R*)-enantiomer was evaluated as grapefruit-like whereas the (*S*)-form was described as sweat and onion-like. We, therefore, concluded that (*S*)-3-methyl-3-sulfanylhexan-1-ol ((*S*)-**4**) is very likely to be the most important contributor to the typical and repulsive sweat malodor (See ***Appendix 7.1)***.

Our ongoing studies in this area will focus on: the elucidation of chemical structures for other sulfur containing peaks and the identification of biochemical pathways leading to VSC

in sweat. To this end, the preparation of putative precursors is underway in our laboratory, as is the characterisation of candidate enzyme systems expressed in *S. haemolyticus* Ax 4.

4. Experimental part

*General.*Unless otherwise stated, all bacteriological media were purchased from *Difco* (USA). *Corynebacterium xerosis* DSMZ 207 43 (corresponding to the ATCC 373) was provided by the *DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*, D-Braunschweig) and was chosen as a routine reference strain as described in [3]. Commercially available chemicals (reagents and solvents) of adequate quality were used without further purification. Reactions were carried out under Ar. Org. extracts were washed to neutrality with 1_M HCl soln., and/or sat. $NaHCO_3$ soln. sat. NaCl soln., dried ($MgSO_4$), and evaporated. Anal. GC: *Agilent 6890* instrument coupled to atomic emission detection (AED, from *Jass*, Germany); He as carrier gas; fused-silica cap. columns *SPB-1* and *CP Chirasil-DexCB*®, 25m x 0.25 mm i.d. with 0.25 µm film (from *Chromapak*). EI-MS: *Agilent 6890-GC* system coupled to *HP MSD-5973* quadrupole mass spectrometer; electron energy *ca.* 70 eV; fragment ions *m/z* (rel. int. in % of the base peak, *SPB-1* and *Supelcowax*® 10, 25 m x 0.25 mm i.d. with 0.25 µm film, all from *Supelco*). The retention indices (*I*) were determined relative to the retention time (t_R) of a series of alkanes with linear interpolation by means of a standard GC temp. program (50° for 5 min, then 5°/min to 240°, and 20 min at 240°). MD-GC (MultiDimensional-Gas Chromatography): *Agilent 6890*. Enantiomer sniffing: first dimension *DMePeBETACDX (OV1701*): 9 m x 0.25 mm x 0.25 µm from *Mega*; second dimension: a deactivated fused silica capillary (for technical details, see [24]). Determination of enantiomeric ratios: first dimension: fused capillary column, *CP-Sil5CD*® 30 m x 0.32 mm x 1 µm, from *Chromapack*; second dimension: *DMePeBETACDX (OV1701*) 9 m x 0.25 mm x 0.25 µm, from *Mega. Prep*. GC-O: *Varian Star 3600* equipped with glass columns and heated sniff ports (200°); 5% *SP*-1000 on *Supelcoport* (100 - 120 mesh) and 10% *SP*-2100 (*Supelco*). Optical rotations: *Perkin-Elmer 241* polarimeter; cell thermostatted at 20° (l = 0.1, in $CHCl_3$). ^{1}H-and ^{13}C-NMR spectra: *Bruker AMX-360* spectrometer; in $CDCl_3$; δ values in ppm downfield from Me_4Si (= 0 ppm), *J* in Hz; assignments by COSY45 and HMCQ experiments.

Prep. Chiral HPLC: *Spectra Physics, SP 8800* ternary pumps, UV detection at 220 nm with *Waters 490 E*, column *Chiralcel OD* 25 cm x 2 cm i.d. from *Diacel Chemical Industries*, elution with hexane/i-PrOH 9 :1 at 5 ml/min flow rate.

Human sweat collection. Apocrine and eccrine secretions were collected from axillae through excessive sweating. Volunteers were asked to collect the sweat that developed from underarm with a plastic goblet whilst taking a sauna or cycling on an exercising bike. Secretions were immediately sterilised through a double filter consisting of 1-µm membrane followed by 0.2-µm sterile filter (*Syringe Prefilter Plus, Nalgene*^{TM}). The time between sweat collection and filtration was not longer than 15 min. Samples were stored at -20° until use. Secretions were olfactively assessed, and the total protein content was determined by the *Bradford* assay using bovine serum albumin as standard. Only non-odorous and high-protein-containing samples (> 0.2 mg/ml) were used in the following experiments.

Microbiological sampling of human axillae. Bacterial isolates were collected from the underarms of six European volunteers (50% men and 50% women aged 26 to 40 years). Deodorants and antiperspirants were prohibited 3 d prior to the sampling. Quant. cultures were obtained with the *Williamson* and *Kligman* detergent scrub technique [31]. This method employs 1 ml of 0.1% *Triton-X-100*, pipetted into a sterile steel cylinder placed on the axilla; the fluid is stirred vigorously for 1 min with *Teflon*® rod and removed. Dilutions were made in 0.05% buffered *Triton* and plated on the following media: tryptic soya broth (TSA) for total aerobic bacteria; heated blood agar for coagulase-negative staphylococci (*Bio-Merieux*); blood tellurite agar (*Merck*) and Tryptic Soya Broth containing 2 g/l of olive oil for lipophilic *Corynebacterium spp*. and brain heart infusion containing KNO_3 for *Bacillus spp*. The first three media were incubated aerobically at 37° for 48 h while the brain heart infusion plates containing KNO_3 were incubated anaerobically in a *Gas-Pak* jar (from *Merck*) system at 37° for 7 d. In the latter, case samples were pasteurised for 10 min at 80° before incubation. Bacteria were classified in four families according to the classification of *Leyden et al*. [3]; micrococci, lipophilic diphtheroids, *Propionibacterium spp*., and others containing *Gram*-negative and sporulating *Gram*-positive bacteria.

Bacterial characterisation. Bacterial identification was carried out using gallery *API* (*Bio-Merieux)*. Strains of interest were typed by the *Pasteur Institute* (Paris) by 16S rDNA sequencing.

Method for regenerating axillary malodor. Bacteria isolates were grown aerobically in the appropriate liquid media at 37° until OD_{600}=1.0. Cells were harvested by centrifugation at 3000 g, 10 min, washed once with sterile 0.1_M phosphate buffer (pH 6.0), and resuspended in fresh buffer. The concentration of individual strains incubated with the sweat precursor was 1 x 10^7 cfu/ml, following preliminary olfactive evaluations with various concentrations of bacteria. The cell suspensions were concentrated four times before mixing 50 µl with 400 µl of axillary secretions. A closed bottle was used to reproduce a semi-occluded environment that minimized evaporation of water. The optimum conditions for underarm odor production were similar to those in the human axilla, *e.g.*, 37°, pH 6. The malodor formation was detected after 3 h incubation at 37°. However, a more realistic axillary malodor was generated after 12-h incubation.

Sensorial analysis of hydrolysed secretion samples. Olfactive evaluations were performed by an expert panel constituted of one *Firmenich* creative perfumer, *Matthijs van deWaal*, an expert in axillary malodor, and 30 trained olfactive assessors. The panel was based on an intensity scale from 0 (none) to 10 (very strong). Six descriptors were carried out to describe the malodor: acid, chicken broth, onion, sweat, butter, and floral. Two negative controls were evaluated at the same time: one containing no bacteria and another without axillary sweat. Only bacteria selected for their ability to generate characteristic axillary malodor were fully characterised. From panel scores, the mean and the standard deviation were calculated for each attribute. Principal component analyses (PCA) were conducted using the means from the descriptive analyses. Data were treated with the *FIZZ Software* (*Biosystèmes*, France) and *Excel 2000*.

Extraction and purification of hydrolysed secretion samples. 500 ml of bacterially hydrolysed axillary secretions (pH 7.5) was spiked with 6N HCl (1.6 ml), to adjust the pH to 1. Extraction was carried out with 3 x 250 ml of CH_2Cl_2. 6.21 g of solid was recovered by lyophilisation. The org. phase was washed with 5 x 30 ml of sat. aq. $NaHCO_3$ soln., dried (Na_2SO_4), and then filtered. Any solvent was removed by distillation on a *Vigreux* column under Ar to a volume

of 2 ml, which was separated into 1-ml aliquots. These latter were finally stored at -20° as the neutral phase. The basic H_2O phase was acidified with 6N HCl, extracted with CH_2Cl_2, dried (Na_2SO_4), and filtered. The solvent was again removed by *Vigreux* column distillation under Ar to 1 ml of volume, and the concentrated extract was stored at -20° as the acidic phase.

*(R)/(S)-S-[1-Methyl-1-(2-oxoethyl)butyl] Ethantioate (**3**).* A 1:3 mixture (Z)/(E)-*3-methylhex-2-en-1-ol* (**1**; 10 g, 88 mmol) provided by the *Firmenich* chemical plant (CH-La Plaine), was treated at r.t. with MnO_2 (100 g) in CH_2Cl_2 (200 ml) overnight. The soln. of crude known aldehyde **2** [32] was filtered and concentrated. MeCOSH was added (10 g, 132 mmol), and the mixture was heated for 4 h at 100°. The mixture was then distilled on a 15-cm *Vigreux* column to yield **3** (8.4 g, 45%). B.p. 85 – 88°/15 mbar. Compound **3** is not very stable and was directly reduced after distillation. GC/MS: *SPB-1* column. ^{1}H-NMR: 9.81 (*dd*, *J* = 2.5, 2.5, 1 H); 3.03 (*dd*, *J* = 2.5, 15.8, 1 H); 2.90 (*dd*, J = 2.5, 15.8, 1 H); 2.27 (*s*, 3 H); 1.89 - 1.63 (*m*, 2 H); 1.52 (*s*, 3 H); 1.44 - 1.32 (*m*, 4 H); 0.91 (t, J = 7, 3 H). ^{13}C-NMR : 201.1 (*d*, CHO); 196.3 (*s*, COS); 51.8 (*s*, C(1')); 51.4 (*t*, CH2CHO); 42.5 (*t*, C(2')); 31.3 (*q*, *Me*-COS); 25.4 (*q*, *Me* - C(1')); 17.4 (t, C(3')); 14.2 (q, Me(4')).MS: 188 (0.5, M^+), 145 (38), 128 (26), 103 (25), 95 (26), 69 (92), 56 (38), 43 (100).

*(R)/(S)-3-Methyl-3-sulfanylhexan-1-ol (**4**).* Compound **3** (940 mg, 5 mmol) was added to a suspension of $LiAlH_4$ (285 mg, 7.5 mmol) in Et_2O (40 ml). After 4 h, the mixture was poured onto aq. HCl with ice and worked up with Et_2O. The crude oil (770 mg) was purified by flash chromatography (SiO_2 (40 g); pentane/Et_2O 7:3). Pure **4** (570 mg) was obtained in 77% yield. Retention indices were determined (ISPB-1 1153, ISPWax 1888) on *Chiralsil-DexCB*® (oven temp. programme started at 100°, heated at 5°/min to 200°) (*S*)-**4**: (t_R 12.32 min, (*R*)-**4**: (t_R 12.51 min. ^{1}H-NMR (+D_2O): 3.80 - 3.90 (*m*, CH_2(1)); 1.80 - 1.95 (m, CH_2(2)); 1.40 - 1.60 (*m*, CH_2(4), CH_2(5)); 1.38 (*s*,Me-C(3)); 0.95 (*t*, Me(6)); ^{13}C-NMR: 60.0 (*t*, C(1)); 47.6 (*t*, C(4)); 46.9 (*s*, C(3)); 46.2 (*t*, C(2)); 30.3 (*q*, *Me*-C(3)), 17.9 (*t*, C(5)); 14.4 (*q*, C(6)).MS: 150 (0.5, $[M + 2]^+$), 148 (11,M^+), 115 (22, [M-SH]), 114 (28), 97 (65), 81 (25), 71 (55), 69 (40), 55 (100), 41 (40).

*rac-3-(Acetylthio)-3-methylhexyl Acetate (**5**).* Compound **4** (50 mg, 0.34 mmol) was treated with Ac_2O (200 mg, 2 mmol) in presence of pyridine (0.4 ml) at r.t. overnight. After the usual workup, 65 mg of crude **5** (83% yield) was separated by prep. chiral HPLC. ^{1}H-NMR: 4.18 (*t*,

J = 7.4, 2 H); 2.29, 2.07 (*m*, 2 H); 2.24 (*s*, 3 H); 2.03 (*s*, 3H); 1.81 - 1.59 (*m*, 2 H); 1.43 (*s*, 3 H); 1.42 - 1.31 (*m*, 2 H); 0.91 (*s*, 3 H). ^{13}C-NMR : 196.5 (*s*, Me - COS); 171.0 *(s, Me-CO2); 61.6 (t, C(1')); 53.8 (s, C(3')); 42.1 (t, C(4')); 36.9 (t, C(2')); 31.5 (q, Me* - COS); 25.2 (*q*, Me - C(3')); 21.0 (*q*, Me - CO2); 17.5 (*t*, C(5')); 14.4 (*q*, C(6')). MS: 232 (1, M^+), 173 (5), 157 (28), 97 (100), 81 (25), 71 (55), 69 (20), 55 (80), 43 (60).

Reduction of (S)-**5** *and (R)*-**5**. The enantiomers *(S)*-**5** ($[\alpha]_D$ = -3.0, (*c* = 1.5, $CHCl_3$)) and (*R*)-**5** ($[\alpha]_D$ = 2.9 (*c* = 1.3, $CHCl_3$)) (18 mg, 0.077 mmol) were separately reduced with $LiAlH_4$ (10 mg, 0.26 mmol) in Et_2O at r.t. for 4 h.Workup gave 5 mg of each of the pure enantiomers, which had the same t_R values on achiral columns, indices I_{SPB-1} 1153; on a chiral column (*Chiralsil-DexCB*®, oven temp. 100° and 5°/min to 200°) (*S*)-**4**: t_R12.32 min $(\alpha]_D$ = - 1.8 (*c* = 0.5, CHCl)), (*R*)-**4**: t_R 12.51 min $(\alpha]_D$ = +2.3 (*c* = 0.5, $CHCl_3$)). Absolute configurations were assigned according to [25].

Bibliography

[1] C. Froebe, A. Simone, A. Charig, E. Eigen, *J. Soc. Cosmet. Chem.* **1990**, *41*, 173–185.

[2] P.J. Rennie, D.B. Gower, K.T. Holland, *British Journal of Dermatology* **1991**, *124*, 596–602.

[3] J.J. Leyden, K.J. McGinley, K. Heolzle, J.N. Labows, A.M. Kligman, *J. Invest. Dermatol.* **1981**, *77*, 413–416.

[4] D. Taylor, A. Daulby, S. Grimshaw, G. James, J. Mercer and S. Vaziri, *Int. J. Cosmetic Science.* **2003**, *25* (3),137–145.

[5] K.T. Holland, R.A. Bojar, *Am. J. Clin. Dermatol*. **2002**, *3* (79), 445–449.

[6] T. Aoki, *J. Invest. Dermatol*. **1962**, *38*, 41–44.

[7] D.B. Gower, A.I. Mallet, W.J. Watkins, L.M. Wallace, J.P. Calame, *J. Steroid Biochem. Molec. Biol*. **1997**, *63*, 81–89.

[8] C. Austin, J. Ellis, *J. Steroid Biochem. Molec. Biol*, **2003**, *87*, 105–110.

[9] A.G. James, J. Casey, D. Hyliands, H. Johnston, G. Mycock, Generation and tunover of volatile fatty acids by axillary bacteria, 22nd IFSCC Congress Edinburgh, **2002**.

[10] X.N. Zeng, J.J. Leyden, J. Brand, A.I. Spielman, K. McGinley, G. Preti, *J. Chem. Ecol.* **1992**, *18*, 1039–1055.

[11] A. Spielman, X.N. Zeng, J.J. Leyden, G. Preti, *Experimentia* **1995**, *51*, 40–47.

[12] X.N. Zeng, A. Spielman, B.R. Vowels, J.J. Leyden, K. Biemann, G. Preti, *PNAS* USA **1996**, *93*, 6626–6630.

[13] A.I. Spielman, G. Sunavala, J.A. Harmony, W.D. Stuart, J.J. Leyden, G. Turner, B.R. Vowels, W.C. Lam, S. Yang, G. Preti, *Arch. Dermatol.* **1998**, *134*, 813–818.

[14] A. Natsch, H. Gfeller, P. Gygax, J. Schmid, G. Acuna, *J Biol. Chem.* **2003**, *278*, 5718–5727.

[15] A.J. Cooper, *Adv. Enzymol. Relat. Areas Mol. Bio.* **1998**, *72*, 199–238.

[16] W. Dekant, S. Vamvakas, K. Berthold, S. Schmidt, D. Wild, D. Henschler, *Chem Biol Interact.* **1986**, *60* (1), 31–45.

[17] H. Wakabayashi, M. Wakabayashi, W. Eisenreich, K.H. Engel, *J. Agric. Food Chem.* **2003**, *51*, 4349–4355.

[18] K.E. Seefeldt, B.C. Weimer, *J. Dairy Sci.* **2000**, *83*, 2740–2746.

[19] H. Wakabayashi, M. Wakabayashi, W. Eisenreich, K.H. Engel, *J. Agric. Food Chem*. **2004**, *52*, 110–116.

[20] S. Lyon, C. O'Neal, H. Van Der Lee, B. Rogers, Amino acid beta lyase enzyme inhibitors as deodorants, International Patent N° WO 91/05541.

[21] J. Labows, G. Preti, E. Hoelzle, J. Leyden, A. Kligman, *J. Chromatogr.* **1979,** *163* (3), 294–299.

[22] M. Van de Waal, Y. Niclass, R. Snowden, G. Bernardinelli, S. Escher, *Helv. Chimica Acta,* **2002**, *85*, 1246–1260.

[23] E. Eigen, C. Froebe, Deodorant compositions comprising inhibitors of odour-producing axillary bacterial exoenzymes, United States Patent 5,676,937.

[24] A. Chaintreau, F. Begnaud, Multidimensional gas chromatography using a double-cool strand interface, 27th International Symposium on Capillary Chromatography, Riva del Garda, **2004** (in press).

[25] G. Acuna, M. Gautschi, F. Kumli, J. Schmid, J. Zsindely, Mercapto-alkanol flavor compounds US 6,610,346B1, **2003**.

[26] Y. Okamoto, Y. Kaida, *J. Chromatogr. A.* **1994**, *666*, 403–419.

[27] P. Darriet, T. Tominaga, E. Demole , D. Dubourdieux, Mise en évidence dans le raisin de *Vitis vinifera* (var. Sauvignon) d'un précurseur de la 4-mercapto-méthylpentan-2-one, *C.R. Acad. Sci.* Paris, Bio. Pathol. Végétale **1993**, *316*, 1332–1335.

[28] T. Tominaga, C. Peyrot des Gachons, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 5215–5219.

[29] S. Widder, C. Sabater Lüntzel, T. Dittner, W. Pickenhagen, *J. Agric. Food Chem.* **2000**, *48*, 418–423.

[30] T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2000**, 48, 2874–2876.

[31] P. Williamson, A.M. Kligman, *J. Invest. Dermatol.* **1965**, *45*, 498–503.

[32] N.Khan, D.E. Loeber, T. P. Toube, B.C.L. Weedon. *J. Chem.Soc., Perkin Trans*. 1, **1975**, 1457.

3. Identification of sulfur odor precursors

Identification of the Precursor of (*S*)-3-Methyl-3-Sulfanylhexan-1-ol, the Sulfury Malodor of Human Axilla-Sweat

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(Supporting material in ***Appendix 7.2*****)**

Abstract

A careful study of human axillary microflora led us to the identification of a new strain of *Staphylococcus haemolyticus*. The role in axillary malodor formation of this microorganism was compared to those of *Corynebacterium xerosis* and *Staphylococcus epidermidis*, upon incubation on sterile human ecrine and aprocrine axilla sweat. *S. haemolyticus* was responsible for the strongest sulfury malodor and the generation of the volatile sulfur compound (VSC), (*S*)-3-methyl-3-sulfanylhexan-1-ol (**3**). In this study we investigated the non-volatile precursors of VSCs. Human axillary sweat was collected, fractionated and analysed by HPLC-APCI-MS (High Pressure Liquid Chromatography coupled to Atmospheric Pressure Chemical Ionisation Mass Spectrometry). The precursor of 3-methyl-3-sulfanylhexan-1-ol **3** was identified as [1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine (Cys-Gly-(*S*)-conjugate), **12**. Because Cys-Gly-(*S*)-conjugates are key intermediates in the glutathione biodetoxification pathway, other derivatives of **12**, specifically glutathione-(*S*)-conjugate **11** and Cys-(*S*)-conjugate **13** were prepared. Compounds **11** and **13** were not detected by HPLC-MS of sterile sweat. Synthetic homologues **11**, **12**, and **13** were incubated with *C. xerosis*, *S. heamolyticus* and *S. epidermidis*. We observed efficient conversion of precursors **12** and **13** to form VSCs when incubated with *S. haemolyticus*, with a clear preference for **12**. *C. xerosis and S. epidermidis* were less efficient in cleaving Cys-Gly-(*S*)-conjugate **12** to form the corresponding thiol **3** (See ***Appendix 7.2***). Incubation of glutathione-(*S*)-conjugate **11** never led to the formation of **3** under the experimental conditions employed.

Keywords: Human Axilla, 3-Methyl-3-Sulfanylhexan-1-ol, *Staphylococcus haemolyticus*, Cysteinylglycine Conjugate.

1. Introduction

Significant progress has recently been made in the study of the composition of human underarm malodor. Three classes of compounds have been identified as major contributors to axillary malodor; one class is of steroidal origin [1][2] while another class of compounds is composed of short chain fatty acids such as (*E/Z*)-3-methyl-2-hexenoic acid. This acid has been described as the predominant olfactory contributor to male sweat malodor [3]. (*E/Z*)-3-Methyl-2-hexenoic acid is produced by the cleavage of the water soluble precursors [4] *N*-*α*-3-methyl-2-hexenoyl-(*L*)-glutamine **1** and *N*-*α*-3-hydroxy-3-methylhexanoyl-(*L*)-glutamine **2** by a bacterial *N*-acyl-aminoacylase [5]. Finally, a third group consists of volatile sulfur compounds (VSCs). We recently described 3-methyl-3-sulfanylhexan-1-ol **3** as the predominant VSC in axillary odor, possessing a typical sweat/onion-like character [6]. Independent research groups reported this observation at the same time [7][8].

Thiol and thiomethyl metabolites, in which sulfur is derived from glutathione, have been identified as excretion products of a variety of xenobiotics [9-11]. Usually, glutathione-(*S*)-conjugates undergo sequential enzymatic hydrolysis to yield Cys-(*S*)-conjugates which can be excreted as such, for example felinine [12], or after acetylation of the mercapturate [13] or after thioether bond cleavage yielding the thiol and pyruvic acid [14][15]. Degradation of glutathione-(*S*)-conjugates in plants occurs in the vacuoles and the first step is cleavage of the glycine residue by a carboxypeptidase [16], then γ-transglutaminase release the Cys-(*S*)-conjugate. In some plants Cys-Gly-(*S*)-conjugate dipeptides may also exist and may be intermediates in the formation of Cys-(*S*)-conjugate [10]. The formation of VSCs from cysteine conjugates has been described in wine [17][18], passion fruit [19], Allium species [20] and others [21]. In *Vitis vinifera L. Sauvignon Blanc* wine [22] and in tropical fruits [19], (*S*)-3-(hexan-1-ol)-glutathione was identified as well as (*S*)-3-(hexan-1-ol)-cysteine which gave, after *β*-elimination promoted by bacterial *β*-lyase, a very potent odorant, 3-sulfanylhexan-1-ol **4** [18].

The generation of VSCs in sweat has been postulated as resulting from the action of a *β*-lyase on a Cys-(*S*)-conjugate without clear specification of the exact nature of the non-volatile precursor or the VSC(s) produced [23]. Cysteine conjugate **13** has been postulated to be the direct precursor of **3**, with the release of **3** resulting from the cleavage of C-(*S*) bond by a C-(*S*)-*β*-lyase present in *Corynebacterium* [8].

In the current study we examine the mechanism of VSC release in human sweat by identifying the immediate precursor of key compound **3** in sterile sweat. We also assess the underarm microflora *Staphylococcus haemolyticus*, *Corynebacterium xerosis* and *Staphylococcus epidermidis* [6] for the ability to form 3-methyl-3-sulfanylhexan-1-ol **3** when incubated with synthetic precursors.

2. Results

2.1 Sweat collection and fermentation

Sterile axillary sweat [6] was separated into five fractions by HPLC with a *Lobar® RP-18 SiO_2* column. An aliquot of each odorless fraction was incubated with *Staphylococcus haemolyticus* and only the third fraction produced the typical smell of VSCs. This *Fraction* 3 was extracted with AcOEt and analysed by GC-MS: (*R*/*S*)-3-methyl-3-sulfanylhexan-1-ol (**3**) was detected. This was a clear indicator for the presence of VSC precursors in Fr. 3 (*Fig. 1*,b). *N*-*α*-3-hydroxy-3-methylhexanoyl-(*L*)-glutamine (M + 1 = 275), **2**, and minor peak *N*-*α*-3-methyl-2-hexenoyl-(*L*)-glutamine (M + 1 = 257), **1**, known to be present in sweat [5], were detected in total sterile sweat (*Fig. 1*,a) and were recovered in Fr. 2.

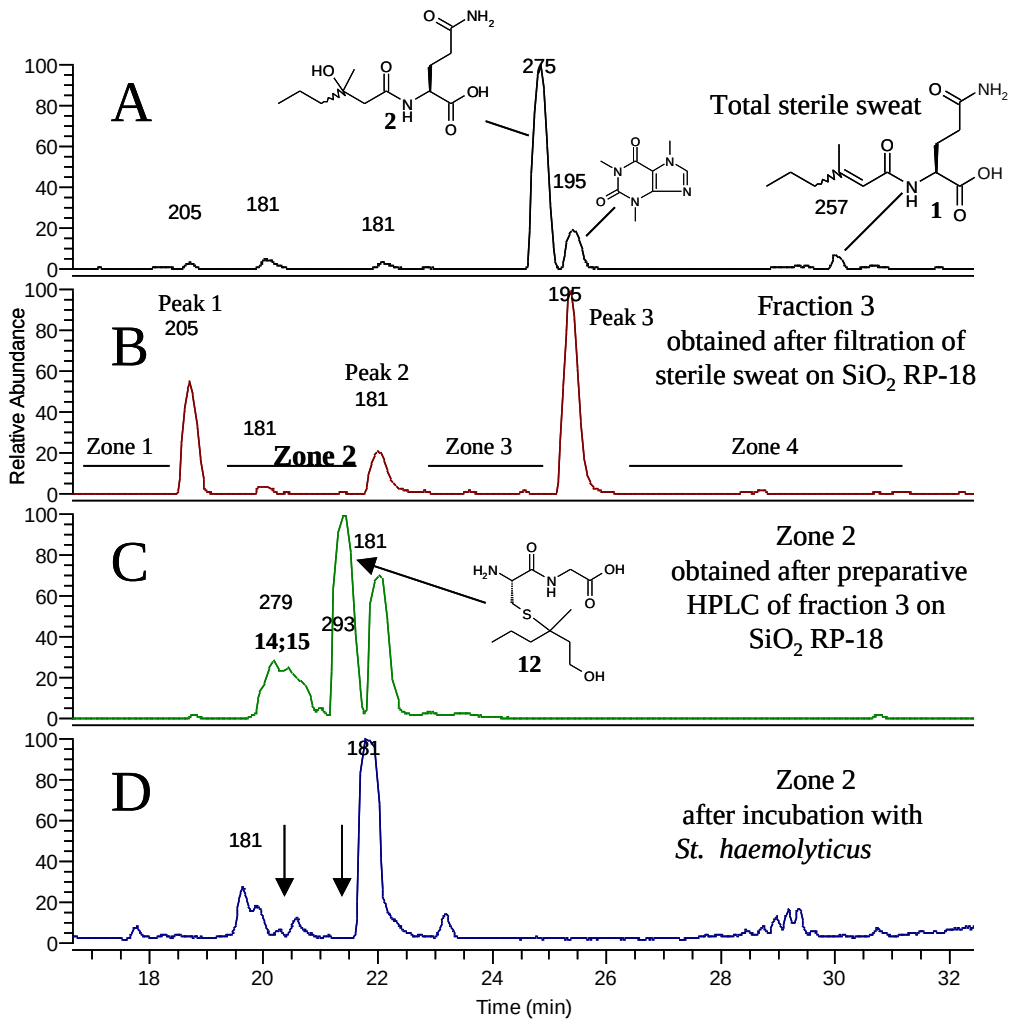


Fig. 1. HPLC-APCI-MS. A: trace of total sweat; B: trace of fraction 3; C: "zone 2" which contained all the precursors and D: "zone 2" after fermentation, arrows show missing peaks. Small numbers are M + 1 values.

Fr. 3 was further separated into sub-fractions using a preparative HPLC column. Portions of the collected zones and peaks were treated with *S. haemolyticus* and the only corresponding fraction of that generate a typical sweat malodor was from "zone 2" (*Fig. 1*,c). It was clear from the HPLC-MS trace of incubated fraction zone 2 that the peaks possessing M + 1 ions corresponding to 279 and 293 had been transformed by *S. haemolyticus*. After extraction and GC-MS analysis of the bacterially treated "zone 2" fraction, we observed on GC-MS a small broad peak with a molecular ion of *m/z* 134, at a retention index corresponding to I_{SPB1} 1080 which we attributed to *syn* (and/or) *anti* 2-methyl-3-sulfanylpentan-1-ol **5** (*Fig. 2*,a). A larger peak with molecular ion of *m/z* 134 and a

retention index I_{SPB-1} 1093 corresponded to 3-sulfanylhexan-1-ol (**4)** [7][8], while the major product formed had a molecular ion of *m/z* 148 at I_{SPB-1} 1149 that was attributed to (*R/S*)-3-methyl-3-sulfanylhexan-1-ol (**3**; *Fig. 2*,a). It was also possible to establish, after injection on a chiral column mounted on a GC equipped with an AED (Atomic Emission Detector), that the major enantiomer formed was the (*S*)-3-methyl-3-sulfanylhexan-1-ol (*S*)-**3**, with an enantiomeric excess of 65%. In the case of (*R/S*)-3-sulfanylhexan-1-ol (**4)** it was not possible to measure precisely the enantiomeric ratio due to poor separation of the enantiomers but the (*S*)-**4** enantiomer was clearly the major isomer formed (*Fig. 2*,b).

Further examination of the data (*Fig. 1*,c) allowed identification of the major compound detected by HPLC-MS in positive mode. The major precursor had a molecular ion, $[M + 1]^+$, of *m/z* 293 and further fragmentation resulted in the formation of an ion having an MS^2 of *m/z* 276, what then produced a fragment by MS^3 at *m/z* 179, interpreted as the loss of 3-methylhexanol. In negative APCI-MS mode, the molecular ion, $[M - 1]^-$ at *m/z* 291, of the major precursor fragmented to form a second generation ion at *m/z* 143, corresponding to *β*-elimination of 3-methyl-3-sulfanylhexan-1-ol, and was consequently identified as being Cys-Gly-(*S*)-conjugate (**12)**. The identity was confirmed by chemical synthesis. Because the incubation of zone 2 produced VSCs **4** and **5** (*Fig. 2*, traces A and B), we postulated that the possible precursors of these compounds may be the peaks with M + 1 = 279 (*Fig. 1*, trace C), that were not detected after incubation. A diastereoisomeric mixture of Cys-Gly-(*S*)-conjugates **14** and **15** originating from (*E*)-2-hexenal and (*E*)-2-methyl-2-pentenal, respectively, were prepared. Their retention times fit exactly the cluster of peaks having $[M + 1]^+$ at *m/z* 279 on *Fig. 1*,c and, therefore **14** and **15** are also present in sweat. The preparations of authentic samples of VSCs **3**, **4** and **5** were performed as described previously [6][24].

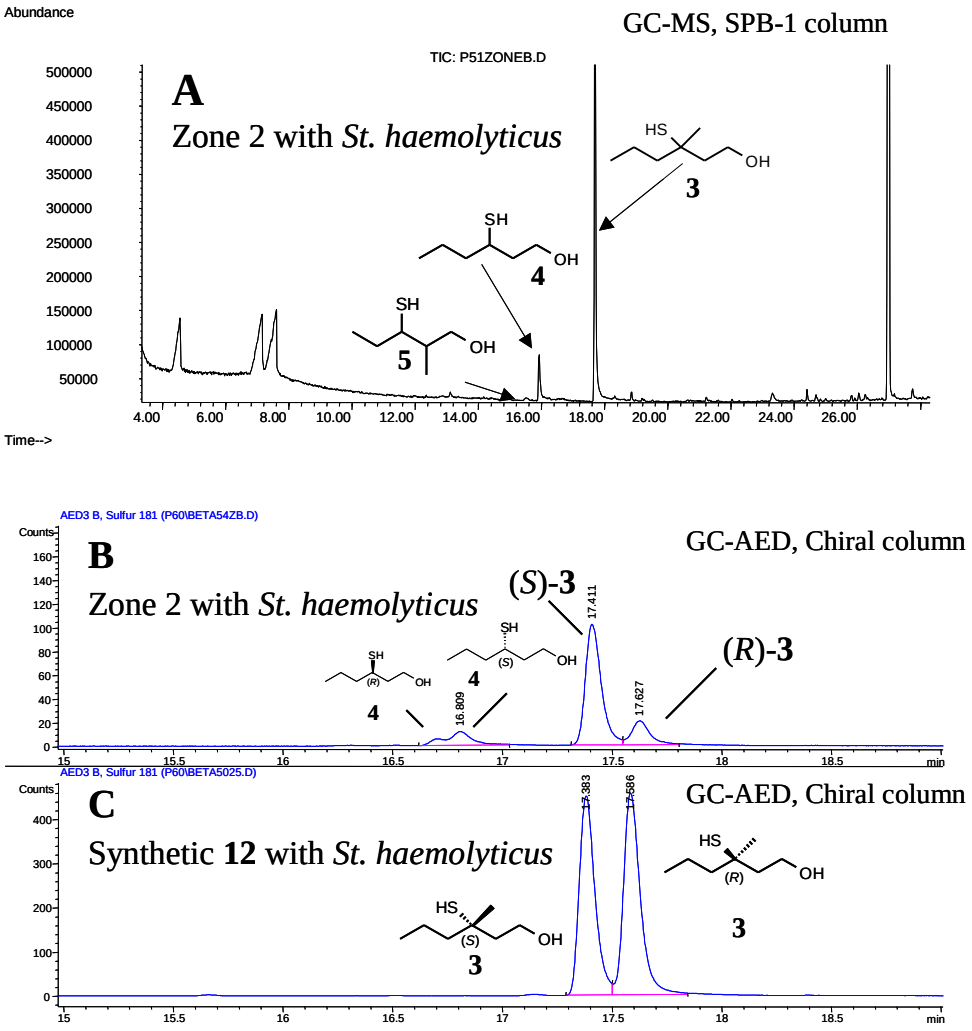


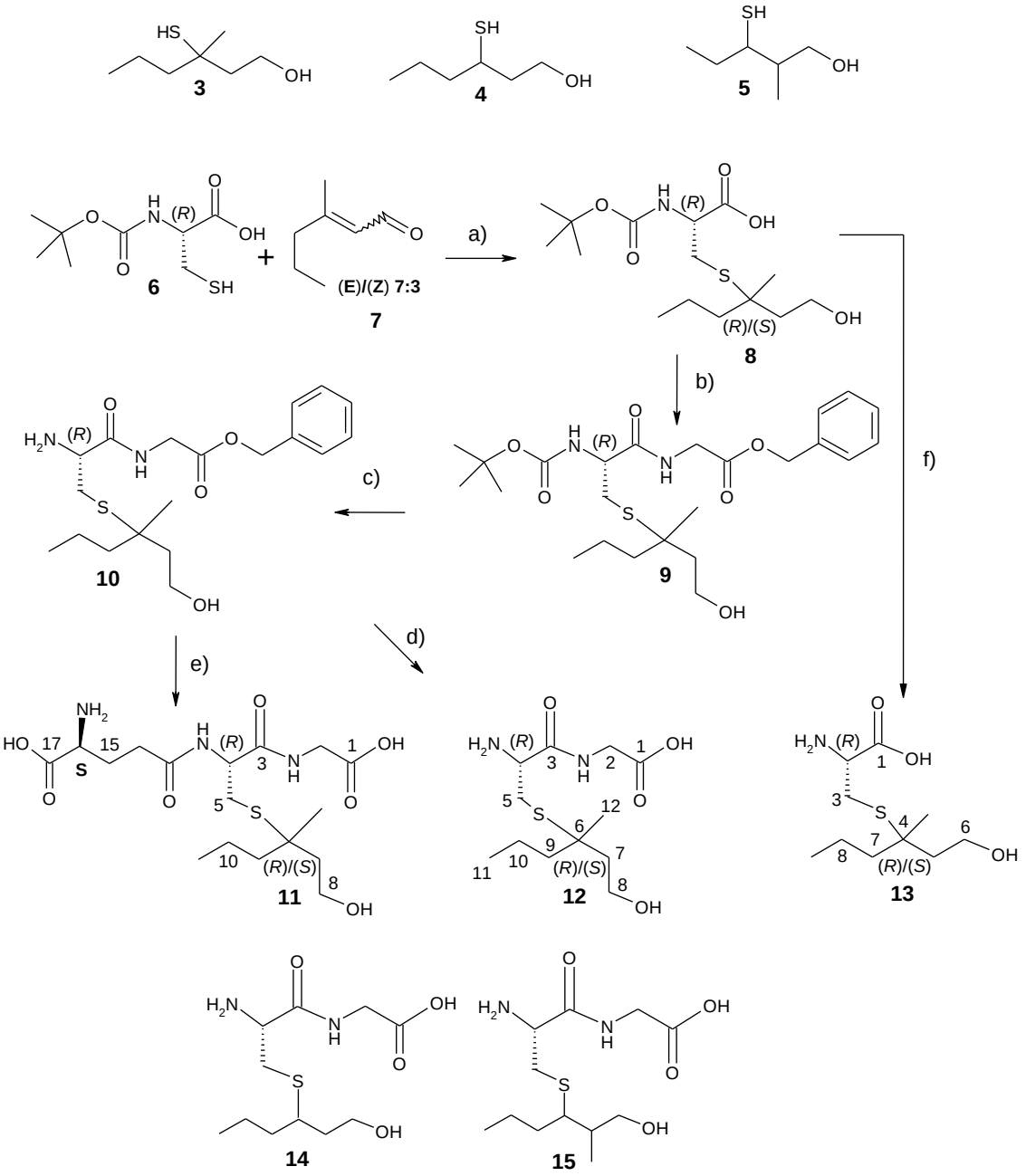
Fig. 2. Trace A: GC-MS column$_{SPB-1}$ of the bioconversion of "sweat fraction zone 2" with *S. haemolyticus*. Trace B: same as *A* but on a GC-AED chiral column$_{betadexcdx}$. Trace C: bioconversion of synthetic **12** with *S. haemolyticus*.

2.2. Synthesis of Cysteine Conjugates

The postulated sulfur precursor(s) of 3-methyl-3-sulfanylhexan-1-ol (**3)**, probably results *via* the glutathione detoxification pathway. Generally this pathway begins with the addition of glutathione to aldehydes by xenobiotics, in this case the corresponding molecule was **11**. Subsequently the glutamyl portion is cleaved, in our case leading to **12**, and finally the glycine part is removed, which corresponds to the formation of **13** (*Scheme 1*). In order to synthesize the putative precursors the 1,4-addition of cysteine to (*E*)-3-methyl-2-hexenal (**7)** was

performed by the addition of (*ter*-butoxy)carbonyl (Boc)-(*L*)-cysteine to **7** followed by reduction with $NaBH_4$ [6][8]. Cys-(*S*)-conjugate **13** was obtained after deprotection of **8** with TFA (trifluoroacetic acid) in CH_2Cl_2. To prepare **9** the coupling of H-Gly-OBz to **8** was performed in CH_2Cl_2 in the presence of PyBOP and DIPEA (diisopropylamine), under standard conditions for peptide synthesis [25].

Scheme 1. Preparation of (*S*)-cysteine conjugates. (a) $CsCO_3$, CH_3CN, $NaBH_4$ (b) H-Gly-Obz, CH_2Cl_2, PyBop, DIPEA (c) TFA, CH_2Cl_2 (d) NaOH aq., MeOH (e) i) Boc-Glu-OBz, CH_2Cl_2, PyBop, DIPEA, ii) TFA, CH_2Cl_2, iii) NaOH aq., MeOH (f) TFA, CH_2Cl_2.



The α-amino-protecting group of **9** was removed to prepare **10** and subsequently **12** and the glutathione-(*S*)-conjugate **11** was prepared as shown in *Scheme 1*. Diastereoisomeric mixtures were obtained throughout, confirmed by ^{1}H and ^{13}C-NMR spectra of **9** and **11**. It was not possible to directly determine the diastereomeric ratios for **12** and **13**, since we were only able to separate the two diastereoisomers of **11** (1:1 ratio) by capillary electrophoresis. The retention time by HPLC-MS and the MS fragmentation pattern for **12** were similar to those of the major compound in sweat zone 2 (*Fig. 3*).

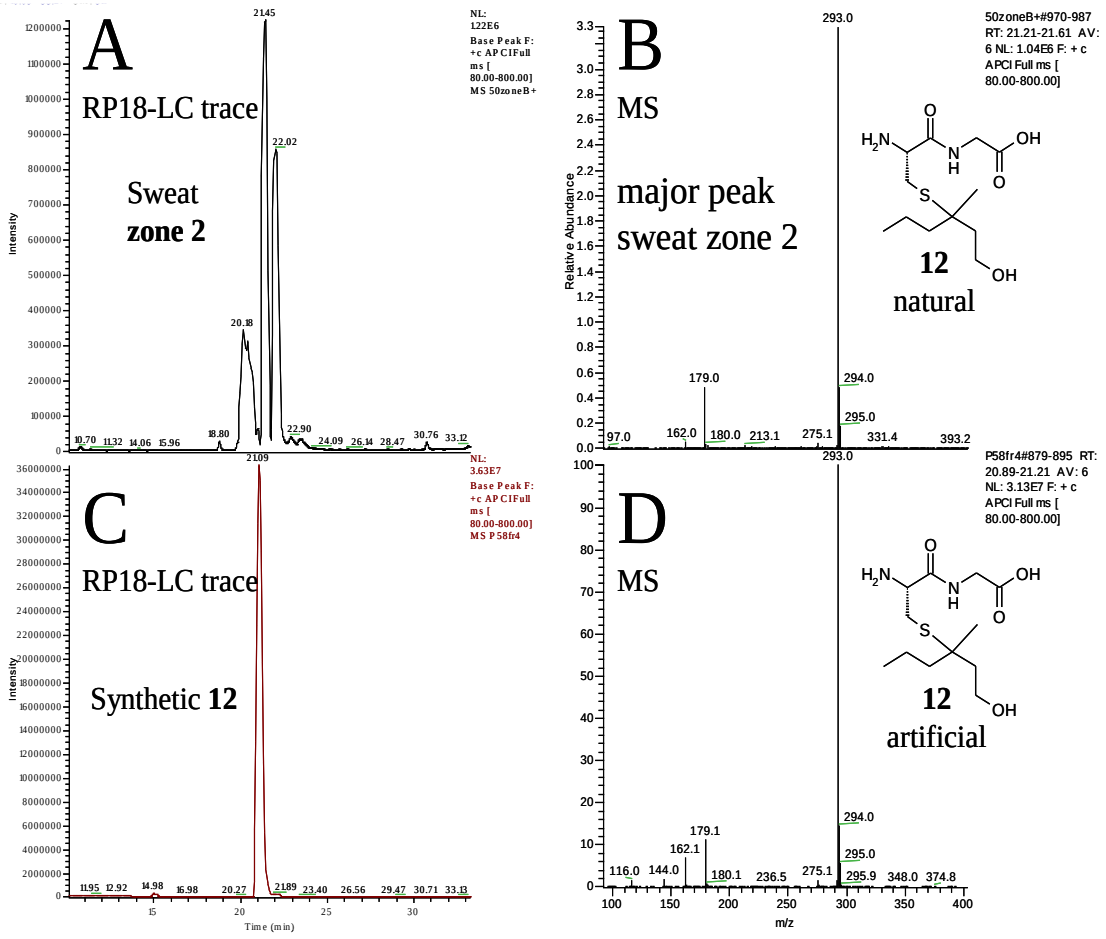


Fig. 3 HPLC-APCI-MS data, positive mode, A: LC-MS Total Ion Chromatogram of collected sweat zone 2 and B: mass spectrum of major peak of sweat zone 2. C: LC-MS Total Ion Chromatogram of synthetic **12** and D: mass spectrum of synthetic **12**.

*2.3. Production of (*R/S*)-3-methyl-3-sulfanylhexan-1-ol (***3***) from synthetic* ***12***, ***11***, ***13*** *upon incubation with* Staphylococcus haemolyticus, Corynebacterium xerosis *and* Staphylococcus epidermidis.

Precursor (*R-R/S*)-**12** was incubated with *S. haemolyticus*. After 18 h at 37°, the sweat odor associated with VSC was clearly perceived while no odor was present in controls corresponding to (*R-R/S*)-**12** incubated in buffer alone and *S. haemolyticus* incubated without the precursor. The fermented broth was then extracted with ethyl acetate containing an internal standard, and injected on a chiral CG column coupled to an atomic emission detector (AED; *Fig. 2*,c). A racemic mixture of (*R/S*)-3-methyl-3-sulfanylhexan-1-ol (*R/S*)-**3** was obtained with a chemical yield in excess of 75%, based on the amount of precursor incubated.

Cys-(*S*)-conjugates (*R-R/S*)-**13** were incubated under the same conditions. They also proved to be substrates for *β*-lyases produced by *S. haemolyticus* and *C. xerosis* but not *S. epidermidis*. The yield of the bio-conversion of **13** into thiol **3** was always much lower than that of **12** (*Table 1*).

Table 1. Incubation of precursors **11**, **12**, and **13** in the presence of microorganisms identified in sweat. Yields (% molar of the thiol **3** calculated from the precursor) were determined from the GC-AED peak surface with octanethiol as an internal standard. Variations were less than 2% over three repetitions with the same batches of bacteria collected at the end of exponential growth. N.d. = not detected.

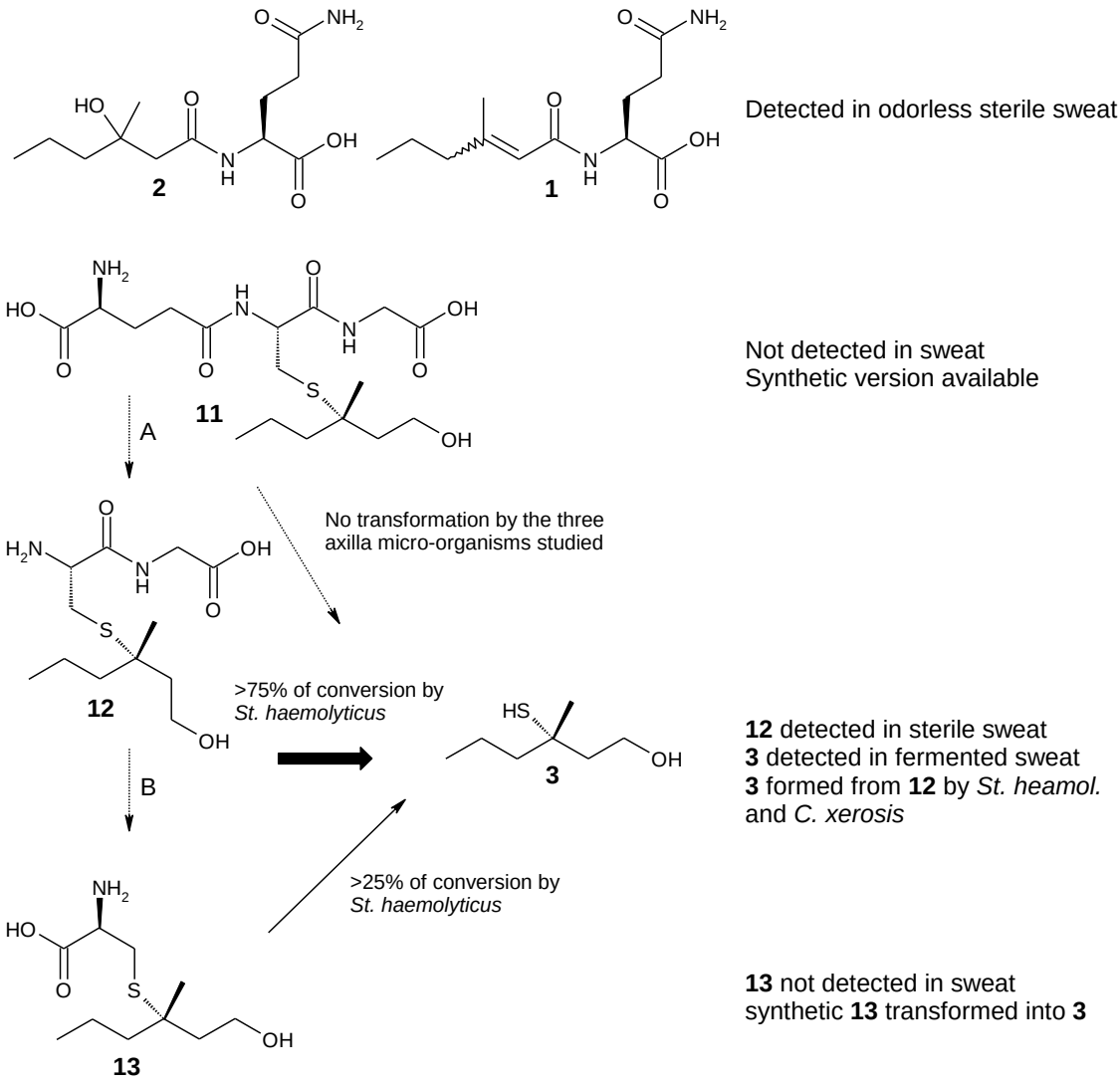
	Precursor **11**		Precursor **12**		Precursor **13**		Sweat zone 2	
	Yield [%]	*(R)*/*(S)* Ratio	Yield [%]	*(R)*/*(S)* Ratio	**1** Yield [%]	*(R)*/*(S)* Ratio	**1** Axillary Sweat [µg/l]	*(R)*/*(S)* Ratio
S. haemolyticus $1.7\ 10^9$ *(cfu/ml)*	n.d.	-	75	1:1	25	1:1	18	1:4
C. xerosis $1.1\ 10^9$ *(cfu/ml)*	n.d.	-	20	1:1	8.5	1:1	-	-
S. epidermidis $1.1\ 10^8$ *(cfu/ml)*	n.d.	-	17	7:3	n.d.	-	-	-

2. Discussion

Microbial aminoacyclases are known to metabolise **1** and **2** to generate (*E/Z*)-3-methyl-2-hexenoic acid, which has a strong sweat malodor, similar to other short chain organic acids. On the other hand glutathione adds to α, β-unsaturated carbonyls, for example aldehydes, via a glutathione-(*S*)-transferase [10][11], which is an ubiquitous natural detoxification pathway. Because of the structural similarity of **1**, **2** and **12** they probably share a common mechanism of formation in the axilla, but this remains obscure. Glutathione-(*S*)-conjugates have been reported from Sauvignon white wine and were described as the primary precursors of VSCs like **4** [22]. In this case, the first step of the VSC release is cleavage of the γ-glutamyl moiety by a γ-glutamyl *trans*-peptidase followed by the action of a carboxypeptidase to generate Cys-(*S*)-conjugates, which are found in wine and have been described as the substrates for β-lyase [18]. In sterile sweat glutathione-(*S*)-conjugate **11** was not detected, therefore the action of the *trans*-glutaminase to produce Cys-Gly-(*S*)-conjugate **12**, found in sterile sweat, takes place before excretion of the sweat on to the surface of the skin (*Scheme 2*,A).

Cys-(*S*)-conjugates such as **13** were not detected in Fr. 3 (*Fig. 1*). Synthetic **13** was less efficient than **12** for the formation of the free thiol **3** by *S. haemolyticus* (*Scheme 2*, B). The transformation of compounds having $[M + 1]^+$ at *m/z* 279 (*Fig. 1*,c) as well as the formation of VSCs **4** and **5** could well indicate that as in the case of **3**, Cys-Gly-(*S*)-conjugates are their precursors.

Scheme 2 Summary of the chemical pathway to **1** from sterile sweat precursors.



The incubation of sweat zone 2 with *S. haemolyticus* produced **3** with an enantiomeric excess of 65% in favor of (*S*)-**3** and a racemic mixture of (*R/S*)-**3** from synthetic (*R,R/S*)-**12** (*Fig. 2*), the naturally occurring diastereisomer should be predominantly (*R,S*)-**12**.

It has been clearly stated [8] that *Staphylococci* and specifically *S. epidermidis* do not have *β*-lyase activity and that only *Corynebacterium spp*. is able to release thiols from Cys-(*S*)-conjugates. In our hands *C. xerosis* produced a weaker sulfury type odor compared to *S. haemolyticus* and this was proven by quantification of **3** after incubation of synthetic **12** with both types of microorganisms (*Table 1*, see ***Appendix 7.2***).). *S. epidermidis* did not produce

sulfur compound **3** from synthetic **13** as already reported [8], but was able to transform **12** into **3** in yields comparable to *C. xerosis*.

Understanding the complexity of the bio-generation of sulfur compounds remains a priority for our laboratory, and more data that elucidates the molecular biology and enzymology of these bioconversions will be published subsequently.

3. Experimental Part

General. Unless otherwise stated, all bacteriological media were purchased from Difco (USA). *Corynebacterium xerosis* DSMZ 207 43 (corresponding to the ATCC 373) was provided by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and was chosen as a routine reference strain, other bacterial strains were handled and cultured as previously described in the literature [3]. Commercially available chemicals (reagents and solvents) of adequate quality were used without further purification. Reactions were carried out under Argon. Organic extracts were washed to neutrality with 1 M HCl soln. and/or saturated aqueous $NaHCO_3$ solutions, saturated aqueous NaCl solutions, dried with $MgSO_4$ and evaporated. *Analytical GC*: An *Agilent* 6890 instrument coupled to Atomic Emission Detection (AED from *Jass*, Germany); He as carrier gas; fused-silica capillary columns *OV1701*, *DMePeBetacdx*®, 10 m x 0.25 mm i.d. with 0.25 µm film (from Mega, *Brechbühler* AG, Switzerland). *GC-MS*: *Agilent* 6890-GC system coupled to *HP-MSD-5973* quadrupole mass spectrometer; electron energy *ca.* 70 eV; fragment ions *m/z* (rel. int. in% of the base peak, *SPB-1*, 30 m x 0.25 mm i.d. with 0.25 µm film, all from *Supelco*). The retention indices (*I*) were determined relative to the retention time (t_R) of a series of *n*-alkanes with linear interpolation by means of a standard GC temperature program (50° for 5 min, then 5°/min to 240° and 20 min at 240°) with a precision of 0.5%. ^{1}H-, ^{13}C NMR, Spectra *Brucker*-AMX-360 spectrometer; in $CDCl_3$; δ values in ppm downfield from Me_4Si (= 0 ppm), *J* in Hz; assignments by COSY45 and HMQC experiments. The HPLC analytical column was a Nucleodur C18 Pyramid® 250 mm x 2 mm i.d., the preparative column was a Nucleodur C18 Pyramid® 250 mm x 10 mm (Machery-Nagel, Switzerland). HPLC-MS analyses were performed using an *Agilent* 1100 LC-MS system equipped with a B1312A binary pump and a G1314A UV detector. Mass Spectrometer

experiments were performed on a *Finnigan* LCQ, classical ion-trap spectrometer, with an atmospheric-pressure chemical ionization (APCI) source, using a spray voltage of 4.5 kV, capillary temperature at 250°, N_2 gas at a flow rate of 70 (Finnigan arbitrary units). Positive and negative ion mode mass spectra (scan range 50–800 Da) were recorded simultaneously. MS^2 and MS^3 were recorded when specified. The HPLC solvents for elution were CH_3CN, and water that contained 0.02% formic acid. The LC gradient started at 100% water for 5 min then increased from 0% CH_3CN to 15% CH_3CN in 20 min then from 15% CH_3CN to 50% CH_3CN in 10 min at a flow rate of 0.3 ml/min and in the preparative mode a flow rate of 6 ml/min was used.

Human sweat collection. Apocrine and ecrine secretions (287 ml, 0.25 mg/ml protein) were collected from axillae through excessive sweating from 42 volonteer, Caucasian, male subjects. These secretions were immediately sterilised through a double filter consisting of 1-µm membrane followed by 0.2-µm sterile filter, frozen as previously reported [6] and then lyophilyzed (2.2 g). After re-dilution in water (5 ml), this concentrate was filtered on *LiChroprep RP-18 Lobar*® (Merck art. 10625) 310 mm, i.d. 25 mm, at an approximate flow rate of 4 ml/min. The elution was performed with water (6 portions of 200 ml each, pooled to yield Fr. 1, freeze-dried to give 2.0 g dry weight), water/ethanol 9:1 (Fr. 2, freeze-dried to yield 0.3 g dry weight), water/ethanol 4:1 (Fr. 3, dried to give 0.16 g dry weight), water/ethanol 7:3 (Fr. 4, gave 4 mg dry weight), water/ethanol 1:1 (Fr. 5, 30 mg dry weight) and ethanol (Fr. 6, 25 mg dry weight). Each fraction was analysed by LC-MS, then one tenth of each fraction was treated with *S. haemolyticus* (5 ml, 1.10^8 cfu/ml).

General method of the fermentation process. Bacteria isolates were grown aerobically in the appropriate liquid media at 37° until OD_{600} = 1.0. Cells were harvested by centrifugation at 3000 g, 10 min, washed once with sterile 0.1 M phosphate buffer (pH 6.0) and re-suspended in fresh buffer. The concentration of individual strains incubated with the sweat precursor was 1.10^8 cfu/ml. These freshly prepared bacteria (5 ml) were incubated with 0.1 ml of precursors (5 mg in 10 ml water), or the sweat fraction, at 37°, pH 6 in a closed bottle. After incubation times of 1, 4 and 18 h 1 ml of ethyl acetate containing octanethiol and octadecane at 0.02 mg/ml was added to a vial containing the sample and the fermentation broth was centrifuged at 16 000 g. The organic phase was directly injected on a GC $_{SPB1}$ column coupled

to MS and on a GC $_{DMePeBetacdx}$ chiral column coupled to AED detection. These experiments were repeated three times, two blanks were done with the microorganism without the precursor and one with the precursor in the growing buffer only. No formation of sulfur compounds was detected in the blanks. The response factor for 1 µl injected of a solution containing 0.01 mg/ml of 1-octanethiol was calculated in comparison with 1 µl injected of a racemic solution of 0.01 mg/ml of 3-methyl-3-sulfanylhexan-1-ol. The addition of both peak areas of analyte of interest (*R*/*S*) gave 280 (± 5) and 1-octanethiol 303 (± 3) peak surface over four injections, so we used, as an approximation, a response factor of 1. The correlation factor for the linearity of the detector response to1 µl injected on both columns, was 0.998 in the range of 1 to 10 ppm of (*R/S*)-3-methyl-3-sulfanylhexan-1-ol, **1**. With 1-octanethiol used as the internal standard, from different preparations of microorganisms, the variation in the chemical yield of **3** was ± 2%. As we cannot exclude adsorption of **3** on cellular materials our yields are considered as minimum yields.

Identification of S-*[1-(2-hydroxyethyl)-1-methylbutyl]*-(L)-*cysteinylglycine* **12**. Fr.3 was lyophilised (16 mg) and was further purified by preparative HPLC. We collected zones between peaks and peaks separately. The fractions and zones were injected on LCMS, extracted as described below and injected on GC-MS. Zone 2 (< 0.5 mg) had three APCI+ signals: a broad signal from 19.5 to 21 min having $[M + 1]^+$ at 279, at 21.5 min with $[M + 1]^+$ at 293, followed at 22 min with a peak of $[M + 1]^+$ at 181. APCI–traces were also recorded and MS 3 were also automatically recorded. Zone 2 produced three sulfur compounds seen on GC-MS: **5** < 1%: I_{SPB-1} 1080, MS: 134 (40, M^+), 100 (80), 74 (98), 71 (70), 55 (65), 41 (100); **4** = 5%: I_{SPB-1} 1093, MS: 134 (5), 100 (50), 67 (40), 57 (60), 55 (100); **3** = 94 %: I_{SPB-1} 1149, MS: 148 (4), 114 (20), 97 (70), 71 (60), 55 (100). Injection on GC $_{DMePeBetacdx}$ chiral column: (*S*)-**3** (r.t. 17.6 min) 62.4%, (*R*)-**3** (r.t. 17.62 min) 16.2%, (*R/S*)-**3** = 0.005 mg (± 0.001), ee of *S* = 65%. Compound (*R*)-**4** (r.t. 16.79 min), (*S*)-**4** (r.t. 16.80 min), (*R/S*)-**4** = 14.7%, (*S*)-major, not quantified due to peak overlapping.

Preparation of S-*[1-(2-hydroxyethyl)-1-methylbutyl]*-(L*)-cysteinylglycine* **12**. (2*R*)-(*S*)-[1-(2-hydroxyethyl)-1-methylbutyl]-tBoc-Cys **8**, prepared according to [5], (679 mg, 2 mmol) was diluted in CH_2Cl_2 (6 ml) and H-Gly-OBzl (741 mg, 2.2 mmol), PyBOP (Benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluorophosphate) (1.14 g, 2.2 mmol) in the presence of DIEA (diisopropylethylamine) (774 mg, 6 mmol) were stirred over night at room temperature. The usual work-up was followed by the deprotection of the α-amino group of (2*R*)-(*S*)-[1-(2-hydroxyethyl)-1-methylbutyl]-Boc-Cys-Gly-OBzl **9** in CH_2Cl_2 (6 ml) with TFA (9 ml) for 1.3 h at r.t.. The crude oily product **10** (1 g) was diluted in water (70 ml), MeOH 50 ml and NaOH (2 g) and heated 1 h at 60°. The pH was then adjusted to 4 with aqueous HCl (1 M). Methanol was removed under vacuum, then the mixture was lyophilysed. The solid was re-diluted in water (1 ml) and flash chromatographed on SiO_2-RP18 (column of i.d. 5 cm, height of silica was 12 cm). The elution started with water and product **12** was eluted with water and ethanol in 4 to 1 ratio. We obtained **12** (340 mg, yield 58%). HPLC, r.t. 22.2 min. APCI+ : M + 1 = 293, MS^2 276, MS^3 179, MS^4 162, MS^5 144, MS^6 116, APCI– : M – 1 = 291, MS^2 143, MS^3 99, 58. ^{1}H-NMR in D_2O: 4.16–4.19 (*m*, 1 H, C(4)); 3.95 (*d*, *J* = 17.5, 1 H, C(2)); 3.74 (*t*, *J* = 7.0, 2 H, C(8)); 3.70 (*d*, *J* = 17.5, 1 H, C(2)); 3.09 (*dd*, *J* = 13.5, 5.5, 1 H, C(5)), 2.99 (*dd*, *J* = 13.5, 8.0, 1 H, C(5)); 1.83 (*t*, *J* = 6.5, 2 H, C(7)); 1.50–1.55 (*m*, 2 H, C (9)); 1.38 (*m*, 2 H, C (10)); 1.29 (*s*, 3 H, C(12)); 0.95 (*t*, *J* = 7.0, 3 H, C(11)). ^{13}C-NMR: 178.8 (*s*, C(1)); 171.0 (*s*, C(3)); 61.1 (*t*, C(8)); 55.8 (*d*, C(4)); 51.9 (*s*, C(6)); 46.2 (*t*, C(2)); 44.8 (*t*, C(9)); 43.7 (*t*, C(7)); 30.6 (*t*, C(5)); 28.3 (*q*, C(12)); 19.9 (*t*, C(10)); 16.5 (*q*, C(11)).

*Preparation of (*L*)-γ-glutamyl*-S-*[1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine* **(11)**. Compound **10** (1.1 g, 3 mmol), diluted in CH_2Cl_2 (9 ml), N-α-t-Boc-D-glutamic acid α-benzyl ester Boc-D-Glu-OBzl (1.1 g, 3.3 mmol), and Pybop (1.72 g, 3.3 mmol) in the presence of diisopropylethylamine (1.16 g, 9 mmol) were stirred overnight at r.t.. The usual work-up was followed by deprotection of the α-amino group in CH_2Cl_2 (5 ml) with trifluoroacetic acid (10 ml) for 2 h at r.t.. The crude oily product (1.3 g, 1.5 mmol) was treated in MeOH (50 ml) and water (50 ml) with sodium hydroxide (2 g) for 2 h at 60°. The solution was acidified with aqueous HCl (1 M) and the solvent was removed under vacuum. The solid was re-diluted in water (1 ml) and flash chromatographed on SiO_2-RP18 (column of i.d. 5 cm,

height of silica was 12 cm). The elution started with water and the product **11** was eluted with water and ethanol in a 7 to 3 ratio. We obtained **11** (130 mg, yield 10%). (Note: removal of OBn with H_2 on Pd/C failed and the bad yield is due to saponification of the benzyl esters). HPLC r.t. 27.7 min. APCI+: $[M + 1]^+$:422, MS^2 308, MS^3 178; APCI$^-$: $[M - 1]^-$:420, MS^2 272 and 254, MS^3 113. ^{1}H-NMR in D_2O: 4.58–4.52 (*m*, 1 H, C(4)); 3.95 (*d*, *J* = 17.5, 1 H, C(2)); 3.90 (*d*, *J* = 18.0, 1 H, C(2)); 3.80–3.77 (*m*, 1 H, C(16)); 3.72 (*t*, *J* = 7.0, 2 H, C(8)); 3.00 (*dd*, *J* = 13.5, 5.0, 1 H, C(5)), 2.85 (*dd*, *J* = 13.5, 8.0, 1 H, C(5)); 2.56–2.46 (*m*, 1 H, C(14)), 2.21–2.46 (*m*, 1 H, C(15)); 1.81 (*t broad*, *J* = 6.5, 2 H, C(7) two diastereoisomers, ratio 1:1), 1.53–1.46 (*m*, 2 H, C (9)); 1.40–1.31 (*m*, 2 H, C (10)); 1.26 (*s broad*, 3 H, C(12) two diastereoisomers, ratio 1:1); 0.89 (*t*, *J* = 7, 3 H, C(11)). ^{13}C-NMR: 177.6 (*s*, C(1)); 177.0 (*s*, C(17)); 175.3 (*s*, C(13)); 175.3 (*s*, C(3)); 62.9 (*t*, C(8)); 56.9 (*d*, C(16)); 56.4 (*d*, C(4)); 51.4 (*s*, C(6)); 45.1 (*t*, C(2)); 44.9 (*t*, C(9)); 43.9 (*t*, C(7)); 34.3 (*t*, C(14)); 31.6 (*t*, C(5)); 29.1 (*t*, C(15)); 28.4 (*q*, C(12)); 19.9 (*t*, C(10)); 16.6 (*q*, C(11)).

*Preparation of (*S*)-[1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteine (***13)**. Compound **8** (1.6 g, 6.8 mmol) was diluted in CH_2Cl_2 (13 ml) and stirred during 2 h in the presence of trifluoroacetic acid (30 ml). The crude product was concentrated under vacuum, re-diluted in water and purified on *LiChroprep RP-18 Lobar*® with a gradient of water and ethanol. We obtained **13** (1.38 g, yield 86%). HPLC r.t. 22.35 min. APCI$^+$: $[M + 1]^+$: 236, APCI$^-$: $[M - 1]^-$:234. ^{1}H-NMR in D_2O : 3.89–3.92 (*m*, 1 H, C(6)); 3.10 (*m*, 1 H, C(2)); 2.98 (*m*, 1 H, C(3)); 1.84 (*t*, *J* = 7.0, 2 H, C(5)); 1.50–1.54 (*m*, 2 H, C(7)); 1.35–1.42 (*m*, 2 H, C(8)); 1.30 (*s*, 3 H, C(10)); 0.92 (*t*, *J* = 7.0, 3 H, C(9)). ^{13}C-NMR: 175.4 (*s*, C(1)); 61.2 (*t*, C(6)); 57.1 (*d*, C(2)); 51.5 (*s*, C(4)); 44.9 (*t*, C(7)); 43.8 (*t*, C(5)); 30.7 (*t*, C(3)); 19.9 (*t*, C(8)); 28.4 (*q*, C(10)); 16.5 (*q*, C(9)).

Preparation of S*-[1-(2-hydroxyethyl)-1-butyl]-(*L*)-cysteinylglycine (***14)**. It was prepared in the same way as described for **12** from (*E*)-2-hexenal (3.2 g, 6.8 mmol). We obtained (*R-R/S*)-**14** (650 mg, yield 34%). HPLC, r.t. 21.7 and 21.9 min. APCI$^+$: $[M + 1]^+$: 279, MS^2 262, MS^3 162, APCI$^-$: $[M - 1]^-$: 277. ^{1}H-NMR in D_2O: 4.18–4.22 (*m*, 1 H, C(4)); 3.95 (*d*, *J* = 17.5, 1 H, C(2)); 3.73 (*t*, *J* = 7.0, 2 H, C(8)); 3.71 (*d*, *J* = 17.5, 1 H, C(2)); 3.04–3.12 (*m*, 2 H, C(5)); 2.88 (*m*, 1 H, C(5)); 1.87–1.74 (*m*, 2 H, C(7)); 1.52–1.60 (*m*, 2 H, C (9)); 1.38–1.42 (*m*, 2 H, C (10)); 0.95 (*t*, *J* = 7.0, 3 H, C(11)). ^{13}C-NMR: 178.7 (*s*, C(1));

171.0 (*s*, C(3)); 62.0 (*t*, C(8)); 55.8 (*d*, C(4)); 46.2 (*t*, C(2)); 45.7 (*d*, C(6)); 39.2 (*t*, C(9)); 39.0 (*t*, C(7)); 33.1 (*t*, C(5)); 22.2 (*t*, C(10)); 16.0 (*q*, C(11)).

Preparation of S-*(1-ethyl-3-hydroxy-2-methylpropyl)-(*L*)-cysteinylglycine* (**15).** It was prepared in the same way as described for **12** from (*E*)-2-methyl-2-pentenal (3.2 g, 6.8 mmol). We obtained a diastereoisomeric mixture of (*R*-*R*/*S*-*R*/*S*)-**15** (1.2 g, yield 58%). HPLC r.t. 21.4 and 21.7 min. mixture of 4 diastereoisomers (minor diastereoisomer)*, same MS: $APCI^+$: $[M + 1]^+$: 279; $APCI^-$: $[M - 1]^-$: 277. ^{1}H-NMR in D_2O: 4.20–4.14 (*m*, 1 H, C(4)); 3.95 (3.96)* (*d*, *J* = 17.5, 1 H, C(2)); 3.71 (3.70)* (*d*, *J* = 18.0, 1 H, C(2)); 3.62–3.58 (*m*, 2 H, C(8)); 3.00–3.20 (*m*, 2 H, C(5)); (2.80–277)* 2.70–2.75 (*m*, 1 H, C(6)); (2.05–2.01)*2.00–1.97 (*m*, 1 H, C(7)); 1.67–1.42 (*m*, 2 H, C(9)); 0.98 (mixture of *d* and *t*, 2 x 3 H, C(10 and 11)); (0.89)* (*d*, *J* = 7.0, 3 H, C(11)). ^{13}C-NMR: 178.8 (*s*, C(1)); 171.0 (*s*, C(3)); 67.5 and 67.2 (*t*, C(8)); 56.0 and 55.9 (*d*, C(4)); 54.4 and 54.2 (*d*, C(6)); 46.2 (*t*, C(2)); 41.6, 40.6 and 40.4 (*d*, C(7)); 35.0 and 34.9 (*t*, C(5)); 29.3 and 29.2 and 26.2 (*t*, C(9)); 15.6 and 15.5 (*q*, C(12)); 14.4, 14.2 and 14.1 (*q*, C(10)).

Preparation of volatile sulfur compounds (*R*)-**3**, (*S*)-**3**, (*R*/*S*)-**4**, **5**. The preparation of (*R*)- and (*S*)-3-methyl-3-sulfanylhexan-1-ol **3** was effected according to [6], compound **4** was available internally [24]. Mixture of *syn* and *anti* **5** was prepared according to [26], $I_{SPB\text{-}1}$ 1078 and $I_{SPB\text{-}1}$ 1080 for *syn* and *anti*, resp.

Bibliography

[1] D. B. Gower, A. I. Mallet, W. J. Watkins, L. M. Wallace, J-P. Calame, *J. Steroid Biochem. Molec. Biol.* **1997**, *63*, 81.

[2] C. Austin, J. Ellis, *J. Steroid Biochem. Molec. Biol.* **2003**, *87*, 105.

[3] X. N. Zeng, J. J. Leyden, J. Brand, A. I. Spielman, K. McGinley, G. Preti, *J. Chem. Ecol.* **1991**, *17*, 1469.

[4] X. N. Zeng, J. J. Leyden, J. Brand, A. I. Spielman, K. McGinley, G. Preti, *J. Chem. Ecol.* **1992**, *18*, 1039.

[5] A. Natsch, H. Gfeller, P. Gygax, J. Schmid, G. Acuna, *J. Biol. Chem.* **2003**, *278*, 5718.

[6] M. Troccaz, C. Starkenmann, Y. Niclass, M. van de Waal, A. J. Clark, *Chem. Biodiversity* **2004**, *1*, 1022.

[7] a) Y. Hasegawa, "The Characterization of Human Body Odour", Advances in Flavours and Fragrances, RSC/SCi Conferences 12–14 May 2004, Manchester. b) Y. Hasegawa, M. Yabuki, M. Matsukane, *Chem. Biodiversity* **2004**, *1*, 2042.

[8] A. Natsch, J. Schmid, F. Flachsmann, *Chem. Biodiversity* **2004**, *1*, 1058.

[9] W. B. Jakoby, *Adv. In Enzymology* **1978**, *46*, 383.

[10] G. L. Lamoureux, D. G. Rusness, Glutathione: Chemical biochemical and medicinal aspects, **1989**, *vol III B*, 154.

[11] D. A. Dickinson, H. Jay Forman, *Ann. N. Y. Acad. Sci.* **2002**, *973*, 488.

[12] W. Hendriks, A. Woolhouse, M. Tarttelin, P. Moughan, *Bioorg. Chem.* **1995**, *23*, 89.

[13] W. B. Jakoby, J. Stevens, M. W. Duffel, R. A. Weisiger, *Rev. Biochem. Toxicol.* **1984**, *6*, 97.

[14] J. Stevens, W. B. Jakoby, *Mol. Pharmacol.* **1983**, *23*, 761.

[15] M. Tateishi, S. Suzuki, H. Shimizu, *J. Biol. Chem.* **1978**, *253*, 8854.

[16] A. E. Wolf, K. J. Dietz, P. Schroder, *FEBS Letters*, **1996**, *384*, 31.

[17] P. Darriet, T. Tominaga, E. Demole, D. Dubourdieux, C. *R. Acad. Sci. Paris, Bio. Pathol. Végétale* **1993**, *316*, 1332.

[18] T. Tominaga, C. Peyrot des Gachons, D. Dubourdieu, J. Agric. Food Chem. **1998**, *46*, 5215.

[19] T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2000**, *48*, 2874.

[20] J. E. Lancaster, M. J. Boland, CRC Onion and allied crop vol. III, **1990**, *vol. III*, pp. 33–72.

[21] C. Vermeulen, L. Gijs, S. Collin, *Food Rev. Intern.* **2005**, *21*, 69.

[22] C. Peyrot des Gachons, T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2002**, *50*, 4076.

[23] S. B. Lyon, B. C. O'Neal, H. van de Lee, B. Rogers, **1993** US Patent 5,213,791 (Chem. Abstr. 115: 99001).

[24] W. Pickenhagen, H. Bronner-Schindler, *Helv. Chimica Acta* **1984**, 67, 947.

[25] J. Johnes, Oxford International Series of Monographs on Chemistry, Clarendon Press Place, **1994**, pp. 23; ISBN0-19-855839-2.

[26] S. Widder, C. Sabater Lüntzel, T. Dittner, W. Pickenhagen, *J. Agric. Food Chem.* **2000**, 48, 424.

4. Gender and body odor : it is all in the chemistry

Gender-specific differences between the concentrations of non-volatile (*R*)/(*S*)-3-methyl-3-sulfanylhexan-1-ol and (*R*)/(*S*)-3-hydroxy-3-methyl-hexanoic acid odor precursors in axillary secretions

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Abstract

The volatile fatty acid, (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid ((*R*)/(*S*)-HMHA), and the human specific volatile thiol, (*R*)/(*S*)-3-methyl-3-sulfanylhexan-1-ol ((*R*)/(*S*)-MSH), were recently identified as major components of human sweat malodor. Their two corresponding precursors were subsequently isolated from sterile and odorless axillary secretions. The purpose of this work was to analyze these two odor precursors in 49 male and female volunteers over a period of three years to elucidate to which extent they are implicated in the gender specific character of body odor. Surprisingly, the ratio between the acid precursor **1**, a glutamine conjugate, and the sulfur precursor **2**, a cysteinylglycine-*S*-conjugate, was three times higher in men than in women with no correlation with either the sweat volume or the protein concentration. Indeed, women have the potential to liberate significantly more (*R*)/(*S*)-MSH, which has a tropical fruit and onion-like odor than (*R*)/(*S*)-HMHA (possibly transformed into (*E*)/(*Z*)-3-methyl-2-hexenoic acid) which has a cheesy, rancid odor. Parallel to this work, sensory analysis on sweat incubated with isolated skin bacteria (*Staphylococcus epidermidis Ax3*, *Corynebacterium jeikeium* K411 or *Staphylococcus haemolyticus Ax4)*, confirmed that intrinsic composition of sweat is important for the development of body odors and may be modulated by gender differences in bacterial compositions. Sweat samples having the highest "sulfur" intensity were also found to be the most intense and the most unpleasant.

Keywords**:** odor precursor, axillary odor, cysteinylglycine-*S*-conjugate, thiol, gender

1. Introduction

The generation of malodor on various sites of the human body, e.g. foot, mouth or armpit, is mainly caused by microbial transformation of odorless natural secretions into volatile odorous molecules [1]. In humans, sebaceous, eccrine and apocrine sweat glands provide an important source of nutrients for bacterial growth. The strong and unpleasant odor emanating from the underarm region has been mainly correlated to the high number of bacteria, such as *Corynebacteria* and *Staphylococci* species, present in this humid and semi-occluded environment [2-7].

More recent studies have deepened our understanding of axillary chemistry. In addition to the two pheromone-like steroids, androstenone (5-*α*-androst-16-en-3-one) and androstenol (5-*α*-androst-16-en-3-*α*-ol), the major and specific constituents of human sweat malodor are the volatile fatty acid, (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid ((*R*)/(*S*)-HMHA) [8] and, the sulfanylalkanol, (*R*)/(*S*)-3-methyl-3-sulfanylhexan-1-ol ((*R*)/(*S*)-MSH) [9-12]. (*R*)/(*S*)-HMHA is released from a glutamine conjugate by the action of a zinc dependent aminoacylase from *Corynebacteria* [13], while (*R*)/(*S*)-MSH is derived from a cysteinylglycine-*S*-conjugate (possibly via a cysteinyl-*S*-conjugate) by the action of *Staphylococci* [14]. In addition to the *Staphylococcus epidermidis* strain, the most abundant microorganism present in axilla, *Corynebacterium jeikeium*, pocessing the aminoacylase [11] and *Staphylococcus haemolyticus*, possessing C-S lyase activities [14] were selected for sensory test in this study.

These last findings confirmed the importance of bacteria, more precisely bacterial enzymes, and sweat odor precursors for the generation of body odors. It is commonly known that gender, along with ethnicity, emotional, physiological and environmental factors may influence the quantity and the quality of sweat [15-20], and some studies have led to the conclusion that individuals have a distinct body-odor type, which is partially determined by their inherited major-histocompatibility-complex (MHC) alleles [21][22]. Unfortunately, our knowledge of the importance of skin bacteria or sweat composition to explain the individual and gender specificity of human body odor is limited, although differences in microflora composition, such as the greater ratio of *Corynebacteria* to *Staphylococci* in male compared to female axilla, have been reported [2][23]. In this study we have chosen to investigate whether there are gender-specific

differences between the type of malodor precursors present in axillary sweat secretions, and whether sweat samples origins correlate with detectable differences in odor. The first part of this work was therefore to collect enough male and female axillary secretions to quantify the precursors of (*R*)/(*S*)-HMHA and (*R*)/(*S*)-MSH, and the second part was to carry out sensory tests.

2. Materials and Methods

2.1 Collection of odorless axillary secretions

Human apocrine and ecrine secretions were collected from the axillae of 49 caucasian volunteers (24 men and 25 women) who used a sauna over three winter seasons from November 2004 to April 2007. A small plastic goblet was used to collect the droplets of sweat that develop from underarm whilst taking a sauna. Secretions were immediately sterilized through a double filter consisting of a 1µm membrane, followed by a 0.2-µm sterile filter, and frozen separately at –20°C as reported previously [10]. The summer season was avoided due to changes in diet and activities of the volunteers, and higher temperature and humidity variations [24]. Each volunteer provided between a minimum of one sample and a maximum of eight samples per season and was asked to refrain from using any deodorant or perfume both the day before and the day of sweat collection. Individual male or female sweat samples were classified as High Protein (HP) if odorless and the protein concentration was superior or equal to 0.15 mg/ml, and as Low Protein (LP) if odorless and the protein concentration was inferior to 0.15 mg/ml. 0.15 mg/ml corresponds to the average protein concentration of male and female sweat samples. One third of the samples were immediately rejected due to the presence of other descriptors (perfume or human sweat odors).

2.2 Biochemical analyses of collected samples

Protein concentration was measured in each collected samples by the micromethod of Bradford with the Coomassie protein assay reagent (Bio-Rad laboratories, Inc., Hercules, CA, USA) and with the Bovine albumin 5% (Becton Dickinson and Company, Sparks, MD, USA) as standard [25]. The pH of sweat secretions was measured with a Mettler Toledo pH meter (InLab 423 electrode). Glucose concentrations were determined with a glucosimeter (YSI 2700Select Biochemistry analyser with a dextrose/lactate membrane).

2.3 Quantification of odor precursors

Volatile sweat precursor concentrations in sterile and individual axillary secretion samples were determined by UltraPerformance Liquid Chromatography coupled to a Mass Spectrometer (UPLC-MS). Separations and quantifications of sweat samples were performed on an Acquity BEH-C18 column (2.1 mm i.d. x100 mm; 1.7 µm) (Waters Corporation, Milford, MA, USA). The elution solvents were water with 0.1% formic acid (solvent A) and acetonitrile (CH_3CN) containing 0.1% formic acid (solvent B). The gradient profile started at 8% of solvent B (0.5 min), increased to 30% (8.4 min) and to 90% of solvent B in 2.1 min. The flow rate was 0.3 ml/min and the retention times (t_R) expressed in minutes. The mass spectrometer was a Thermo Finnigan Triple Stage Quadrupole (TSQ) quantum ultra® triple quad spectrometer (Thermo Electron Corporation, Somerset, NJ, USA) with an electrospray ion source operated in positive mode (ESI^+). The spray voltage was fixed at 4.0 kV and the capillary temperature at 349 °C. The sheath gas was nitrogen at a flow rate of 60 (Finnigan arbitrary units). The auxiliary gas was nitrogen at a flow rate of 5 (Finnigan arbitrary units). Analyses were performed in SIM mode according to $[M + 1]^+$ measurements for N-*α*-3-hydroxy-3-methylhexanoyl-(*L*)-glutamine **1** ($C_{12}H_{22}N_2O_5$, MW: 274.32) and *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine **2** ($C_{12}H_{24}N_2O_4S$, MW: 292.40). Calibration curves were generated with pure and diluted synthetic compounds in solvent A [10][14]. (*R*/*S* 1:1)-MSH, (*R*/*S* 1:1)-HMHA were synthesized as reported previously [10][9].

2.4 Odor Detection Threshold of sweat volatiles

Air-dilution olfactometers were used to determine the Odor Detection Threshold (ODT) of major sweat volatiles and its corresponding dose-response curve, which corresponds to the plot of the perceived odor intensity (response) versus the gas phase concentration (dose) [26]. The body odor volatile was first diluted in propylene glycol and injected continuously into a chamber at 130°C, where vaporization occurs instantaneously at the end of the tube. Nitrogen as carrier gas is used to avoid oxidative processes in the concentrated odor flow, which is then diluted with humidified air to obtain the final gas phase concentration. The sniffing outlet delivers a continuous and constant odorized air flow. Sensory evaluations were performed by 30 non-trained Firmenich employees (15 men and 15 non-pregnant women between 17 and 60 years of age) [27]. Dose-response curves were determined for each volatile with 8 concentrations varying from its volatility value to 1.10^{-6} µg/l air. The volatility is defined as the gas phase concentration at 22 °C/730 mmHg (± 30) expressed by the concentration of the volatile in the air above the pure liquid or solid at equilibrium.

2.5 Bacterial transformation of axillary secretions

Three underarm bacterial isolates, *Corynebacterium jeikeium* K411 [5], *Staphylococcus haemolyticus* Ax4 [10], and *Staphylococcus epidermidis* Ax3 [10], were grown aerobically at 37 ° C in liquid Brain Heart Infusion (BHI from Becton Dickinson and Company, Sparks, MD, USA) containing 0.5% tween 80 until OD_{600} was equal to 1.0. *S. haemolyticus* and *C. jeikeium* were confirmed to be sulfur and acid malodor producer after incubation with sweat (conversion yield of **2** was between 50 to 80% for *S. haemolyticus* and *C. jeikeium*. Conversion yield of precursor **1** was between 0.1 to 0.5 % for *C. jeikeium*, data not shown). Cells were harvested by centrifugation for 10 min at 3000 x g, washed once with sterile 0.1 M phosphate buffer (pH 6.0), and suspended in fresh buffer to a final four-fold concentration. The cell suspension (50 µl) was mixed with 400 µl of male or female, High Protein sweat (HP) or Low Protein sweat (LP) odorless axillary secretions and incubated 17 hours at 37 °C. Axillary secretions comprise a mixture of individual collected odorless samples after carrying out biochemical analyses. A

closed glass bottle was used to reproduce a semi-occluded environment that minimized water evaporation and maximised the sweat malodor generation by bacterial transformation.

2.6 Sensory evaluation of incubated sweat samples

Twelve external and trained assessors (one man and eleven non-pregnant women between 25 and 55 years of age) evaluated incubated sweat samples in blind-test conditions. The sweat samples were prepared as previously described and consisted of a male sweat sample and a female sweat sample incubated with either *Staphylococcus epidermidis, Staphylococcus haemolyticus* or *Corynebacterium jeikeium*. The six samples consisted of several pots and each pot was not opened more than twice during the session. Samples were evaluated at 37 °C in a balanced order in a sequential monadic way. Two replicates were carried out with either HP or LP on two consecutive days. The assessors evaluated the samples according to several attributes: overall sweat odor intensity, "sulfur" odor intensity, "acid" odor intensity, and odor unpleasantness. Attributes were evaluated on an unstructured 0–10 linear scale (0 = not perceptible; 10 = very strong intensity or 0 = not at all unpleasant; 10 = very unpleasant). To evaluate the "sulfur" odor intensity and "acid" odor intensity, the assessors were first given references to smell, and then rated the attributes of samples on the basis of these references. The "sulfur" and "acid" odor references were glass pots containing 0.002% (*R/S* 1:3)-MSH in dipropylene glycol (DIPG) / mQ water (50/50) ("sulfur" odor reference) and 0.1% (*E/Z* 3:1)-3-methyl-2-hexenoic acid in DIPG / mQ water (1/10) ("acid" odor reference) [28]. The sensory tests were performed using FIZZ 2.30C system (Biosytèmes, Couternon, France). ANOVA were carried out using XLSTAT 2008 2.03 (Addinsoft, Paris, France) followed by Duncan's post-hoc analysis ($\alpha = 0.05$).

3. Results

3.1 Biochemical analysis of sterile axillary secretions

Results of sweat sampling are summarized in *Table 1*. Average analytical results on mixed samples and variations in individual samples per gender per year and per protein concentrations are presented. 191 Male and 113 female samples, which correspond to 2252 and 265 ml of sweat

respectively, were collected over a period of three winter seasons from 2004 to 2007 following a 15 minute sauna session. As previously noticed, the average sweat yield was five times greater for males at 11.8 ± 1.2 ml (mean ± SEM) vs females at 2.4 ± 0.5 ml whereas the average protein content was similar (0.22 ± 0.06 vs 0.16 ± 0.03 g/l for male and female respectively) (See ***Appendix 7.3***). The glucose content was 2 to 5 times higher for males than for females (3.6 ± 0.9 g/l and less than 1.0 ± 0.1 mg/l in male and female secretions, respectively), which is an important source of carbon for Gram positive bacterial growth. The average pH was lower in females (7.5 ± 0.3 vs 8.0 ± 0.3) which is consistent with results of previous reports [29][30].

Table 1. Chemical analysis of odorless human sweat samples collected over three winter seasons from November 2004 to April 2007. Average data on sterile odorless sweat secretions (HP or LP) per year and per gender are represented. 24 male and 25 female volunteers participated in this study. All the volunteers gave at least one sample. Precursors concentrations and precursor ratios were average by individual sweat donor and per protein concentrations before mean calculation**. LP** = Low protein sweat (< 0.15 mg/ml); **HP** = High protein sweat (≥ 0.15mg/ml sweat). **N** = number of samples. Acid precursor **1 =** *N*-*α* -3-hydroxy-3-methylhexanoyl-(*L*)-glutamine; sulfur precursor **2** = *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine. Standard Error of the Mean (**SEM**) = (SD/√Nr-1); **Nr** (number of repetitions) = 6; **SD** = Standard Deviation.

Gender	Year	Protein Level	N	Volume (ml)	Protein (mg/ml) (SD)	pH (SD)	Glucose (mg/l) (SD)	Acid precursor 1 (µg/sample)	Sulfur precursor 2 (µg/sample)	Acid precursor 1 (mg/l)	Sulfur precursor 2 (mg/l)	Ratio acid precursor 1 / sulfur precursor 2 (1)/(2)
Male	2004-05	LP	67	750.4	0.09 (0.01)	9 (0.14)	3.0 (0.5)	212.8	2.2	19	0.2	95
	2005-06	LP	66	844.8	0.146 (0.02)	8 (0.20)	2.0 (0.8)	1100.8	6.0	86	0.47	182.98
	2006-07	LP	17	265.2	0.08 (0.01)	7 (0.15)	1.5 (0.5)	1185.6	7.5	76	0.48	158.33
	2004-05	HP	2	17.0	0.53 (0.03)	8 (0.07)	6.0 (1.0)	136.0	1.5	16	0.18	91.43
	2005-06	HP	21	226.8	0.202 (0.01)	8 (0.20)	6.0 (0.5)	1144.8	9.1	106	0.84	126.19
	2006-07	HP	18	147.6	0.27 (0.02)	8 (0.17)	3.0 (0.7)	984.0	4.8	120	0.59	203.39
Average (male samples)				11.8	0,22	8	3.6	794.0	5.2	70.5	0.46	142.89
SEM				1.24	0.06	0.24	0.9	198.1	1.2	8.77	0.05	20.71
Female	2004-05	LP	15	48.0 (15)	0.08 (0.02)	9 (0.16)	<1	179.2	3.3	56	1.02	54.9
	2005-06	LP	22	63.8 (22)	0.06 (0.02)	7 (0.20)	<1	174.0	4.3	60	1.47	40.82
	2006-07	LP	20	74.0 (20)	0.13 (0.01)	7 (0.14)	<1	791.8	13.6	214	3.66	58.4
	2004-05	HP	10	13.0 (10)	0.19 (0.03)	8 (0.15)	<1	72.8	2.7	56	2.1	26.67
	2005-06	HP	25	22.5 (25)	0.24 (0.02)	7 (0.14)	1.0 (0.5)	139.5	5.8	155	6.43	24.11
	2006-07	HP	21	44.0 (21)	0.28 (0.03)	7 (0.12)	1.0 (0.3)	833.9	13.6	398	6.49	61.36
Average (female samples)				2.4	0,16	7.5	<1.0	365.2	7.2	156.5	3.53	44.38
SEM				0.49	0.03	0.31	0.1	156.1	2.3	27	0.49	7.3

3.2 Odor precursor analysis in sterile axillary secretions

The analytical method was optimized with the two major sweat precursors present in sweat samples. Thus, racemic precursors **1** and **2** were prepared and injected on UPLC in SIM mode (Single Ion Monitoring mode) (Fig. 1). The diastereoisomers were not separated by UPLC, and thus the peak area corresponds to the quantification of both diastereoisomers. The odor precursor quantification was very sensitive and precise using this method. The lowest detected concentration of **2** in individual sweat samples was 0.02 µg/ml after four repetitions with only a 2% variation (0.02 ± 0.0004 µg/ml), whereas the lowest concentration of **1** was 25 µg/ml with a 0.3% variation.

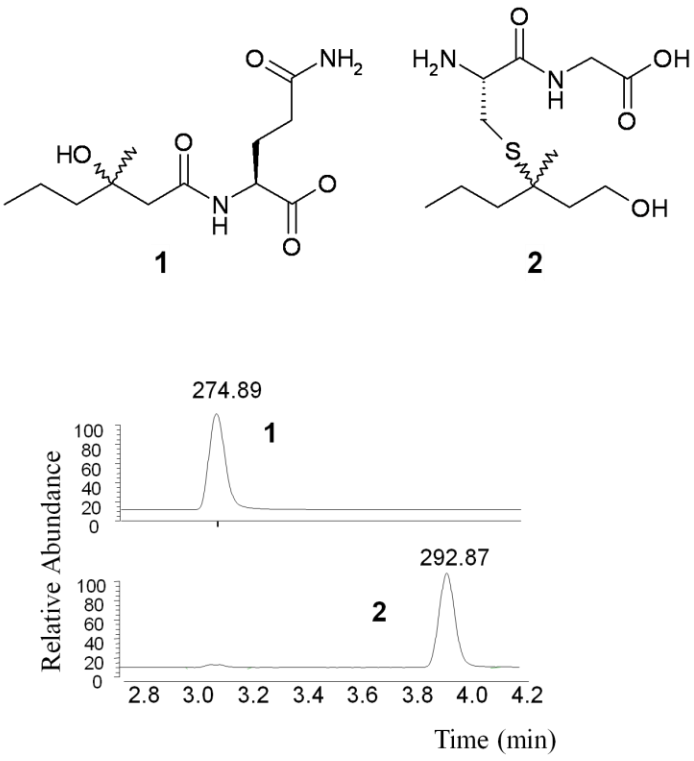

Fig. 1. Structure of major sweat precursors **1** (*N*-α-3-hydroxy-3-methylhexanoyl-(*L*)-glutamine) and **2** (*S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine) and the UPLC trace (UPLC coupled to a Thermo Finnigan TSQ quantum in Single Ion Monitoring (SIM) mode with electrospray ion source operated in positive mode (ESI^+)) of a standard solution containing 1 µg/ml of each precursor.

The mean quantity (± SEM) of **1** in male sweat samples was 1021 (± 121) µg with a maximum of 2790 µg while, in the female sweat sample, the average quantity was 606 (± 86) µg of **1** with a maximum of 2290 µg per sample. The mean quantities (± SEM) of **2** were 5 (± 1) and 9 (± 1) µg in males and females, respectively, with a maximum of 17 and 22 µg. Although huge variations were observed between individuals, for each individual, the ratio of **1** and **2** was consistent over the three years. To our surprise, this ratio was three times higher in

men (value of 143) than in women (value of 44), and independent of the sweat volume or the protein concentration (*Table 1*, *Fig. 2*).

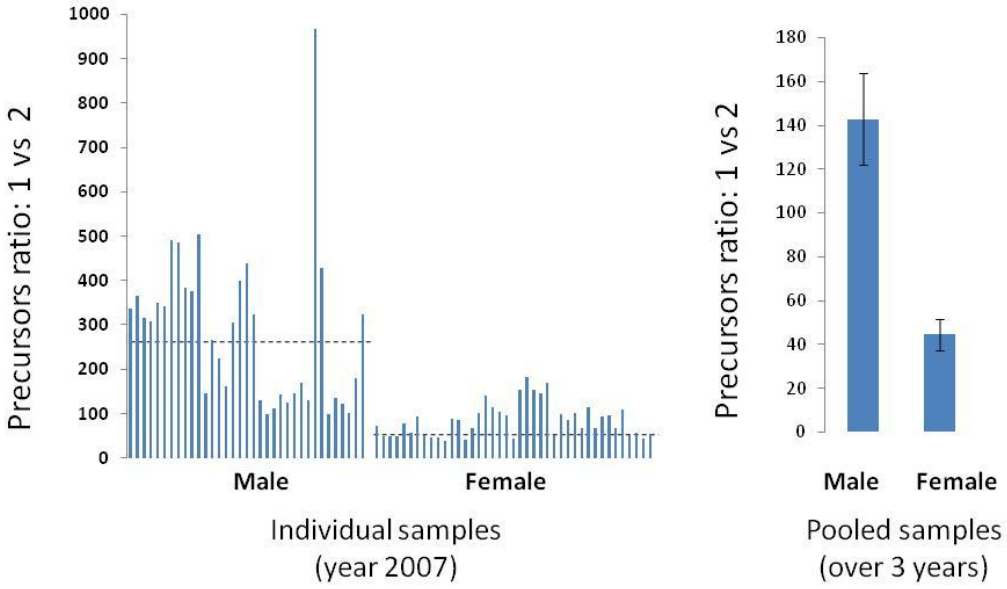

Fig. 2. Ratio between the acid precursor **1** (*N*-*α*-3-hydroxy-3-methylhexanoyl-(*L*)-glutamine) and the sulfur precursor **2** (*S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine) in sterile male and female sweat secretions (collected from the axillae of 24 men and 25 women).
Ratios were calculated on individual samples collected during the season 2007 (76 samples) and on 304 samples collected between November 2004 and April 2007 (mean ± SEM). Dotted lines represent the average results per gender in 2007. Precursors were quantified by direct injection on Ultraperformance Liquid Chromatography (UPLC) on a Water Acquity system coupled to a Thermo Finnigan TSQ quantum in Single Ion Monitoring (SIM) mode.

3.3 Odor Detection Threshold of Major Axillary Components

The Odor Detection Threshold (ODT), *i.e.* the lowest detectable odor level expressed in liquid or gaseous concentration units, was calculated for three sweat odor volatiles in both male and female subjects (*E*)/(*Z*)-3-methyl-2-hexenoic acid, its hydrated form (*R*)/(*S*)-HMHA, and (*R*)/(*S*)-MSH. The Odor Detection Threshold reflects the olfactive impact of the body odor volatiles. The average ODT and the corresponding dose-response curves calculated from the data generated from 15 males and 15 females are shown in Fig. 3 and *Table 2*. The most common descriptor for synthetic (*R*)/(*S*)-HMHA was animalic, cheesy and rancid whereas the sulfur molecule (*R*)/(*S*)-MSH was judged to be fruity and onion-like. (*R*)/(*S*)-MSH was perceived at a concentration 100x lower than (*R*)/(*S*)-HMHA, a fact that may explain its important organoleptic contribution in human malodor (Odor Detection Thresholds of 2.27 x 10^{-6} µg/l air and 1.80 x 10^{-4} µg/l air for the two molecules respectively). Similar results were found for both male and female assessors. The low odor detection threshold of sulfur containing molecules has already been reported in several studies and summarized in the review of Vermeulen [31].

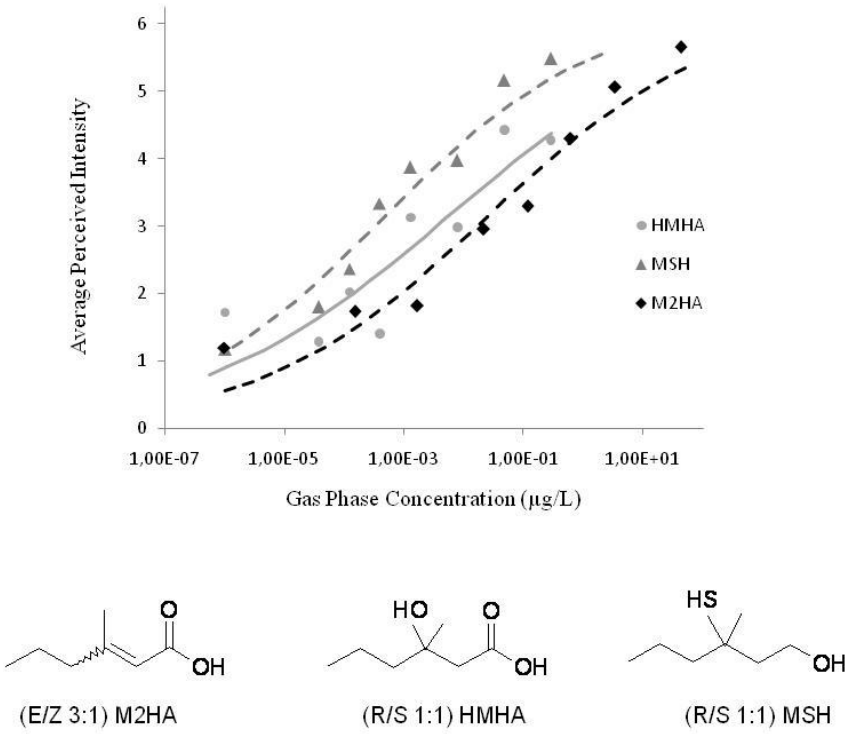

Fig. 3. Dose-response curve of the major sweat volatiles: (*R*)/(*S*)-HMHA, (*E*)/(*Z*)-3-methyl-2-hexenoic acid ((*E*)/(*Z*)-M2HA) and *(R)/(S)*-MSH. Olfactive evaluations were performed by 30 Firmenich employees (15 women and 15 men).

Table 2. Odor detection thresholds of the major sweat volatiles: (*R/S* 1:1)-3-hydroxy-3-methylhexanoic acid, (*E/Z 3:1*)-3-methyl-2-hexenoic acid and (*R/S* 1:1)-3-methyl-3-sulfanylhexan-1-ol. Olfactive evaluations were performed by 30 Firmenich employees (15 women and 15 men). Odor Detection Thresholds were determined for women and men subjects together and separately. Odor Detection Thresholds of (*R*) and (*S*) isomers and (*E*) and (*Z*)-3-methyl-2-hexenoic acids were not significantly different (data not shown).

Chemical name	Volatility (µg/l air)	Odor Detection Threshold (µg/l air)		Descriptors
		Men (N=15)	Women (N=15)	
(*E*)/(*Z*)-3-methyl-2-hexenoic acid	40.00	$2.59.\ 10^{-4}$	$1.44.\ 10^{-4}$	Perspiration, acidic, rancid, cheese
(R)/(S)-3-hydroxy-3-methylhexanoic acid	0.29	$1.49.\ 10^{-4}$	$2.00.\ 10^{-4}$	Perspiration, animal, cheese, rancid
(*R*)*/*(*S*)-3-methyl-3-sulfanylhexan-1-ol	132.00	$2.31.10^{-6}$	$1.53.10^{-6}$	Perspiration, sulfur, onion, grapefruit

3.4 Sensory analysis

Sensory tests were performed with incubated sweat samples: equal volumes of sterile sweat from males and females were separately fed with a fixed number of *S. haemolyticus*, *C. jeikeium* or *S. epidermidis* (gram-positive skin bacteria) and incubated at 37 °C overnight prior to analysis. *S*amples were rated according to their "sulfur", "acid", "sweat" odor intensity and their unpleasantness, on a linear 0–10 scale. The significant influence of bacterial species, gender, and sweat protein concentration is represented by the probability value associated to Fisher's F value (*Table 3*). Indeed, the protein concentration in sweat had no significant effect on the perceived sweat intensity, sulfur and acid character ($P > 0.1$). However, our results confirmed previous findings that *C. jeikeium* and *S. haemolyticus* generate the most sulfurous and intense odors in comparison to *S. epidermidis* [10]. This effect was stronger after incubation with female rather than with male axillary secretions. Regarding the "sulfur" and sweat odor intensity, the Fisher F

value of the analysis of variance indicates a much higher effect of gender compared to the bacterial type (F value of 8.6 vs. 3.1 for the "sulfur" odor and 11.0 vs. 4.3 for the sweat odor intensity). Female sweat samples generated significantly higher sweat and sulfur odor intensity (99% confidence level) (*Table 3, Table 4*) In addition, samples having the highest "sulfur" intensity, such as female sweat samples, were also found to be the most intense and the most unpleasant. Although sweat volatiles are somewhat unpleasant, the assessors (92% female) found the male odors to be the least unpleasant ($P < 0.05$).

Table 3. Analysis of variance (ANOVA) with Duncan's post-hoc analysis ($\alpha = 0.05$) on sensory data. Sensory analysis was performed with incubated pooled sweat samples collected from November 2006 to April 2007 from Male or Female (35 male samples and 41 female samples) with various microorganisms (*C. jeikeium, S. haemolyticus, S. epidermidis*). **DF**: degrees of freedom. **F**: Fisher's F. **Pr>F**: probability associated to the Fisher's F value. ***** Differences are significant when P<0.05 (*95% confidence level, **99% confidence level, ***99.9% confidence level). Olfactive evaluation was performed by 12 trained assessors (eleven women and one man). Sweat descriptors are:"sulfur","acid" and overall sweat odor intensity and overall unpleasantness. Variables are: Protein Level in sweat secretions (**HP** or **LP** sweat), Bacterial Type (*C. jeikeium, S. haemolyticus, S. epidermidis*) and Sweat Gender of the samples (**F**emale or **M**ale).

Descriptors	Variables	DF	F	Pr > F
Sweat odor intensity	Protein level	1	1.800	0.182
	Sweat gender	1	11.000	0.001***
	Bacterial type	2	4.300	0.006**
"Acid" odor intensity	Protein level	1	0.500	0.479
	Sweat gender	1	4.900	0.028*
	Bacterial type	2	0.600	0.647
"Sulfur" odor intensity	Protein level	1	0.100	0.740
	Sweat gender	1	8.600	0.004**
	Bacterial type	2	3.100	0.028*
Overall unpleasantness	Protein level	1	2.000	0.157
	Sweat gender	1	11.100	0.001***
	Bacterial type	2	0.800	0.504

Table 4. Sensory analysis performed with incubated sweat samples from Male or Female. Pooled sweat samples collected from November 2006 to April 2007 (*Table 1*) were used for the sensory test. Olfactive evaluation was performed in duplicate by 12 trained assessors. Values are average data obtained with HP and LP sweat samples and with various microorganisms (*C. jeikeium, S. haemolyticus, S. epidermidis*). CI = 95% Confidence Interval. *95% CI = Mean* ± (*1.96 * Standard Error)*, the value 1.96 comes from our understanding of the normal curve with assumption that the means are normally distributed. Attributes were evaluated on an unstructured 0–10 linear scale: 0 = not perceptible; 10 = very strong intensity or 0 = not at all unpleasant; 10 = very unpleasant. C.j = *C. jeikeium; S.h = S. haemolyticus; S.e = S. epidermidis.*

	Female sweat samples				Male sweat samples			
	C.j	*S.h*	*S.e*	Average	*C.j*	*S.h*	*S.e*	Average
Sweat odor (CI)	6.60 (0.74)	6.53 (0.68)	5.72 (0.69)	6.28 (0.71)	6.18 (0.77)	4.75 (0.76)	4.90 (0.66)	5.35 (0.75)
"Acid" odor (CI)	4.00 (1.02)	4.90 (0.95)	4.87 (0.94)	4.62 (0.97)	4.17 (0.90)	4.10 (0.92)	3.70 (1.06)	3.99 (0.95)
"Sulfur" odor (CI)	5.72 (0.69)	5.86 (0.94)	4.10 (0.92)	5.22 (0.99)	4.62 (1.00)	4.01 (0.97)	3.47 (1.08)	4.03 (1.01)
Unpleasantness (CI)	6.96 (1.00)	7.57 (2.11)	6.55 (0.89)	7.02 (0.91)	6.35 (0.86)	6.00 (0.88)	5.81 (0.88)	6.06 (0.78)

4. Discussion

Most of the studies to elucidate gender and individual differences in axillary sweat odor, its chemistry and odor intensity, have confirmed the key role of skin bacteria, more particularly bacterial enzymes, in the sweat transformation process [32][5][4]. Consequently, the vast majority of deodorants utilize antimicrobial agents which are also often enzyme inhibitors; the rationale is that, by decreasing the bacterial numbers present in the axilla, the biogenesis of odor can be generally decreased [33][34]. Individuals were classified as to have intense, acrid or "acid" body odor, with respect to the composition of their microflora [2]. In this study we have found that the same microflora may generate distinctively different body odors with respect to the composition of underarm secretions (see *Table 3*). After incubation with the axillary bacteria *S. haemolyticus*, female sweat has a much more intense and "sulfur" odor intensity than male sweat. Indeed, analysis of individual differences in the composition of sweat may help us find new strategies to design individualized products to compete with body odors.

The content of the major acid and sulfur odor precursors, **1** and **2** were analyzed by ultra high pressure liquid chromatography, coupled to a Thermo Finnigan TSQ quantum, in limited amount of freshly secreted filter-sterilized sweat samples from 49 male and female volunteers. In previous reports, the sulfur precursor **2** had been shown to be transformed into (*R*)/(*S*)-MSH by *S. haemolyticus* and *Corynebacteria* [14][35]. For the first time we have shown that this precursor was found in higher amounts in female underarm secretions compared to males (5.1 mg/ml of **2** in females vs 0.5 mg/ml of **2** in males). As expected huge variations were found between individuals, but to our surprise the ratio of **2** to **1** was constant in males and females over the three-year period of the study. This ratio was equal to 143 ± 21 (mean ± SEM) in male axillary samples and 44 ± 21 in female samples. These results indicate that females have the potential to liberate significantly more sulfur volatiles, such as (*R*)/(*S*)-MSH which has a tropical fruit and onion-like odor in underarm, relative to (*R*)/(*S*)-HMHA (possibly converted into (E)/(Z)-3-methyl-2-hexenoic acid) which possesses a cheesy, rancid odor. Similarly to the results of Khun and Natsch [36], our results indicate that the most probable hypothesis for these gender differences may be genetic or hormonal [35]. Savelev et al. have showed recently that both genes and environmental factors determine characteristic odor of an individual [20].

Sensory tests have shown a higher "sulfur" odor intensity of female compared to male sweat after incubation with individual skin bacteria. The "sulfur" odor induces negative emotions (unpleasantness) towards female sweat samples, suggesting the repulsive effect of (*R*)/(*S*)-MSH in sweat. More intense odors tend to be more unpleasant for women as previously reported [22]. Olfactive evaluation of synthesized body odor volatiles confirmed the low odor detection threshold of thiols and (*R*)/(*S*)-MSH was perceived at a concentration 100x lower than (*R*)/(*S*)-HMHA, with the same sensitivity for males and females (similar odor thresholds), which explains its important contribution in body odors.

In conclusion, chemical analysis of freshly secreted male and female sweat samples and statistical analysis on data generated by sensory tests, with individual bacteria, have clearly shown that male and female excrete thiol and acid precursors **1** and **2** in different concentration ratio and that-intrinsic composition of sweat is important for the development of body odors and may be modulated by gender differences in bacterial compositions.

Bibliography

[1] J.N. Labows, J.T. Reilly, J.J. Leyden, G. Preti, In: Antiperspirants and Deodorants. Laden K, editor. New York: Marcel Dekker. **1999**. p. 53–83.

[2] J.N. Labows, K.J. Mc Ginley, A.M. Kligman, *J. Soc. Cosmet. Chem.* **1982**, *34*, 193–202.

[3] A.G. James, D. Hyliands, H. Johnston, *J. Cos. Sci.* **2004,** *26*, 149–156.

[4] J. Leyden, K.J. McGinley, E. Holzle, J.N. Labows, A.M. Kligman, *J. Investig. Dermatol.* **1981**, *77*, 413–416.

[5] D. Taylor, A. Daulby, S. Grimshaw, G. James, J. Mercer, S. Vaziri, *J. Cos. Sci.* **2003**, *25*, 137–145.

[6] C. Austin, J. Ellis, *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 105–110.

[7] P.J. Rennie, D.B. Gower, K.T. Holland, A.I. Mallet, W.J. Watkins, *J. Cos. Sci.* **2007**, *12*, 197–207.

[8] A. Natsch, S. Derrer, F. Flachsmann, J. Schmid, *Chem. Biodiv.* **2006**, *3*, 1–20.

[9] Y. Hasegawa, M. Yabuki, M. Matsukane, *Chem. Biodiv.* **2004**, *1*, 2042–2050.

[10] M. Troccaz, C. Starkenmann, Y. Niclass, M. Van de Waal, *Chem. Biodiv.* **2004**, *1*, 1022–1035.

[11] A. Natsch, J. Schmid, F. Flachsmann, *Chem. Biodiv.* . **2004**, *1*, 1058–1072.

[12] M. Gautschi, A. Natsch, F. Schroder, *Chimia*. . **2007**, *61*, 27–32.

[13] A. Natsch, H. Gfeller, P. Gygax, J. Schmid, G. Acuna, *J. Biol. Chem.*, **2003**, *278*, 5718–5727.

[14] C. Starkenmann, Y. Niclass, M. Troccaz, A.J. Clark, *Chem. Biodiv.* . **2005**, *2*, 705–715.

[15] A.V. Rawlings, *Int. J. Cos. Sci*. **2006**, *28*, 79–93.

[16] T. Akutsu, K. Sekiguchi, T. Ohmori, K. Sakurada, *Chem Senses* **2006**, *31*, 557–563.

[17] S.J. Dixon, Y. Xu, R.G. Brereton, H.A. Soini, M.V. Novotny, E. Oberzaucher, K. Grammer, D.J. Penn, *Chemometr. Intell. Lab. Syst.* **2007**, *87*, 161–172.

[18] D.J. Penn, E. Oberzaucher., K. Grammer, G. Fisher, H.A. Soini, D. Wiesler, M.V. Novotny, S.J. Dixon, Y. Xu , R.G. Brereton, *J R Soc Interface.* **2007,** *4*, 331–340.

[19] J. Havlicek, P. Lenochova, *Chem. Senses* **2006**, *31*, 747–752.

[20] S.U. Savelev, S. Antony-Babu, S.C. Roberts, H. Wang, A.S. Clare, L.M. Gosling, M. Petrie, M. Goodfellow, A.G. O'Donnell, A.C. Ward, *J Chem Ecol*, **2008**, *34*, 1253–1257.

[21] S.C. Roberts, L.M. Gosling, T.D. Spector, P. Miller, D.J. Penn,.M. Petrie, *Chem Senses.* **2005**, *30*, 651–656.

[22] C. Wedekind, T. Seebeck, F. Bettens, A.J. Paepke, *Proc. R. Soc. Lond. B.* **1995**, *260*, 245–249.

[23] P.J.H. Jackman, W.C. Noble, *Clin. Exp. Dermatol*. **1983,** *8*, 259–268.

[24] D. Hopwood, M.D. Farrar, R.A. Bojar, K.T. Holland, *Acta Derm Venereol*. **2004**, *85*, 363–364.

[25] M.M Bradford, *Anal. Biochem*. **1976**, *72*, 248–254.

[26] C. Vuilleumier, M. van de Waal, H. Fontannaz, I. Cayeux, P.A. Rebetez, Perfumer & Flavorist. **2008**, *33*, 54-61.

[27] E.L. Cameron ,*Chem. Senses.* **2007,** *32*, 775–782.

[28] X.N. Zeng, J.J. Leyden, H.J. Lawley, K. Sawano, I. Nohara, G. Preti, *J. Chem. Ecol.* **1991**, *17*, 1469–1492.

[29] F. Herrmann, L.A. Mandol, *J. Invest. Dermatol*. **1955**, *24*, 225–246.

[30] C. Ehlers, U.I. Ivens, M.L. Moller, T. Senderovitz, J. Serup, *J. Skin Res*. Technol. **2001,** *7*, 90–94.

[31] C. Vermeulen, L. Gijs, S. Collins, *Food Reviews Int.* **2005**, *21*, 69–137.

[32] X.N. Zeng, J.J. Leyden, A.I. Spielman, G. Perti, *J. Chem. Ecol.* . **2006**, *22*, 237–257.

[33] S.A. Makin, M.R. Lowry, Deodorant Ingredients. In: Antiperspirants and Deodorants. Laden K, editor. New York: Marcel Dekker. **1999**. p. 169–214.

[34] N. Dayan, P. Wertz, D. Drake, *J. Cosmet. Sci*. **2007**, *58*, 184–185.

[35] R. Emter, A. Natsch, *J. Biol. Chem.* **2008**, *283*, 20645–20652.

[36] F. Kuhn, A. Natsch, *J. R. Soc. Interface*, doi:10.1098/rsif.2008.0223. **2008**.

5. Enzymes involved in sulfur compounds biosynthesis

Properties of Recombinant *Staphylococcus haemolyticus* Cystathionine β-Lyase (*metC*) and Its Potential Role in the Generation of Volatile Thiols in Axillary Malodor

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(Supporting material in ***Appendix 7.4*****)**

Abstract – Enzymes implicated in cysteine and methionine metabolism such as cystathionine β-lyase (CBL; EC 4.4.1.8), a pyridoxal-5'-phosphate (PLP)-dependent Carbon-Sulfur lyase, have been shown to play a central role in the generation of sulfur compounds. This work describes the unprecedented cloning and characterization of the *met*C-cystathionine β-lyase from the axillary isolated strain *S. haemolyticus* AX3, in order to determine its activity and its involvement in amino acid biosynthesis and in the generation of sulfur compounds in human sweat. The gene contains a sequence capable to effect β-elimination, and also a cysteine/methionine metabolism enzyme pattern. The recombinant enzyme was shown to cleave cystathionine into homocysteine and to convert methionine into methanethiol at low levels. No odor was generated after incubation of the recombinant enzyme with sterile human axillary secretions; sweat components were found to have an inhibitory effect. These results suggest that the generation of sulfur compounds by *Staphylococci* and the β-lyase activity in human sweat are mediated by enzymes other than the *metC* gene or by the concerted activities of more than one enzyme (see ***Appendix 7.4***).

Keywords: Cystathionine β-lyase, *metC* Gene, *S. haemolyticus*, Axillary Malodor

1. Introduction

Cystathionine *β*-lyase (CBL; EC 4.4.1.8, commonly referred to as *β*-cystathionase or cysteine lyase) is a pyridoxal-5'-phosphate (PLP)-dependent carbon-sulfur (C-S) lyase which principally cleaves L-cystathionine to homocysteine, pyruvate, and NH_3, and forms the penultimate step in microbial and plant methionine biosynthesis [1][2]. The enzyme catalyzes the split of the *α* C-N (deamination) and of the *β* C-S bonds through a *β*-elimination. It belongs to the large family of cysteine-S-conjugate β-lyases [1][3]. Cystathionine β-lyases have been purified from several plants and bacteria such as *Arabidopsis thaliana* [4][5], *Escherichia coli* [6], *Salmonella enterica* [7], *Bordetella avium* [8] and *Lactococcus lactis* [9]. In the *Staphylococcus haemolyticus* genome, a putative metC-CBL was identified by a bioinformatics-based approach, but has never been fully characterized (source: Database of the Genomes Analysed at Nite, DOGAN) [10].

Besides its central role in amino acid metabolism, the cystathionine *β*-lyase has been described as contributing to the formation of important sulfur containing volatiles [9][11][12], and also as being responsible for the off-flavor deterioration of unblanched brocolli [13][14]. In *Lactococcus lactis*, CBL was shown to degrade cystathionine into cysteine and α-ketobutyrate, and to degrade methionine into methanethiol (γ C-S linkage), a precursor of important flavor compounds in cheese [9].

A number of pyridoxal-dependent enzymes involved in the metabolism of cysteine, homocysteine, and methionine have been shown to be evolutionarily related [6]. The PLP group appears to be attached to a lysine residue located in the central section of the enzyme. The sequence around this residue is highly conserved and has been used as a signature pattern to detect this class of enzymes. This conserved domain was named Cys_Met_Meta_PP (domain identification at the NCBI). It is characteristic of enzymes with a pyridoxal attachment site involved in cysteine and methionine metabolism (Cys/Met metabolism enzyme) [8][15]. Cystathionine *β*-lyase (CBL) is part of this enzyme family including cystathionine *γ*-lyase (CGL), cystathionine *γ*-synthase (CGS), methionine *γ*-lyase (MGL), *O*-acetylserine/*O*-acetylhomoserine sulphydrylase (OAH/OAS sulphydrylase), and *O*-succinylhomoserine sulphydrylase. Interestingly, in *Corynebacteria spp*. and in *Escherichia. coli*, it has been reported that *malY* genes encoding an aminotransferase were also found to have CBL activities [16–18]. The *malY* gene was reported to complement a *metC* mutation in a methionine auxotroph *E. coli* [18].

Axillary strains such as *Corynebacterium striatum, Corynebacterium jeikeium* and *Staphylococuss haemolyticus* were found to generate sulfanylalkanols *via β*-elimination of an odorless *S*-conjugate present in sweat [19][20]. Indeed, the presence of a pyridoxal phosphate dependent *β*-lyase similar to cystathionine *β*-lyase was proposed. *Natsch et al.* and *James et al.* mentioned the role of a MalY type C-S *β*-lyase (*malY* gene products abolishing endogenous induction of the maltose system) from *C. striatum* and *C. jeikeium* having similarities to a bacterial aminotransferase involved in the generation of thiols from a cysteinyl-*S*-conjugates [16][17].

The purpose of this work was to determine the role of Cys/Met metabolism enzymes from *metC* gene, present in *S. haemolyticus* for both amino acid biosynthesis and thiol formation in sweat. Protein sequence comparisons and database searches were used to identify potential C-S lyases to be cloned and expressed in *Escherichia coli*. The possible role of these enzymes, especially *metC*- cystathionine *β*-lyase, in thiol release was evaluated by incubation with amino acid substrates, sterile odorless sweat, and the previously described cysteinyl and cysteinylglycine *S*-conjugated sweat precursors [19].

2. Results

2.1 Cys/Met metabolism enzymes in Staphylococcus haemolyticus

The complete genome of S*taphylococcus haemolyticus JCSC1435* was sequenced in 2005 by *Takeshi* and co-workers [10]. From genome analysis, four genes under the locus names SH2548 (cystathionine *γ*-synthase; CGS), SH2635 (putative cystathionine *γ*-synthase, pCGS), SH2636 (putative cystathionine *β*-lyase; pCBL) and SH0593 (*O*-acetylhomoserine sulphydrylase) were identified to possess the Cys/Met metabolism enzyme pyridoxal-phosphate attachment site (PROSITE conserved domain PS00868; *Table 1*). Interestingly, the pCBL SH2636 from *Staphylococcus haemolyticus* was also found to contain similarities in its amino acid sequences with the pfam motif PF01212 corresponding to a *β*-eliminating lyase pyridoxal-phosphate attachment site [3] (E value < 0.0006; consensus pattern: [YV]-x-D-x(3)-M-S-[GA]-K-K-D-x-[LIVMF]-[LIVMAG]-x-[LIVM]-G-G with the second K being a pyridoxal-P

[3] http://motif.genome.jp

attachment site. The Expectation value (E-value) represents the number of times the protein database match may have occurred just by chance. The cut-off value was set to 1.10^{-4}.).

2.2 Phylogenetic and evolutionary analyses

A phylogenetic tree has been generated to better understand the evolutionary history of Cys/Met metabolism enzymes from different bacterial sources, and in an effort to predict their respective activities (*Fig. 1*). Multiple amino acid sequence alignements have shown that there is a high degree of similarity at a protein level between the previously described *metC* gene enzymes CGS SH2548, pCGS SH2635, and pCBL SH2636 from *S. haemolyticus*, and the corresponding enzymes in other *Staphylococci* (higher than 80% identity in amino acid sequences). Cystathionine *γ*-synthase or cystathionine *β*-lyase from different *Staphylococci* species may have common ancestors and may play a primary role in amino acid biosynthesis.

Similarities were also found between the pCBL from *S. haemolyticus* and CBL BLi01290 from *Bacillus licheniformis* (46% identity) and CBL L0181 from *Lactococcus lactis* (45% identity). In contrast, less similarities were found between pCBL from *S. haemolyticus* and CBL from *E. coli* (27.5% identity), which may reflect the functional and catalytic differences of the proteins.

Table 1. Search results of protein sequence from S. haemolyticus JCSC1435 containing the Cys/Met metabolism enzymes pyridoxal-phosphate attachment site [a]).

Locus Name/Accession Number	Enzyme classification	Gene coordinates	Motif match in protein sequence [b])	Principal catalysed reaction [c])	Conserved domain [d])
SH0593/YP_252508	CysD, *O*-acetylhomoserine sulfhydrylase	609300–608014	DIVIHSATKFIGGHG	*O*-acetyl-*L*-homoserine + methanethiol → *L*-methionine + acetate	Met 17
SH2548/YP_254463	MetB, cystathionine *γ*-synthase	2566044–2564899	DIVLHSATKYIGGHS	*O*-Succinyl-*L*-homoserine + *L*-Cysteine → *L*-Cystathionine + Succinate	Met C
SH2636/YP_254551	Unnamed protein product; similar to cystathionine *β*-lyase	2639002–2640177	DIVVHSATKFLGGHS	n *L*-Cystathionine + H_2O → *L*-Homocysteine + NH_3 + Pyruvate	MetC
SH2635/YP_254550	Unnamed protein product; similar to cystathionine *γ*-synthase	2637902–2639005	DIVLHSATKYIGGHN	*O*-succinyl-*L*-homoserine + *L*-cysteine → *L*-cystathionine + succinate	MetC

[a]) PROSITE pattern PS 00868 corresponding to the defined sequence [DQ]-[LIVMFY]-x(3)-[STAGCN]-[STAGCIL]-T-K-[FYWQI]-[LIVMF]-x-G-[HQD]-[SGNH]. [b]) Obtained from The Institute for Genome Research database (TIGR) and the Comprehensive Microbial Resource (CMR). [c]) Obtained from Kyoto Encyclopedia of Genes and Genomes (Kegg). [d]) Conserved Domain (CD) server from National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

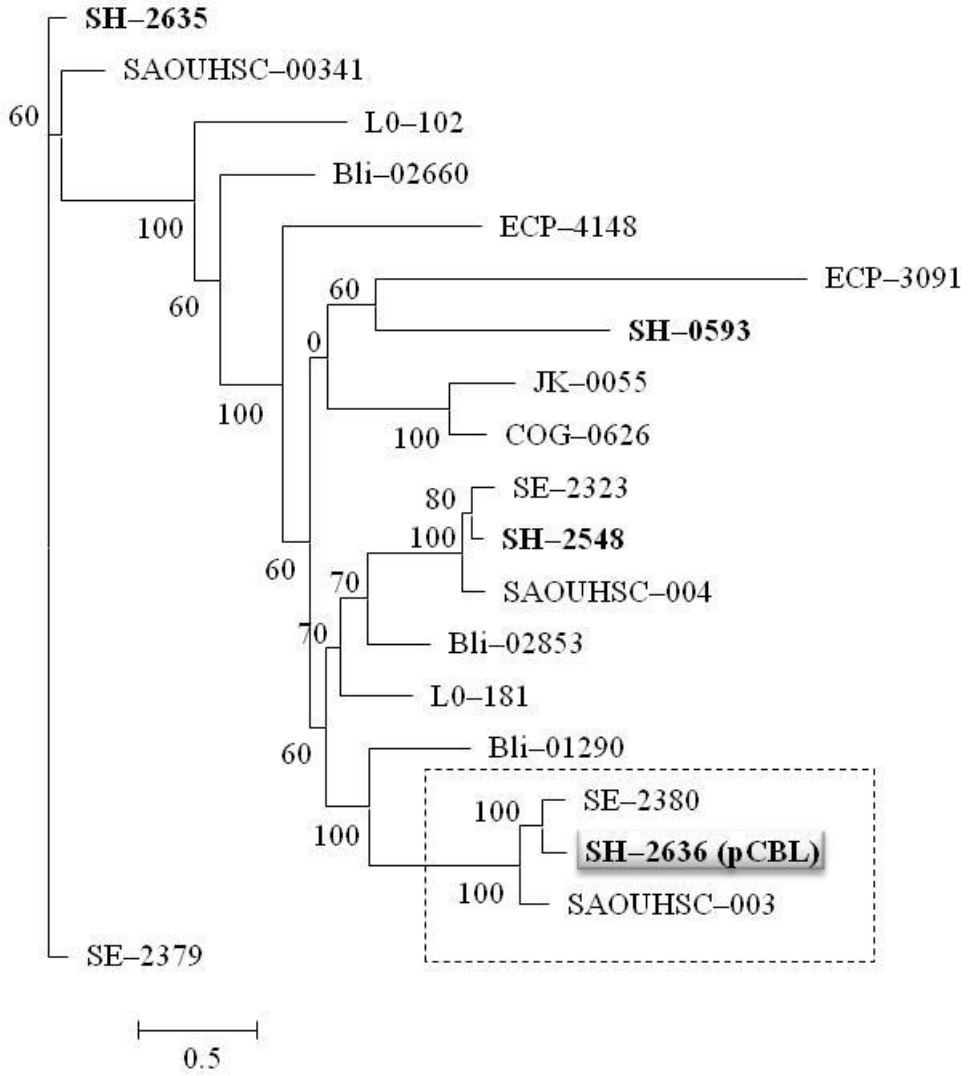
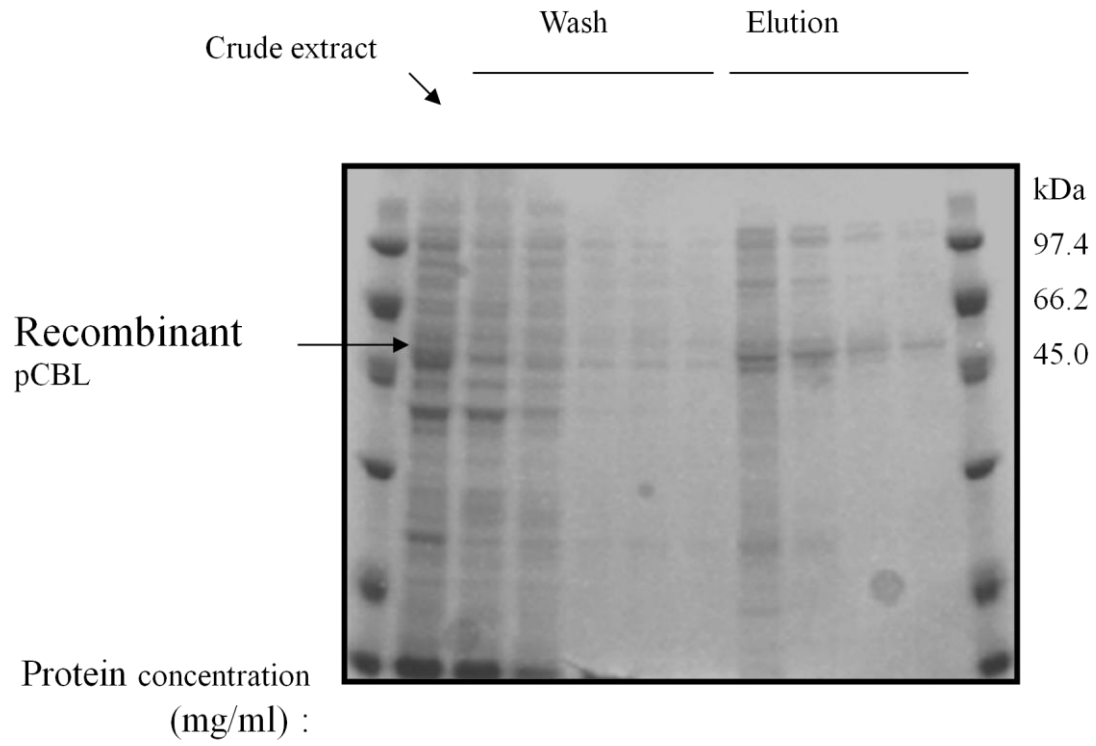


Fig. 1. Phylogenetic analysis (maximum likelihood) of the relationships among Cys/Met metabolism bacterial enzymes. Bootstrap support values are indicated for major nodes only. The placement of *S. haemolyticus* pCBL is indicated by shaded labels. *S. haemolyticus* enzymes are in bold characters. Enzymes from the following bacterial strains are represented: *Corynebacterium glutamicum* ATCC13032*: COG_0626 (Locus Name)* Cystathionine *β*-lyases/cystathionine *γ*-synthases (Enzyme classification); *Corynebacterium jeikeium* K411*:* JK_0055 cystathionine *γ*-synthase; *Escherichia coli 536:* ECP_4148 cystathionine *γ*-synthase; ECP_3091 cystathionine *β*-lyase; *Bacillus licheniformis* ATCC14580: BLi_02660 Cys/Met metabolism PLP-dependent enzymes; BLi_01290 similar to cystathionine *β*-lyase; BLi_02853 similar to cystathionine *γ*-synthase; *Lactococcus lactis subsp. Lactis IL1403:* LO_ 181 cystathionine *β*-lyase MetC; L0_102 cystathionine *γ*-synthase; *Staphylococcus epidermidis* ATCC 12228*:* SE_2380 putative cystathionine *β*-lyase; SE_2379 cystathionine *γ*-synthase; SE_2323 cystathionine *γ*-synthase; *Staphylococcus aureus* NCTC 8325*:* SAOUHSC_00340 *trans*-sulfuration enzyme family protein; SAOUHSC_00341 conserved hypothetical protein; SAOUHSC_00422 *trans*-sulfuration enzyme family protein; *Staphylococcus haemolyticus JCSC1435:* SH_2636 hypothetical protein, similar to cystathionine *β*-lyase; SH_2548 cystathionine *γ*-synthase; SH_2635 hypothetical protein, similar to cystathionine *γ*-synthases; SH_0593 *O*-acetylhomoserine sulfhydrylase.

2.3 Cloning and purification of pCBL SH2636 from S. haemolyticus

The gene encoding for a putative cystathionine *β*-lyase in axillary isolated *S. haemolyticus* AX3 was cloned and expressed in *E. coli* BL21. The *S. haemolyticus* AX3 homologue was 100% identical to the sequence derived from *S. haemolyticus JCSC1435* genome [10]. As observed by SDS/Page, a polypeptide with an apparent molecular weight of 46 kDa (42.7 kDa gene containing a 3.6-kDa-His-tag in vector pEt151) accumulated in IPTG-induced BL21 *E. coli* cells (*Fig. 2,a*). The His-tag was confirmed by *Western* blot analysis (*Fig. 2,b*). A cell-free extract obtained by ultrasonication of the recombinant strain culture was purified on a nickel column under native conditions with a histidine gradient elution. Starting with 50 ml of recombinant bacteria, ca. 12.8 mg (in 8 ml) of crude proteins and 0.3 mg (in 1 ml) of de-salted purified p. CBL were obtained. A native protein gel revealed the presence of a polypeptide of 170 kDa, which might indicate that the protein is in a tetrameric form, as reported in studies using *E. coli* [1].

a)



b)

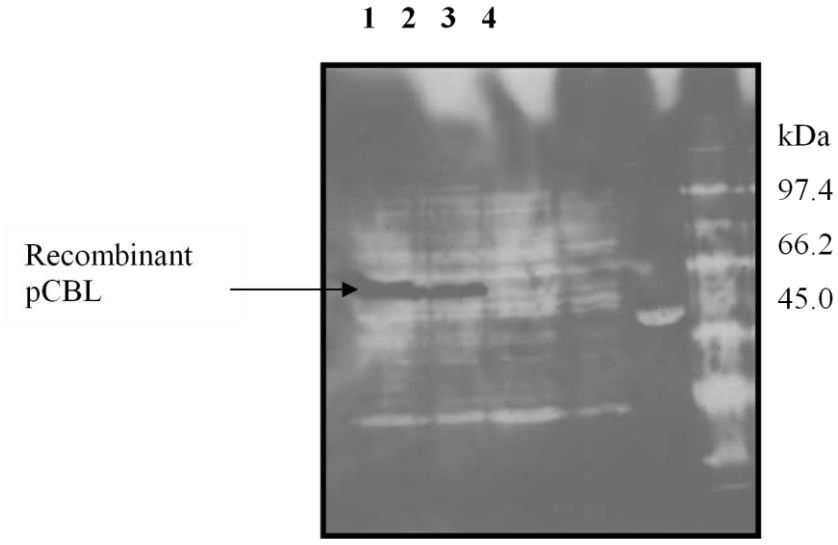


Fig.2. a) *SDS-PAGE* (12.5% polyacrylamide) showing the different purification steps of the recombinant cystathionine *β*-lyase (CBL) from *S. haemolyticus* with ProBond system from Invitrogen. Line 1: induced lysate; line 2: non-induced lysate; lines 3-4-5: sucessive washes; lines 7-8-9-10: elutions (1 ml aliquots). First and last columns: molecular weight markers. Molecular weights (in thousands) are noted on the right. b) Western *blot autoradiography of His-tag recombinant CBL from* S. haemolyticus *expressed in* E. coli BL21 (DE3). Lines 1-2: induced bacteria containing pEt151-CBL plasmid; lines 3-4: non-induced cells. The location of the over-produced proteins is indicated (arrow).

2.4. Activity of the recombinant enzyme

The formation of homocysteine was monitored by quantification of sulfhydryls using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid); DTNB). A correlation was observed between the enzyme concentrations and the reaction velocity (*Fig. 3,a*). In addition, a modification of the standard elution protocol in which histidine was replaced by imidazole increased the activity four fold. The imidazole inhibition effect was not removed by a de-salting column, suggesting that imidazole may bind to the active site.

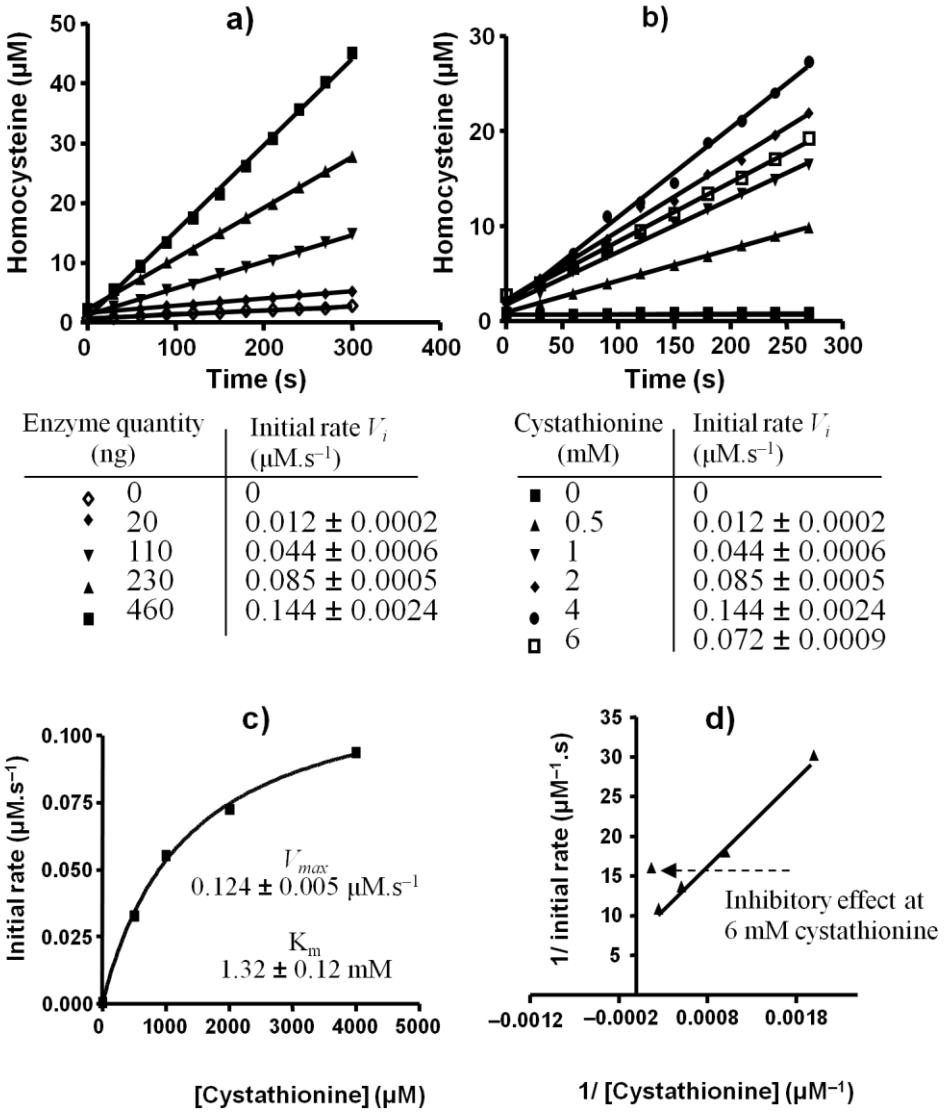


Fig.3. a) Initial rate of recombinant *S. haemolyticus* AX3 CBL enzyme with various enzyme concentrations (4 mM cystathionine). b) Initial rate of homocysteine formation by recombinant *S. haemolyticus* AX3 CBL with various substrate concentrations (230 ng of recombinant enzyme). c) Michaelis-Menten curve with 230 ng of recombinant CBL. d) The Lineweaver-Burk plot. Assay mixture contains 0.2 mM DTNB, 0.1 mM PLP, various purified His-tagged proteins concentrations and various *L*-cystathionine substrate concentrations. Reaction parameters are: 1 ml total volume, 0.1 M TrisCl, pH 8.0, 37°. V_{max} = maximum velocity at saturation; K_m = Michaelis constant is the substrate concentration required to reach half-maximal velocity (V_{max}/2).

The influence of PLP dependent enzyme was measured by varying the concentration between 0 and 100 μM. When PLP was omitted in the reaction mixture, the rate of homocysteine formation decreased to one-third of that in the complete system. As expected by sequence analysis, these results indicated the occurrence of *L*-cystathionine cleavage by recombinant CBL requiring PLP as a cofactor in this organism. The optimum pH and temperature in *Tris*.HCl buffer was found to be 8.5 and 37°, respectively, and four concentrations of *Tris*.HCl (100, 250, 500 mM and 1 M) were investigated. The rate of formation of homocysteine doubles from 100 mM to 1 M. Recombinant enzyme activity appeared to be lost after 4 d of storage at 4°. The addition of 15% glycerol extended the enzymatic lifetime to several days and permitted freezing of the preparation (–20°).

2.5. Substrate specificity

The purified *β*-cystathionase obeyed *Michaelis-Menten* kinetics with cystathionine as a substrate (*Fig. 3,b* and *c*). The substrate had an inhibitory effect at a concentration higher than 6 mM (*Fig. 3,d*). The K_m and V_{max} values for *L*-cystathionine were 1.3 mM and 32 U/mg protein (*Table 2*), respectively. Both methionine and homocysteine were identified as degradation products of cystathionine by HPLC/MS measurements after 15 min. The molar yield of methionine was 0.004% with an initial cystathionine concentration of 4 mM. This activity is relatively low in comparison to homocyteine formation (4% molar yield). Recombinant *Staphylococcus haemolyticus* CBL was able to catalyze an *α/β* elimination equally well, which, in the case of methionine, results in the production of methanethiol which smells like rotten cabbage as previously reported for *Lactococcus lactis* [9]. The maximum rate, V_{max}, was 3.6 U/mg after 15-min incubation with 4 mM methionine at a pH of 5.5. No methanethiol was generated at a pH above 7.0. No activity was found after 30 min incubation with cysteine, cysteinyl or cysteinylglycine-*S*-precursors (*S*-[1-(2-hydroxymethyl)-1-methylbutyl]-*L*-cysteinylglycine and *S*-[1-(2-hydroxymethyl)-1-methylbutyl]-*L*-cysteine) present in sweat secretions. A sulfur odor may only be detected after 24 h incubation at a pH of 8, 37°. These results were confirmed by GC/MS. The maximum conversions were found to be 1% and 5% molar yield after 24 h incubation at 37° with 1.7 μM of C-*S* and CG-*S* precursors respectively. The maximum rates were 0.02 and 0.04 U/mg for the two substrates (*Table 3*). No odors were detected after incubation of the recombinant enzyme with sterile odorless sweat secretion after 3 d.

Table 2. Characteristics of recombinant cystathionine *β*-lyase (CBL) from various microorganisms and plants versus recombinant CBL from *S. haemolyticus*. Reaction conditions are 37°, pH 8 and 0.1 mM pyridoxal phosphate (PLP) when not specified. U/mg = µmol.min^{-1}.mg^{-1}. ND = not determined.

Enzyme host	**K_m (mM)**	**V_{max} (U/mg)**
Escherichia coli [a)]	0.04 ± ND	249.0 ± ND
Arabidopsis thalalianab [b)]	0.22 ± ND	51.9 ± ND
Lactobacillus delbrueckii [c)]	5.13 ± 0.42	13.2 ± 1.01
Staphylococcus haemolyticus	1.20 ± 0.12	32.0 ± 1.3

[a)] 37°C, pH 7 [21]. [b)] 30°C, pH 8.5 [5]. [c)] 37°C, pH 8 [22].

Table 3. Substrate specificity of the recombinant CBL from *S. haemolyticus*. Reaction conditions are: 37°, pH 8 or 5.5, 0.1 mM pyridoxal phosphate (PLP).

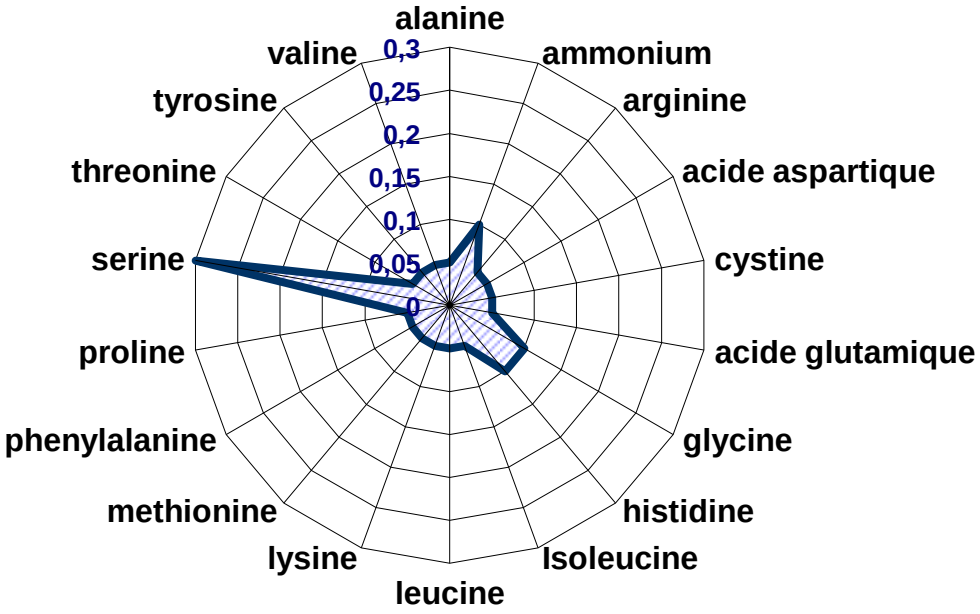
Substrate	**Substrate Concn (µM)**	**pH**	**Sulfur containing Product**	**Relative activity[a)] [%]**
L-Cystathionine	4 000	8	Homocysteine	100
L-Methionine	4 000	5.5	Methanethiol	20
L-Cysteine	4 000	5.5; 8	Hydrogen sulfide	0
Cys-Gly-*S*-conjugate [b)]	1.7	8	Sulfanylhexanol	0.01
Cys-*S*-conjugate [c)]	1.7	8	Sulfanylhexanol	0.22

[a)]Relative activities are calculated by thiol determination as a percentage of the cystathionine rate. [b)]*S*-[1-(2-Hydroxymethyl)-1-methylbutyl]-*L*-cysteinylglycine. [c)]*S*-[1-(2-Hydroxymethyl)-1-methylbutyl]-*L*-cysteine

2.6. *Sweat composition and related enzyme inhibition*

Amino acid analysis of male axillary sweat secretions (heat induced) showed high levels of serine (*Fig. 4*), as previously described by *Farrior* and *Kloos* in 1976 [23]. HPLC Measurements with sterile male sweat gave the following concentrations: 3 mM serine, 0.9 mM glycine, 0.65 mM histidine and 0.75 mM ammonium sulphate. The glucose concentration was 0.010 g/l and the pH 7.2 ± 0.7 (average for ten European volunteers). Sweat secretions were found to inhibit the recombinant *S. haemolyticus* Cystathionine *β*-lyase activity, whereas they did not inhibit thiol formation by incubating the whole cell of *S. haemolyticus* (data not shown). Serine, glycine and ammonium sulphate at sweat concentrations were also found to inhibit the recombinant CBL (*Table 4*).

a)



b)

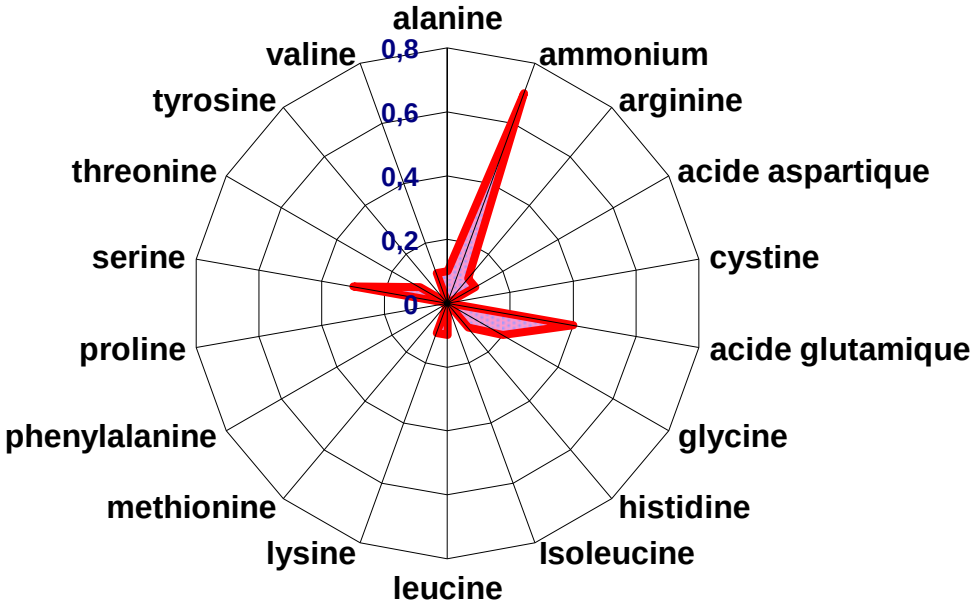


Fig. 4. Amino acid compositions of male axillary sweat secretions (heat stimulation).
a) Free amino acid composition.
b) Total amino acid composition.

Table 4. Effects of inhibitors on recombinant CBL activity.Reaction conditions are: 37°, 230 ng recombinant CBL enzyme, 4 mM cystathionine substrate in 0.1 M TrisCl pH 8.

Inhibitors	**Inhibitor concentrations**	**Inhibition [%][a])**
Glycine	2 mM	0
	4 mM	73
Serine	2 mM	5
	4 mM	86
Methionine	2 mM	0
	6 mM	40
Ammonium Sulfate	4 mM	66
Sterile sweat secretions[b])	50% v/v	47
Inhibitor mixture[c])	50% v/v	40

[a]) Percentage inhibitions are determined for each inhibitor as a percentage of maximum cystathionine rate without inhibitors; cystathionine rate is calculated by thiol determination. [b]) Collected from excessive sweating in a sauna and filter-sterilised. [c]) Consists of 3 mM glycine, 1 mM serine and 1 mM ammonium sulfate.

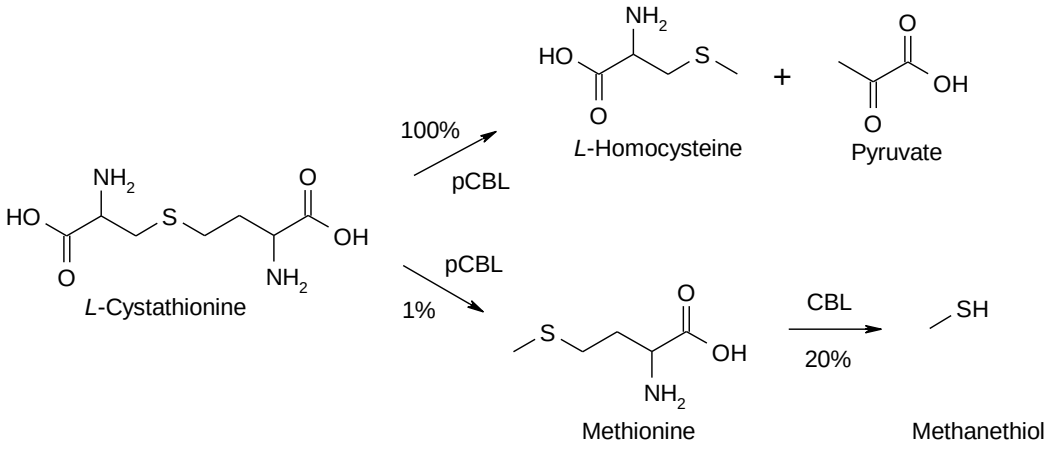
3. Discussion

Amino acid sequence analysis revealed that axillary-isolated *S. haemolyticus* enzymes implicated in cysteine and methionine metabolism have some homologies to those present in *Lactococcus lactis, Bacillus licheniformis* and other skin *Staphylococci* such as *S. aureus* and *S. epidermidis.* Not surprisingly, the pCBL from the axillary-isolated *S. haemolyticus* AX3 has 80% identity at protein level with CBL from *S. epidermidis*. It contains both a *β*-eliminating group and a Cys/Met metabolism enzyme pattern, which suggests that this protein may be implicated in amino acid biosynthesis and in C-S lyase cleavage in sweat secretions.

The recombinant *S. haemolyticus* pCBL enzyme expressed in *E. coli* BL21 exhibits a cystathionine-*β*-lyase activity similar to that described in *Lactococci*. It utilizes amino acids containing a *β* C-*S* linkage as substrates, producing pyruvate, NH_3 and a sulfur-containing molecule (*Scheme*). This enzymatic activity is typical for the gene product of *met*C, a CBL, involved in the biosynthesis of methionine. The optimal pH of the reaction was found to be 8.5, which is higher than found under axilla (range: 4.2–7.5 in thermal sweat [24]). The enzyme is PLP-dependent, as expected by aminoacid sequence homology analysis. Analogous to CBL occurring in *Escherichia coli, Bordetella avium* and *Paracoccus denitrificans,* it is a tetrameric protein with identical subunits of *ca.* 42 kDa in size. The K_m value of 1.2 mM is higher than

reported for *E. coli* enzyme (0.04 mM), indicating a lower affinity of *S. haemolyticus* recombinant CBL to cystathionine (K_m at pH 8, 37°; *Table 3*). The V_{max} value for *L*-cystathionine was 32 U/mg of protein. This low specific activity may be due to the presence of the His-tag on the *N*-terminal part of the enzyme. Like many other enzymes of biosynthetic pathways, it is not endowed with an absolute specificity: the enzyme is also able to catalyze the conversion of cystathionine into methionine, and metionine into MeSH at low levels (*Scheme*). These results are in accordance with the results obtained for other known bacterial CBLs. CBL from *Lactococcus lactis* is responsible for the conversion of methionine into MeSH; this reaction was shown to be 100-fold less efficient than its primary catabolic reaction of transforming cystathionine into homocysteine [25][9]. In *S. haemloyticus* the reaction was five fold less efficient. Sweat components such as glycine, serine and ammonium sulphate were strong inhibitors for the recombinant cystathionine *β*-lyase.

Scheme. *α/β* elimination reaction of recombinant *S. haemolyticus* CBL (pCBL) with cystathionine as substrate. Percentages indicate relative percentage activity in comparison to homocysteine formation.



Most micrococci extracted from the skin have been found to be auxotrophic for methionine [23]. We have shown that, in *Staphylococci*, the biosynthetic route for this amino acid exists but may be inhibited by sweat compositions. Possibly a lyase other than CBL contributes to methionine production (direct sulfhydrylation) mediated by *O*-acetylhomoserine lyase or other bacterial activity. *O*-Acetylhomoserine lyase protein sequence was found to be very similar to the same enzyme present in *C. glutamicum* in which direct sulfhydrylation was

reported [26] [27]. Further cloning and expression studies may be necessary to establish whether or not this pathway is active in *S. haemolyticus.*

Enzymes from the cysteine/methionine metabolism such as CBL or cystathionine *γ*-lyase from *Lactoccocus* bacteria play a central role in the generation of sulfur compounds in cheese as demonstrated and documented in several publications [9][11][12]. The axillary isolated *Bacillus licheniformis* was also known to convert Cysteinyl and CysteinylGlycine-*S*-precursors from sterile sweat secretions into sulfur compounds (data not shown). On the contrary, *Staphylococci* bacteria such as *S. epidermidis* and *S. aureus* are non-related to sulfur axillary malodors in sweat [16][20]. These results raise questions on the relevance of *S. haemolyticus met*C-CBL in sweat odors. Indeed it was not surprising that no sulfur odors were generated after 3 d incubation of the recombinant *S. haemolyticus* CBL with odorless sweat secretions. One reason may be due to the very low level of activity of the enzyme with the two synthesized sulfur sweat precursors, Cysteinyl and CysteinylGlycine-*S*-precursors. Another reason may be due to the relatively low levels of these precursors in sweat samples, which may lead to a concentration of volatiles which is below the detection threshold of instrumentation and even the nose. Similarly, the absence of volatile sulfur amino acid derived compounds, such as MeSH or H_2S, may be due to the relatively low levels of free methionine, the low activity of the enzyme at skin pH, and the inhibitory effect of sweat components. In addition, the recombinant CBL had no activity on cysteine as substrate, as previously described in *Lactococcus helveticus* [12].

Indeed, this relatively low level of activity in sweat secretions and the high homologies between *S. haemolyticus, S. aureus* and *S. epidermidis* Cys/Met enzymes suggest that *met*C-CBL is not responsible for the high amount of sulfur compounds generated in sweat by *S. haemolyticus*. *Natsch et al*. and *James et al* reported the release of sulfanylalkanols by axillary strains *Corynebacterium striatum* and *Corynebacterium jeikeium* from a MalY type cys-conjugate PLP dependent *β*-lyase [16][17]. This enzyme was found to have similarities to a bacterial aminotransferase, but has no significant sequence homology to CBL from *Staphylococcus aureus, Staphylococcus epidermidis* or *Bacillus cereus*. Similar MalY type enzymes have been identified from *S. haemolyticus* by a bioinformatics-based approach (data not shown). Future work, involving the expression and characterisation of these enzymes, may confirm whether a similar mechanism implicating a single enzyme or a multiple enzyme mechanism is responsible for a *β*-elimination reaction leading to the release of sulfanylalkanols by *S. haemolyticus*.

4. Conclusion

In the present work we have cloned and characterized the *met*C-cystathionine *β*-lyase gene from the axillary isolated strain *S. haemolyticus* AX3. To our knowledge, a cystathionine *β*-lyase from *Staphylococci* has not been previously described. *Staphylococci* CBL, whose function within the growing cell is primarily related to amino acid catabolism, appears unlikely to be involved in sulfur compound generation in sweat. Even if it were, the intensive thiol odor following the addition of *S. haemolyticus* to odorless secretions cannot be solely explained by *staphylococci metC*-CBL. Future work, involving the expression and characterization of MalY type enzymes such as the two *S. haemolyticus* PLP aminotransferases, SH0221 and SH2169 with *β*-lyase activity (see *Appendix 7.4*), may confirm whether a single enzyme or a multiple enzyme mechanism is responsible for the release of sulfanylalkanols by *S. haemolyticus* in body odors.

5. Experimental Part

Chemicals. Unless otherwise stated, all chemicals were purchased from Fluka (Switzerland). The two (*S*)-cysteine conjugates, *S*-[1-(2-hydroxymethyl)-1-methylbutyl]-*L*-cysteinylglycine and *S*-[1-(2-cydroxymethyl)-1-methylbutyl]-*L*-cysteine, were prepared as reported in [19]. The odorless human sweat secretions were collected from axillae through excessive sweating from 42 volunteer Caucasian male subjects by heat stimulation. These secretions were immediately sterilized through a double filter consisting of 1-µm membrane, followed by 0.2-µm sterile filter and stored at –20° before use.

Bioinformatics tools. For protein phylogenetic analysis, the amino acid sequences of bacterial enzymes containing the Cysteine/Methionine enzyme pattern (PROSITE Pattern PS 00868: [DQ]-[LIVMFY]-x(3)-[STAGCN]-[STAGCIL]-T-K-[FYWQI]-[LIVMF]-x-G-[HQD]-[SGNH]) were taken into consideration. The results of homology search against public databases with sequenced bacterial genome resulted in 21 amino acid sequences from *Staphylococcus haemolyticus* JCSC1435, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* NCTC 8325, *Corynebacterium glutamicum* ATCC13032, *Corynebacterium jeikeium* K411, *Bacillus licheniformis* ATCC14580, *Lactococcus lactis* IL1403 and *Escherichia coli* 536 were compared. The data analysis consisted of two steps:

first, multiple alignments of amino acid sequences by use of MAFFT [28], then a maximum likelihood tree was calculated in Phyml [29] by use of a JTT model following a gamma distribution model with optimized shape parameters [30]. Branch support was calculated using a boot-strapped data set (100 replicates generated by Seqboot) and the same parameters in Phyml. Motif search on multiple genomes were obtained from *The Institute for Genome Research* database (TIGR) and the *Comprehensive Microbial Resource* (CMR) (http://cmr.tigr.org), and from the free motif scan program MyHits (http://myhits.isb-sib.ch/cgi-bin/index). The web site of NCBI was used to search conserved domain (CD) in amino acid sequences (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Enzyme activities were found in *Kyoto Encyclopedia of Genes and Genomes* website (KEGG).

Cloning and expression of CBL. Purified *g*enomic DNA (gDNA) was obtained from an overnight culture of axillary isolated *S. haemolyticus* according to ChargeSwitch gDNA Mini Bacteria Kit protocol (Invitrogen). Published information on gDNA from *S. haemolyticus* was used to design primers [10], with an extension at the 5'end of the forward primer (CACC). The gene of interest was amplified with *pfu* DNA polymerase, purified, and subsequently ligated into the pET 151/D-Topo Directional vector coding for a cystathionine *β*-lyase containing a His--tag at the *N*-terminal end (Invitrogen). The constructs were verified gene sequencing with T7 primers (Fasteris, Geneva). Transformation of Chemically Competent *E. coli* 10F, gave *E. coli* CBL containing the CBL gene under the transcriptional control of the *T7lac*-promoter. BL21 (DE3) One Shot Chemically Competent *E. coli* transformed with pET 151/D-TOPO were grown at 37° in 50 ml Luria Broth (Difco) supplemented with (50 µg/ml) carbenicillin until OD_{600} reach 0.6. Expression of the recombinant protein was then induced by adding 1 mM IPTG (Isopropyl-*β*-D-thiogalactopyronosid; AppliChem), and the culture was maintained at 28° for 16 h. Cells were harvested by centrifugation at 2000*g* for 15 min and re-suspension in 8 ml 1x native purification buffer *A* (pH 8) (buffer *A*: *ProBond Purification* system from Invitrogen) supplemented with protease inhibitory enzymes EDTA-free (Roche) and 1 mg/ml lysozyme from hen egg white.

Protein purification. The cell-free extract obtained from ultrasonication was purified on a nickel column (*ProBond Purification* system, Invitrogen) under native conditions. The elution was carried out with sodium monoascorbic phosphate buffer (pH 8) containing 250 mM imidazole or 250, 125, and 62 mM histidine for gradient elution. imidazole, was removed by

using a de-salting column (*ZebaTM Desalt Spin Column*, Pierce), and the buffer changed by eluting the column with buffer *A*. The removal of imidazole was confirmed by GC/MS (Agilent technologies; *6890N Network* GC system; *5975* inert mass selective detector).

Western *Blot.* The presence of His_6-tagged protein was confirmed by enzyme-mediated chemiluminescence. Blotting was performed with TransBlot SD-semi-dry transfer cell from BioRad, nitrocellulose membrane from *Sigma Aldrich*, and *Whatman* paper from *BioRad*. Anti-His_6-peroxidase was used as antibody. Detection was performed with *ECL Plus™ Western Blotting Reagents* from *GE Healthcare* (formerly *Amersham Biosciences*). This system utilizes chemiluminescence technology for the detection of proteins.

Enzyme Assay System with recombinant enzyme and kinetic constant determination. The assay used for determination of kinetic parameters was based on the formation of free SH groups by spontaneous disulfide interchange with *Ellman's* reagent (DTNB) according to a modified procedure from *Uren* [31]. The assay mixture contained 0.2 mM DTNB, 20 µM PLP, various cystathionine concentrations (from 2 mM to 10 mM) and purified His-tagged enzyme in a final volume of 1 ml; 50 mM *Tris*.HCl, pH 8.0, was used as buffer. Cystathionine was prepared in 0.04 M HCl. The increase in absorbance at 412 nm was measured at 0.5 min intervals for 15 min in a *M2* spectrometer at 37° (*Molecular devices*). A molar absorption coefficient for the arenethiol of 13200 $l.mol^{-1}.cm^{-1}$ was used to calculate the enzyme activity. One enzyme unit represents the formation of 1 µmol arenethiol min^{-1} under the assay conditions at 37°. Enzymatic activity was calculated using GraphPad Prism v 4.0 for Windows (GraphPad Prism software, San Diego, CA, USA). The Lineweaver–Burk plot ($1/v_i$ against 1/c(substrate)) was employed to calculate K_m and v_{max} values. The values reported represent a mean of three independent experiments ± SD. (*R*)/(*S*)-3-Methyl-3-sulfanylhexan-1-ol was detected by GC/MS (GC: *Agilent 6890* instrument coupled to atomic emission detection from *Jass*, Germany; He as carrier gas; fused-silica cap. columns *SPB-1*).

Protein quantification. Protein concentrations were estimated by the micromethod of Bradford with the *Coomassie* protein assay reagent (Bio-Rad) and with the Bovine albumin 5% (Difco) as the standard [32].

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulphate (SDS)-PAGE was carried out with 7% polyacrylamide gels. The proteins were visualized by *Coomassie* brilliant blue staining. Low molecular mass marker proteins (*BioRad*) were used as references. Native PAGE was performed with 12% *Tris*-Glycine Gel (*Invitrogen*), native *Tris*-Glycine sample buffer (2x), and molecular weight marker Pre-Stained *Protein Ladder* (*Invitrogen*).

Sweat amino acid analysis. Free and total amino acids from sweat secretions were analyzed by UV-HPLC by *UFAG Laboratory* (Switzerland). Detection limit was 0.08 g/l of sweat secretions.

LC/MS Methionine analysis. Amino acid formation was measured using HPLC (*Waters 2790; Allian HP*) with a silica column (*Phenomenex; Luna C18*; 5 μm; 250 x 4.6 mm) combined with MS (*Bruker Daltonics; esquire 3000*). 10-μl samples were injected at a flow rate of 0.2 ml/min under isocratic conditions with 30% solvent *A* (methanol with 0.5% formic acid) and 70% solvent *B* (milli-Q water with 0.5% formic acid). Retention times were 0.6 min, 1.9 min and 3.2 min for cystathionine, homocysteine, and methionine, respectively.

Bibliography

[1] T. Clausen, B. Laber, A. Messerschmidt, *Biological Chemistry.* **1997**, *378,* 321.

[2] H. Lee, B. Hwang, *Applied Microbiology and Biotechnology.* **2003**, *62,* 459.

[3] A. J. L. Cooper, *Advanced in Enzymologie and Related Areas of Molecular Biology.* **1998**, *72,* 199.

[4] U. Breitinger, T. Clausen, S. Ehlert, R. Huber, B. Laber, F. Schmidt, E. Pohl, A. Messerschmidt, *Plant Physiol.* **2001**, *126,* 631.

[5] S. Ravanel, D. Job, R. Douce, *Biochem. J.* **1996**, *320,* 383.

[6] B. Laber, T. Clausen, R. Huber, A. Messerschmidt, U. Egner, A. Muller-Fahrnow, H. D. Pohlenz, *FEBS Letters.* **1996**, *379,* 94.

[7] L. J. Ejim, V. M. D'Costa, N. H. Elowe, J. C. Loredo-Osti, D. Malo, G. D. Wright, *Infect. Immun.* **2004**, *72,* 3310.

[8] C. R. Gentry-Weeks, J. M. Keith, J. Thompson, *J. Biol. Chem.* **1993**, *268,* 7298.

[9] A. C. Alting, W. J. M. Engels, S. van Schalkwijk, F. A. Exterkate, *Appl. Environ. Microbiol.* **1995**, *61,* 4037.

[10] F. Takeuchi, S. Watanabe, T. Baba, H. Yuzawa, T. Ito, Y. Morimoto, M. Kuroda, L. Cui, M. Takahashi, A. Ankai, S. I. Baba, S. Fukui, J. C. Lee, K. Hiramatsu, *J. Bacteriol.* **2005**, *187,* 7292.

[11] B. Weimer, K. Seefeldt, B. Dias, *Antonie van Leeuwenhoek.* **1999**, *76,* 247.

[12] W. J. Lee, D. S. Banavara, J. E. Hughes, J. K. Christiansen, J. L. Steele, J. R. Broadbent, S. A. Rankin, *Appl. Environ. Microbiol.* **2007**, *73,* 3034.

[13] H. Wakabayashi, PhD. Thesis, Technical University of Munich, **2004.**

[14] E. C. Ramirez, J. R. Whitaker, *Food Biochem.* **1998**, *22,* 427.

[15] A. Martel, C. Bouthier, F. Le Goffic, *Biochem. Biophys. Res. Commun.* **1987**, *147,* 565.

[16] A. Natsh, J. Schmid, F. Flachsmann, *Chem. Biodiv.* **2004**, *1,* 1058.

[17] A. G. James, C. Austin, D. Hyliands, D. Cox, D. Taylor, S. Grimshaw, R. Calvert, Cosm'ing conference, June **2007.**

[18] E. Zdych, R. Peist, J. Reidl, W. Boos, *J. Bacteriol.* **1995**, *177,* 5035.

[19] C. Starkenmann, Y. Niclass, M. Troccaz, A. J. Clark, *Chem. Biodiv.* **2005**, *2,* 705.

[20] M. Troccaz, C. Starkenmann, Y. Niclass, M. Van de Waal, *Chem. Biodiv.* **2004**, *1,* 1022.

[21] C. M. Dwivedi, R. C. Ragin, J. R. Uren, *Biochemistry*. **1982**, 21, 3064.

[22] D. Aubel, J. E. Germond, C. Gilbert, D. Atlan, *Microbiology.* **2002**, 148, 2029.

[23] J. W. Farrior, W. E. Kloss, *Microbiology*. **1976**, *22,* 1680.

[24] F. Herrmann, L. Mandol, *The Journal of Investigative Dermatology.* **2007**, 225.

[25] B. Dias, B. Weimer, *Appl. Environ. Microbiol.* **1998**, *64,* 3320.

[26] B. Weimer, K. Seefeldt, B. Dias, *Antonie Van Leeuwenhoek.* **1999**, *76,* 247.

[27] B. J. Hwang, H. J. Yeom, Y. Kim, H. S. Lee, *Am. Soc. for Microbiology*. **2002**, *184,* 1277.

[28] K. Katoh, *Nucleic Acids Res.* 2002, *30,* 3059.

[29] S. Guindon, F. Lethiec, P. Duroux, O. Gascuel, *Nucl. Acids Res.* **2005**, *33,* 557.

[30] D. T. Jones, W. R. Taylor, J. M. Thornton, *Comput. Appl. Bioscience*. **1992**, *8,* 275.

[31] J. R. Uren, *Methods in Enzymology*. **1987**, *143,* 483.

[32] M. M. Bradford, *Anal. Biochem.* **1976**, *72,* 248.

6. Final Conclusions

Final Conclusions

The unique nature of human axillary odor is generally attributed to the bacterial degradation of the apocrine secretion, which is sterile and only faintly odoriferous when initially secreted. This odor may be modulated by several factors, such as gender, ethnicity, hygiene and environmental factors (see *Chapter 1*). Sebum and the breakdown products of keratin and other cell debris, can also serve as substrates for bacterial degradation, and contribute to the overall odor.

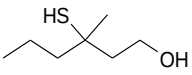
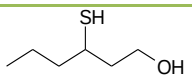
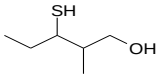
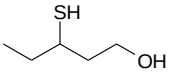
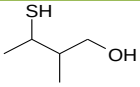
Two classes of chemicals, steroids (*e.g.* 5-α-androst-16-en-3-one and 5-α-androst-16-en-3-α-ol) and aliphatic, branched and unbranched fatty acids ((E)-3-methyl-2-hexenoic acid and 3-hydroxy-3-methylhexanoic acid) have been reported as major contributors to perspiration odor. Few studies concerning Volatile Sulfur Compounds (VSCs) have been forthcoming, principally due to the difficulties in studying high-impact, low-threshold volatiles. This thesis sets out to redress the lack of knowledge in this area, and has identified **(R/S)-3-methyl-3-sulfanylhexan-1-ol** (*R*/*S*-MSH) as the major descriptor for the human axilla-sweat odor profile (see *Chapter 2*). For this purpose, the collection of sterile odorless sweat secretions from a sufficient number of volunteers was necessary (500 ml of sterile sweat was collected from 30 European volunteers). Interestingly, we found that an axillary isolated strain of *S. haemolyticus* (Ax 4) generated the most intense underarm odor after incubation with sweat samples. (S)-3-Methyl-3-sulfanylhexan-1-ol was described as sweat and onion-like, whereas its enantiomer, (R)-3-methyl-3-sulfanylhexan-1-ol, was described as fruity and grapefruit-like. The (S)-form was found to be the predominant enantiomer (75%).

Since then, three groups have independently discovered MSH and other thiols as a major organoleptic component of the human axillary sweat odor (see *Table 1*). Recently, 3-sulfanylalkanols such as 3-sulfanylhexan-1-ol and 2-methyl-3-sulfanylpentan-2-ol have been identified as important odorant molecules in Sauvignon white wine and onion, respectively [1][2][3]. To our knowledge, 3-methyl-3-sulfanylhexan-1-ol is the first human-specific VSC described in the literature that can be assigned as a major descriptor of human underarm odor.

In the past, the contribution of bacteria has been clearly demonstrated in axillary odors. However, there is little evidence in the literature to show how sweat compositions, *e.g.* male or female sweat secretions, influence the bacterial formation of volatile odorants. In Chapter 3, the precursor of 3-methyl-3-sulfanylhexan-1-ol was identified as **[1-(2-**

hydroxyethyl)-1-methylbutyl]-(*L*)-cysteinylglycine (Cys-Gly-(*S*)-conjugate) and not a Cysteine-*S*-conjugate as reported in wine must, or in the glutathione detoxification pathway (see *Chapter 1*).

Table 1. Major volatile thiols present in axillary body odor.

Name	*Structure*	*Odor*	*Bibliography*
(R/S)-3-methyl-3-sulfanylhexan-1-ol*		sweat, onion-like, grapefruit	[4-7]
(R/S)-3-sulfanylhexan-1-ol			[5][6][7]
(R/S)-2-methyl-3-sulfanylpentan-1-ol			[7]
(R/S)-3-sulfanylpentan-1-ol		meaty, onion-like, sulfuric, green, grapefruit	[4][6]
(R/S)-2-methyl-3-sulfanylbutan-1-ol		onion, sweat-like	[4][6]

*Odor Detection Thresholds: 2.27 x 10^{-6} µg/L air (see *Appendix 7.1*).

For the first time, it has been shown that this precursor is present in higher amounts in female underarm secretions compared to males (5.1 mg/ml in females vs 0.5 mg/ml in males) (see *Chapter 4*). As expected, huge variations were found between individuals, but to our surprise, the ratio of the acid to the thiol precursors in fresh axillary secretions from 49 males and females was constant in males and females over the three-year period of the study. The ratio between the acid and the sulfur precursor (a glutamine and a Cys-Gly-(*S*)-conjugate, respectively), was three times higher in men than in women, with no correlation with either the sweat volume or the protein concentration. Indeed, women have the potential to liberate significantly more *R*/*S*-MSH, which has a tropical fruit and onion-like odor, than *R*/*S*-HMHA (possibly transformed into (*E*/*Z*)-3-methyl-2-hexenoic acid), which has a cheesy, rancid odor. Sensory analysis confirmed that the intrinsic composition of sweat is important for the development of body odors, and may be modulated by gender differences in bacterial compositions (see *Chapter 4*). If genetics may explain these chemical and bacterial differences [8][9], we cannot exclude environmental and hygienic factors [10]. These results are not surprising, and may be compared to wine aroma chemistry. Yeasts have a crucial role

in wine fermentation to release volatile sulfanylhexanols. However, the must composition was found to be a good marker to predict the "soil" ("terroir" in French) flavor characteristics [11-13]. With regard to body odors, whereas the presence of Gram positive bacteria is variable, the content of axillary sweat precursors in individuals is also thought to be important.

Following these findings several questions remain to be elucidated:

√ *How are human sulfur volatiles perceived when present in combination with other odorous molecules?* We have found that the odorous volatile sulfur compound, (*R*)/(*S*)-3-methyl-3-sulfanylhexan-1-ol, was perceived at a concentration a hundred times lower than the volatile carboxylic acid (*R*)/(*S*)-3-hydroxy-3-methylhexanoic acid, which thus explains its important contribution to human malodor, even when present at a concentration up to a hundred times lower than the acid (see *Chapter 4, Appendix 7.1*). An additive effect of thiols has been reported in wine [14]; indeed, we could suppose that a similar effect is operative in sweat. It would be interesting to know how human axillary odor is modulated when sulfur volatiles are present in combination with other human odoriferous molecules such as fatty acids and steroids.

√ *Are volatile sulfur compounds and their precursors present in the axillae of other ethnies?* A number of workers have pointed out that substantial racial differences exist in axillary organ size, especially in apocrine glands and body odors (see *Chapter 1*). (*R*)/(*S*)-3-Methyl-3-sulfanylhexan-1-ol was identified in European male and female sweat and in Japanese axillary samples [15]. Sensory tests have shown that the "sulfur" odor induces negative emotions (unpleasantness) towards female sweat samples, suggesting the repulsive effect of (*R*)/(*S*)-MSH (see *Chapter 4*). It would be relevant to extend these studies using other ethnies and study their correlations with genetic characteristics such as Human Leukocyte Antigen (HLA) [16][17].

√ *How is body odor modulated when several bacteria are present simultaneously*?

Bacterial populations in the armpit are characterized by a diversity of species and strains, whose effect depends on individual parameters and compositions of sweat secretions. It is known that the diversity and composition of bacteria contributes to the sensory characteristics of sweat, because the growth of each bacterium is characterized by a specific metabolic activity. However, human skin may be viewed as a microbial ecosystem [18]. When a bacterial strain produces a compound, this can be taken up and used by other bacteria present. Bacterial interactions, gene exchanges and sharing of metabolites could also occur in

the axillary region, which explain the difficulties in reproducing human odors *in-vitro,* but those aspects have never been investigated.

√ *The pheromone effect of thiols.* Apocrine sweat has often been described as a sexual attractant. It has been shown that sulfur-containing volatile compounds such as 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-methyl-3-methylthio-1-butanol, and 3-methyl-3-(2-methyldisulfanyl)-1-butanol, can function as pheromones used for conspecific recognition, and for reproductive purposes in mature cats [19]. However the pheromonal effect of sulfur-containing volatiles in humans has never been studied and is a controversial subject (see *Chapter 1*).

Although of considerable interest to the cosmetic industry, the identification of metabolic mechanisms involved in the generation of VSCs in axillary malodor has proved difficult. A primary reason for this is a lack of knowledge of the enzymes involved. Although Cysteine-Glycine-(S)-conjugates (Cys-Gly-*S*-conjugates) are key intermediates in the glutathione biodetoxification pathway, neither glutathione-(*S*)-conjugate nor Cysteine-(S)-conjugates (Cys-*S*-conjugates) has been detected in sterile sweat samples by HPLC-MS measurements. In addition, incubation of glutathione-(*S*)-conjugate has never led to the formation of a thiol under the experimental conditions employed. During the study of the bio-generation of 3-methyl-3-sulfanylhexan-1-ol, it was shown that enzymatic extracts from *S. haemolyticus* were more active on synthetic Cys-Gly-*S*-conjugates than with synthetic Cys-*S*-conjugates, whereas it is the reverse after incubation with *C. jeikeium* bacterial strain (see *Chapter 3*, *Appendix 7.2*). The focus of this thesis was first to study the enzymes implicated in cysteine and methionine metabolism, such as cystathionine *β*-lyase (CBL; EC 4.4.1.8), a pyridoxal-5'-phosphate (PLP)-dependent Carbon-Sulfur lyase (C-S-lyase) that has been shown to play a central role in the generation of sulfur compounds (see *Chapter 1*, *Chapter 5*). *Staphylococci* CBL, whose function within the growing cell is primarily related to amino acid catabolism, appears unlikely to be involved in sulfur compound generation in sweat (see *Chapter 5*). Even if it were, the intensive thiol odor following the addition of *S. haemolyticus* to odorless secretions cannot be solely explained *by staphylococci metC*-CBL.

The production of sulfur compounds in wine has been shown to be multigenic; 4 genes with C-S lyase activities have been described in *S. cerevisiae* (see *Chapter 1*) [20]. Recently, a MalY-type C-*S* *β*-lyase from *C. jeikeium* and *C. Striatum* with similarities to a bacterial aminotransferase has been reported to be involved in the generation of thiols from a Cys-*S*-

conjugate [21][22]. Interestingly, *Corynebacterium* needs sequential action of a metallopeptidase and a cystathionine *β*-lyase to release the odorous thiol from the Cys-Gly-S-conjugate [23]. Thus, future research, involving gene knockout or the expression of *S. haemolyticus* MalY-type enzymes such as the two PLP aminotransferases, SH0221 and SH2169 with *β*-lyase activity, and which are not present in *S. epidermidis* and *S. aureus*, may confirm whether a single enzyme or a multiple enzyme mechanism is responsible for the release of sulfanylalkanols by *S. haemolyticus* in body odor (see *Appendix 7.4*).

Outlook. Various approaches have been studied to solve the problem of axillary malodor (*Fig. 1*, reviewed in [24]). One approach has been the use of antiperspirants that either inhibit perspiration or decrease bacterial growth by substrate or mineral-binding molecules [25]. A second approach has been the use of perfumes which mask the odor produced. In this respect, the discovery of thiols involved in axillary odors has helped the construction of synthetic body odors in cosmetic perfume performance evaluation [26][27]. The addition of specific scavengers of malodorant sulfur compounds, such as aldehydes, may also be a solution to this problem. Another approach is the use of antimicrobials, which are often enzyme inhibitors; here the reasoning is that, by decreasing the bacterial numbers present in the axilla, the biogenesis of odor can be also be lowered [28]. D-Amino acids and fragrance materials such as musk and sandalwood ingredients have been reported for their antimicrobial activities against aerobic diphteroids responsible for axillary odors [30][31]. Arylsulfatase, esterase or lipase inhibitors, lipooxygenase, aminoacylase and beta lyase inhibitors have also been reported [32][33] (reviewed in [34]). Competitive or non-competitive inhibitors may be synthesised to reduce sulfur compound generation. Similarly, specific *S. haemolyticus* C-S lyase enzyme substrates

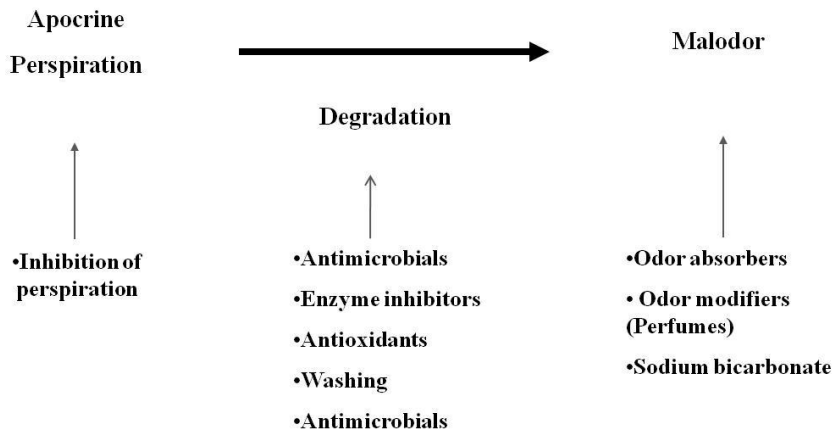


Fig.1: Possible approaches for the control of axillary malodor (modified from [29]).

may be cleaved to release fragrance molecules. In 1991, Lyon *et al.* already suspected *β*-lyase as responsible for the generation of body odors, and patented hydroxylamines and amino-acid derivatives (cysteine and serine derivatives) as inhibitors [32]. The inhibition of cysteine-(S)-conjugate *β*-lyase, a PLP-containing enzyme, was possible by irreversibly binding of the PLP prosthetic group *via* covalent bond formation, or by the generation of reactive intermediates (suicide inhibitors) which react irreversibly with amino-acids present in the active site of the enzyme [34]. We succeeded in synthesizing some inhibitors by using a model comprising the whole cells of *S. haemolyticus* and the synthetic cysteine-glycine-S-precursor *(Fig.2.)*; from those results, the cysteine-glycine-conjugate with R being a 2-propynyl group (generating a reactive intermediate) was the most effective inhibitor (70 % inhibition in the generation of the MSH, see *Appendix 7.5*). However, one of the difficulties of such inhibition may be to find molecules that are non-toxic with respect to the numerous and essential skin PLP-dependent enzymes.

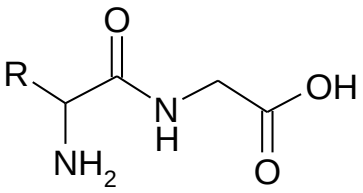


Fig.2. Competitive inhibitors of *S. haemolyticus* enzymes.

Other secondary approaches to reduce the formation of sulfur compounds in the axillary region include the addition of non-resident bacteria such as lactic acid bacteria to compete with sulfur-generating skin bacteria [35]. However, the survival of such bacteria in the armpit is often critical. The addition of antibodies directed against the transport protein of bacterial cells has been patented [36]. However, further experiments are needed to show if such an approach is effective in reducing VSCs. Once the mechanisms of the generation of sulfur compounds have been elucidated, the pharmacokinetic effects of perfumery raw materials and botanical extracts for the design of new bio-active deodorants can be investigated.

In conclusion, this thesis has broadened our understanding of the biochemistry of the axillary region. We have used microbiological, chemical and sensorial assays, together with a collection of sterile sweat from 49 human volunteers over a period of three years. The elucidation of the mechanisms involved in the generation of odoriferous sulfanylalcohols may lead to different strategies for body malodor control. However, the efficiency, toxicity, and overall cost will remain important factors in the development of commercially useful systems for the counteraction of malodor.

Bibliography

[1] P. Darriet, T. Tominaga, E. Demole, D. Dubourdieux, *C.R. Acad. Sci.* Paris, Bio. Pathol. Végétale **1993**, *316*, 1332.

[2] T. Tominaga, C. Peyrot des Gachons, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 5215.

[3] S. Widder, C. Sabater Lüntzel, T. Dittner, W. Pickenhagen, *J. Agric. Food Chem.* **2000**, *48*, 418.

[4] Y. Hasegawa, M. Yabuki, M. Matsukane, *Chem. Biodiv.* **2004**, *1*, 2042.

[5] M. Troccaz, C. Starkenmann, Y. Niclass, M. Van de Waal, A. .J. Clark, *Chem. Biodiv.* **2004**, 1, 1022.

[6] A. Natsch, J. Schmid, F. Flachsmann, *Chem. Biodiversity.* **2004**, *1*, 1058.

[7] C. Starkenmann, Y. Niclass, M. Troccaz, A.. J. Clark, *Chem. Biodiv.* **2005**, *2*, 705.

[8] R. Emter, A. Natsch, *J. Biol. Chem.* **2008**, *283*, 20645.

[9] F. Kuhn, A. Natsch, *J. R. Soc. Interface*. **2008**. doi:10.1098/rsif.

[10] S. U. Savelev, S. Antony-Babu, S. C. Roberts, H. Wang, A. S. Clare, L. M. Gosling, M. Petrie, M. Goodfellow, A. G. O'Donnell, A. C. Ward, *J. Chem. Ecol.* **2008**, *34*, 1253.

[11] T. Tominaga, M. L. Murat, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 1044.

[12] P. Bouchilloux, P. Darriet, R. Henry, V. Lavigne-Cruege, D. Dubourdieu, *J. Agric. Food Chem.* **1998**, *46*, 3095.

[13] M. L. Murat, T. Tominaga, D. Dubourdieu, *J. Agric. Food Chem.* **2001**, *49*, 5412.

[14] E. Sarrazin, S. Shinkaruk, T. Tominaga, B. Benneteau, E. Frérot, D. Dubourdieu, *J. Agri. Food. Chem.* **2007**, *55*, 1437.

[15] M. Yabuki, T. Hagura, K. Takeuchi, Y. Hasegawa. 'Analyses of Human Axillary Odors and Their Precursors in Normal and Stressful Situations'. *AChemS Abstracts* **2006**, *149*. 422-424.

[16] C. Wedekind, S. Furi, *Proc. Biol. Sci.* **1997**, *264*, 1471.

[17] C. Wedekind, T. Seebeck, F. Bettens, A. J. Paepke, *Proceedings of the Royal Society B: Biological Sciences.* **1995**, *260*, 245.

[18] D. J. Bibel, R. J. Smiljanic, D. J. Lovell, *Applied Env. Microbiol.* **1978**, *35*, 1136.

[19] M. Miyazaki, T. Yamashita, Y. Suzuki, Y. Saito, S. Soeta, H. Taira, A. Suzuki, *Chem. Biol.* **2006**, *13*, 1071.

[20] K. S. Howell, M. Klein, J. H. Swiegers, Y. Hayasaka, G. M. Elsey, G. H. Fleet, P. B. Hoj, I. S. Pretorius, M. A. de Barros Lopes, *Appl. Environ. Microbiol.* **2005**, *71*, 5420.

[21] A. Natsch, J. Schmid, F. Flachsmann, *Chem. Biodiv.* **2004**, *1*, 1058.

[22] A. G. James, C. Austin, D. Hyliands, D. Cox, D. Taylor, S. Grimshaw, R. Calvert, Cosm'ing conference, Saint Malo, **2007**.

[23] R. Emter, A. Natsch, *J. Biol. Chem.* **2008**, *283*, 20645.

[24] S. A. Makin, M. R. Lowry. 'Deodorant Ingredients' in 'Antiperspirants and deodorants', Ed. Marcel Dekker Ltd, K. Laden, Cosmetic science and technology series, New-York, 1999, 20 (6), p. 169.

[25] A. Landa., S. Makin, 22nd IFSCC Congress, Edinburg, **2002**.

[26] A. Finke, C. Sabater-Luntzel, S. Widder, H. J. Bertram, to *Symrise GMBH* CO, KG, Holzminden (DE), Int. Pat. WO 2006/3053.

[27] M. Yabuki, Y. Hasegawa, to *Kao Corp*, JP 2005-062159.

[28] E. Eigen, C. Froebe, to *Colgate-Palmolive Company, New-York, N.Y*, US 005676937A.

[29] P. M. Baxter, J. V. Reed, *Int. J. Cos. Science*. **1983**, *5*, 85.

[30] P. J. Hayden, V. S. Goldman, to *Gillette Company, Boston, MA*, US 6060043.

[31] K. Sawano, T. Sato, R. Hattori, *J. Soc. Cosmet. Chem. Jpn.* **1993**, 27, 227.

[32] S. Lyon, C. O'Neal, H. Van Der Lee, B. Rogers, to *Gillette Company, Boston, US* Int. Pat. WO 91/05541.

[33] A, Natsch, H, Gfeller, P, Gygax, J. Schmid, *Int. J. Cos. Sc.* **2005**, *27*, 115.

[34] A. C. Eliot, J. F. Kirsch, *Annu. Rev. Biochem.* **2004**, *73*, 383.

[35] C. Lang, Cosm'ing conference, Saint Malo, **2007.**

[36] B. Rogers, A. Maczulak, to *Gillette Company*, US 5069896.

7. APPENDIX

7.1. Determination of Odor Detection Thresholds of major sweat volatiles

a) *Methods*

Dose-response curves and Odor Detection Thresholds (ODT) of the major sweat volatiles ((*Z/E*)-3M2HA, (*R/S*)-HMHA, isovalerianic acid, (*R/S*)-MSH) were determined by the Olfit® method (*Fig. A1*, *Table A1*) [1]. The dose-response curve corresponds to the plot of the perceived intensity (response) versus the gas phase concentration (dose) in the air-dilution olfactometers. The ODT is the lowest detectable odor level expressed in liquid or gaseous concentration units. It reflects the individual olfactive impact of a chemical.

Olfactive evaluations were performed by 30 non-trained evaluators (15 men and 15 women between 17 and 60 years of age). Statistical calculations were carried out by Fizz (Fizz for Windows 2.10 A; provided by Biosystemes). Dose-response curves were determined for each chemical at concentrations ranging from the volatility down to 10^{-6} µg/l air. Volatility is defined as the gas phase concentration at 22°C/730 mmHg (± 30) expressed by the concentration of the chemical in the air above the pure liquid or solid at equilibrium. Evaluators were asked to rate odor intensity on a 0-10 scale (0: no odor; 10: very intense odor) via Tablets-PC system (Fujitsu Siemens Computer, TabletPC Stylistic ST5020D).

ODT determinations were carried out using six discriminative tests with different concentrations calculated from the dose-response curves (corresponding to low intensities: 1.25, 0.8, 0.35, which surround the threshold value).

b) *Results*

The Dose-response curve and the ODT of the three acid volatiles, (*Z/E*)-3M2HA, (*R/S*)-HMHA and isovalerianic acid were very similar (see *Table A1*, *Fig. A1,a*). The ODT were $1.37x10^{-4}$ ug/l air, $1.80x10^{-4}$ ug/l air and $2.23x10^{-4}$ ug/l air for the (Z/E)-3M2HA, HMHA and isovalerianic acid, respectively. No differences were seen between measured ODT for male and female evaluators (*Table A1*).

The sulfur-containing volatile, (*R*)/(*S*)-MSH was perceived at a concentration a hundred times lower than (*R*)/(*S*)-HMHA, which may explain its important contribution in human malodor (ODT of 2.27 x 10^{-6} µg/L air and 1.80 x 10^{-4} µg/L air for the two molecules

respectively, with the same sensitivity for males and females) (see *Fig. A1,a*). The average perceived intensity in the dose response curves was always higher for the racemic molecules in comparison to their individual enantiomers (*Fig. A1,b,c*). Therefore, the ODT was found to be lower for (*Z/E*)-3M2HA, (*R/S*)-HMHA and (*R/S*)-MSH in comparison to their respective enantiomers (see *Table A1*).

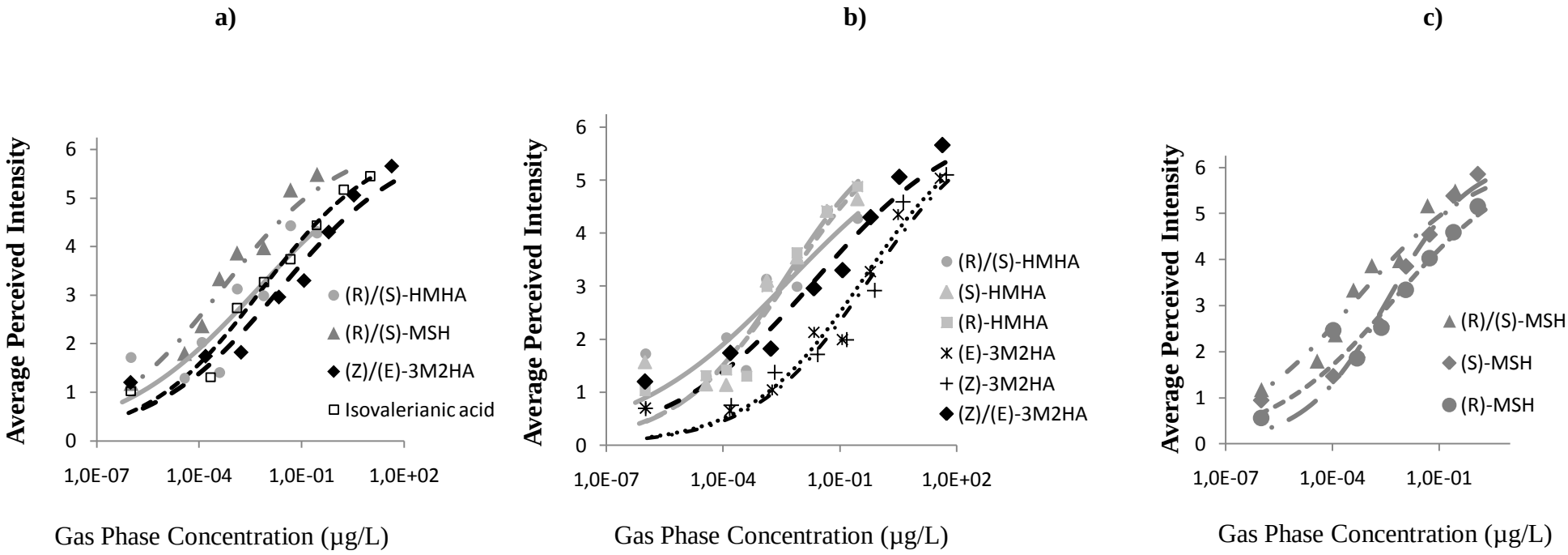


Fig. A1. Dose-response curves of major sweat volatiles determined by the Olfit® method with 30 evaluators. a) *(R)/(S)*-3-methyl-3-sulfanylhexan-1-ol (MSH), isovalerianic acid, (*Z*)/(*E*)-3-methyl-2-hexenoic acid (3M2HA) and its hydrated form *(R)/(S)*-3-hydroxy-3-methylhexanoic acid (HMHA). b) Volatile fatty acids: (*Z*)/(*E*)-3M2HA, *(R)/(S)*-HMHA and their respective enantiomers. c) Volatile sulfur compounds: *(R)/(S)*-3-methyl-3-sulfanylhexan-1-ol (MSH) and its respective enantiomers.

Table A1. Odor Detection Threshold (ODT) of the major sweat volatiles: volatile fatty acids (isovalerianic acid, (*Z*)/(*E*)-3M2HA and its hydrated form *(R)/(S)*-HMHA) and volatile sulfur compound (*(R)/(S*)-MSH). Olfactive evaluations were performed by 30 non-trained panelists (15 females and 15 males). Odor Detection Thresholds were determined for females and males together and separately.

Sweat volatiles	**Volatility (µg/L air)**	**Odor Detection Threshold (µg/L air)**			**Descriptors**
		Group (men and women)	**In men (N=15)**	**In women (N=15)**	
(*Z/E*)-3-methyl-2-hexenoic acid	40	1.37^{-04}	2.59^{-04}	1.44^{-04}	acidic, rancid, cheese, perspiration
(*Z*)-3-methyl-2-hexenoic acid	53	7.80^{-04}	6.79^{-04}	7.53^{-04}	
(*E*)-3-methyl-2-hexenoic acid	37	7.41^{-04}	7.74^{-04}	6.80^{-04}	
(*R/S*)-3-hydroxy-3-methylhexanoic acid	0.29	1.80^{-04}	1.49^{-04}	2.00^{-04}	perspiration, animal, fatty, dirty, sewer
(*S*)-3-hydroxy-3-methylhexanoic acid	*	7.70^{-05}	4.95^{-05}	7.09^{-05}	pungent, dirty, yeast-like
(*R*)-3-hydroxy-3-methylhexanoic acid	*	1.98^{-04}	9.39^{-05}	3.12^{-04}	weak, fatty, fermented fruit-like
isovalerianic acid	1531	2.23^{-04}	2.90^{-04}	1.32^{-04}	
(*R/S*)-3-methyl-3-sulfanylhexan-1-ol	133	2.27^{-06}	2.31^{-06}	1.53^{-06}	perspiration, sulfur, onion, grapefruit
(*R*)-3-methyl-3-sulfanylhexan-1-ol	*	1.21^{-05}	7.77^{-06}	1.37^{-05}	sulfury, burnt, grapefruit
(*S*)-3-methyl-3-sulfanylhexan-1-ol	*	3.00^{-06}	3.00^{-06}	2.43^{-06}	sulfury, perspiration, onion

* Quantities too limited to determine volatilities.

7.2. Biotransformation of cysteinyl-glycine *S*-conjugate by *S. haemolyticus*

a) *Methods*

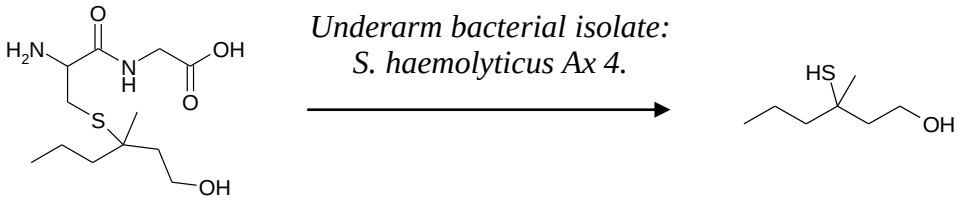
General. Corynebacterium xerosis DSMZ 20743 (corresponding to the ATCC 373) was provided by the DSMZ (*Deutsche Sammlung von Mikroorganisen und Zellkulturen GmbH, Braunschweig, Germany)*. *Staphylococcus aureus* ATCC 6538, *Corynebacterium xerosis* ATCC 373 was provided by the ATCC (*American Type Culture Collection*). *Corynebacterium jeikeium* K411 was obtained from Unilever Research (Portsunlight, England). Other bacterial strains *Bacillus licheniformis* Ax5, *Corynebacterium tuberculostearicum* Ax1, *Staphylococcus epidermidis* Ax 3 and *Staphylococcus haemolyticu*s AX4 are European underarm isolates (see *Chapter 2*). *Staphylococcus haemolyticus* 114126, *Staphylococcus haemolyticus* 121875, *Staphylococcus haemolyticus* 124655 were provided by the Geneva University Hospital (Laboratory of Bacteriology, Geneva, Switzerland) from human skin isolates. The identification of Cys-Gly-*S*-conjugate **1** (S-[1-(2-hydroxyethyl)-1-methylbutyl]-(L)-cysteinylglycine) (*Scheme A1*), Cys-*S*-conjugate (S-[1-(2-hydroxyethyl)-1-methylbutyl]-(L)-cysteine) and (R)/(S)-MSH **2** were effected using a HPLC/MS and a GC SPB-1 column coupled to an AED detector as described previously (see *Chapter 3*).

Bacterial growth conditions and fermentation process. 500 µl of an overnight bacterial solution were grown in 50 ml Brain Heart Infusion liquid broth containing 0.5% Tween 80 at 37°C, 250 rpm until the appropriate optical density. The bacterial solution was twice washed in 20 mM phosphate buffer pH 7.5 (buffer **A**) and re-suspended in buffer **A**. 1 ml of bacterial solution was added to 200 µl (0.05 mg) of **1** and 1 ml of buffer **A**. The mixture was incubated overnight at 37°C, 150 rpm and the yield of the reaction was determined. To evaluate the influence of bacterial interactions on the transformation of **1,** two bacterial solutions at equal concentrations were added to **1** (1 ml of the second bacterial solution was added instead of buffer **A**).

Yield calculation. The influence of different parameters such as time (0-18 hours), pH (4-8), temperature (30-45°), substrate concentration (0.01-0.05 mg/ml), bacterial quantity (10^4-10^{10}

cfu/ml) and bacterial growth state (OD between 0 and 8) on the biotransformation of **1** by *Staphylococcus haemolyticus* was evaluated. The bioconversion yield is defined as the amount of product, *e.g.* (R/S)-MSH **2**, obtained by enzymatic conversion of the precursor **1**. It is given as a relative yield (in percentage), which is the actual yield divided by the theoretical yield (the ideal, or mathematically calculated, yield). The theoretical yield value always relates to the substrate **1** taking into account the molar ratio of the substrate and the stoichiometry of the reaction (*Scheme A1*). The formation of **2** was monitored by GC/MS as defined previously (see *Chapter 2 and 3*).

Scheme A1



1 **2**

Precursor **1:** *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(L)-cysteinylglycine, MW 292.40 g/mol, V*olatile sulfur compound* **2**: (R)/(S)-3-methyl-3-sulfanylhexan-1-ol, MW: 148.1 g/mol).

b) *Results*

The biotransformation of **1** by *S. haemolyticus* is influenced by several factors, such as the precursor concentration, the incubation time, temperature, pH, bacterial number, bacterial state, and the other bacteria present in the underarm ecosystem.

We observed that, under the conditions described, the optimal activity (yield of 75 ± 8 %) was obtained with 0.05 mg/ml of precursor, 45°, pH ≈ 7, 10^9cfu/ml of bacteria (grown until the end of the exponential phase) after 30 min incubation *(Fig.* A2). During this conversion, only small amounts of Cys-*S*-conjugate was generated and was slowly converted into (*R/S*)-MSH **2**

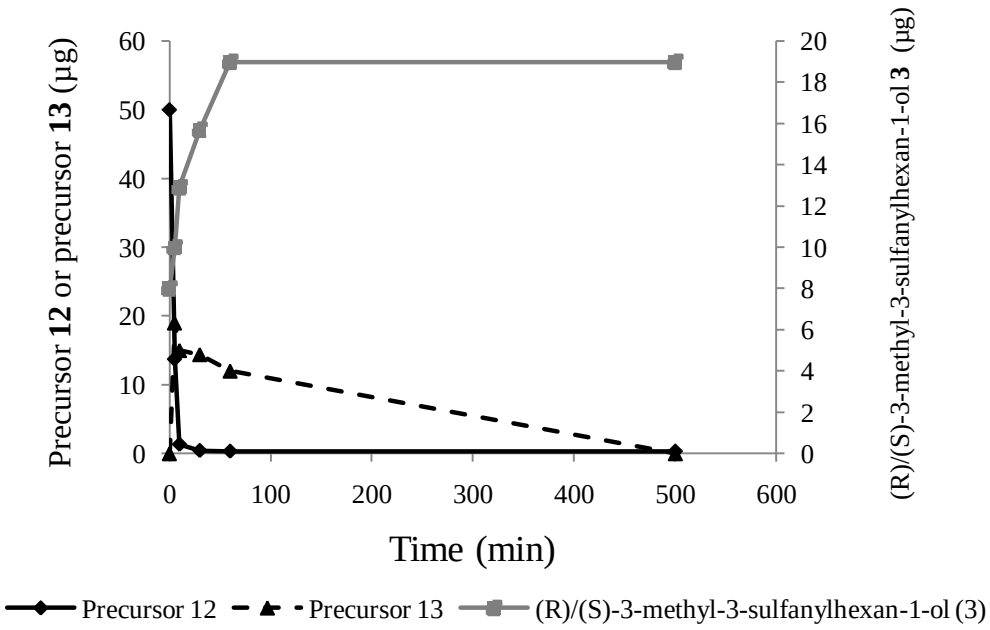


Fig. A2. Conversion of Cys-Gly-*S*-precursor **1** into *(*R/S*)*-MSH **2** by *S. haemolyticus* Ax4.

S. haemolyticus and *B. licheniformis* were the most efficient bacteria for the generation of volatile thiols from precursor **1**, with a yield of 75 ± 8 % and 72 ± 5 %, respectively (*Table A2*). All of the studied *S. haemolyticus* strains had similar activities. The isolated strain of *C. jeikeium* had no or little influence on the biotransformation of **1** (Yield: 2 ± 4 %). Interactions among skin bacteria have been reported *in vitro* and *in vivo* [2]. In this experiment, we first examine of the influence of *S. epidermidis*, *B. licheniformis* and *C. jeikeium* on the formation of **2** by *S. haemolyticus*. The addition of these strains did not significantly affect the activity of *S. haemolyticus* (*Table A2*). A minor additive effect was seen when *S. aureus* was added to *C. jeikeium*.

Table A2. Bioconversion yield of **1** into **2** by underarm bacteria alone or in combination. Reaction conditions: bacterial OD_{600} = 4, temperature: 37°C, timing speed: 250 rpm, reaction time: 18 hours, substrate concentration: 0.05mg, reaction volume: 2 ml.

Bacterial solution	Yield [%] (mean ± SEM)
Corynebacterium xerosis ATCC 373	20.0 ± 10.0
Bacillus licheniformis Ax5	72.0 ± 5.0
Corynebacterium tuberculostearicum Ax1	2.0 ± 8.0
Staphylococcus epidermidis Ax 3	17.0 ± 11.0
Staphylococcus aureus ATCC 6538	0.2 ± 0.9
Corynebacterium jeikeium K411	2.0 ± 4.0
Staphylococcus haemolyticus AX4	75.0 ± 8.0
Staphylococcus haemolyticus 114126	75.0 ± 6.0
Staphylococcus haemolyticus 121875	70.0 ± 5.0
Staphylococcus haemolyticus 124655	68.0 ± 6.0
C. *xerosis* ATCC 373 / *S. epidermidis* Ax 3 (50/50)	41.0 ± 6.0
S. haemolyticus Ax 4 / *B. licheniformis* Ax 3(50/50)	74.0 ± 6.0
S. haemolyticus Ax 4 / *S. epidermidis* Ax 3 (50/50)	75.0 ± 8.0
S. haemolyticus Ax 4 / *C. jeikeium* K411 *(50/50)*	80.0 ± 5.0
C. jeikeium K411 / *S. aureus* ATCC 6538	4.4 ± 3.0

7.3. Free and total amino-acids composition of male and female underarm secretions

a) *Methods*

Collection of odorless axillary secretions (see *Chapter 4*).

Sweat amino-acid analysis. Free and total amino-acids from sweat secretions were analyzed by UV-HPLC by *UFAG Laboratory* (Switzerland). The detection limit was 0.08 g/l of sweat secretions (see *Chapter 5*).

Protein quantification. Protein concentrations were estimated by the micromethod of Bradford with the *Coomassie* protein assay reagent (Bio-Rad) and with the Bovine albumin 5% (Difco) as the standard [3] (see *Chapter 5*).

b) *Results*

Free and total amino-acid analyses of male and female underarm secretions (heat-induced) gave similar profiles (see *Table A3*). The average protein content (mean ± SEM) was 0.22 ± 0.06 g/l for males, and 0.16 ± 0.03 g/l for females. A high concentration of serine (0.3 g/l) was found (see *Table A3*), as previously reported by Farrior and Kloos in 1976 [4]. Glutamic acid is present in both male and female sweat at a concentration of 0.4 g/l as proteins, whereas glycine and histidine occur only in male sweat as free amino-acids, but at lower concentrations.

Table A3. Free and total amino-acid (a.a) profile of male and female underarm secretions (heat-induced) (detection limit: 0.08 g/l).

	Male sweal secretions		Female sweat secretions	
	Free a,a (g/L)	Total a,a (g/L)	Free a,a (g/L)	Total a,a (g/L)
alanine	-	0.1	-	0.1
ammonium (NH4)	0.1	0.7	-	0.4
arginine	-	0.1	-	-
aspartic acid	-	0.1	-	0.1
cystine	-	-	-	-
cysteine	-	-	-	-
glutamic acid	-	0.4	-	0.4
glycine	0.1	0.2	-	0.1
histidine	0.1	0.1	-	-
Isoleucine	-	0	-	-
leucine	-	0.1	-	-
lysine	-	0.1	-	-
methionine	-	0	-	-
phenylalanine	-	0	-	-
proline	-	0	-	-
serine	0.3	0.3	0.2	0.2
threonine	-	0.1	-	-
tyrosine	-	0	-	-
valine	-	0.1	-	-

7.4. Carbon-Sulfur β-lyase (C-S *β-lyase)* similarities in axillary bacteria

a) *PLP dependent aminotransferase superfamily*

Enzymes implicated in cysteine and methionine metabolism are members of the PLP dependent aminotransferase superfamily (clan: *PLP_aminotran* CL0061)[4]. Apart from Cys/Met metabolism enzymes, this family comprises other enzymes such as transaminases, aminotransferases, pyridoxal-dependent decarboxylases, and β-eliminating lyases (tryptophanase, tyrosine phenol-lyase and threonine aldolase). Tryptophanase from *E. coli* and β-lyase from *C. striatum* have been shown to have carbon-sulfur lyase activities against a variety of cysteine-S-conjugates [5-7]. An amino-acid homology search using public protein databases against the genome of *S. haemolyticus* JCSC1435 resulted in 18 genes having aminotransferases, transaminases or beta-eliminating patterns (see *Table A4*). Only one enzyme, SH0221, was not found in *S. epidermidis* and four of them (SH1201, SH1172, SH1209, SH2169*)* have small similarities to *S. epidermidis* or *S. aureus* enzymes (E-value > 0; see *Table A4*). The Expectation value (E-value) represents the number of times the protein database match may have occurred just by chance. The cut-off value was set to 1.10^{-4}. In table A4, a domain is defined as a structural protein unit that consists of at least 50 amino-acids. The unit can fold on its own and have its own function; as well as being able to fold into two more or less independent sections. A motif represents multiple overlapping amino-acids that are grouped together based on evolutionary, biochemical or physical properties.

[4] http://pfam.sanger.ac.uk/clan?id=PLP_aminotran

Protein Name*	Gene	Enzyme classification	Molecular mass (kDa)	Motifs / Domains**	Similar sequence	
SH1018		hypothetical protein, similar to aspartate transaminase	48.3	Aminotransferase class I and II	E=0.0	ref|NP_765182.1| aspartate transaminase protein [S. epidermidis]
					E=0.0	ref|NP_372460.1| hypothetical protein [S. aureus]
					E=0.0	ref|NP_646695.1| ORFID:MW1878~hypothetical protein, similar to aspartatetransaminase protein [S. aureus]
					E=1.0E-107	gb|AAL10665.1| aspartate transaminase-like protein [Bacillus subtilis]
					E=1.0E-104	ref|ZP_00082377.1| hypothetical protein [Geobacter metallireducens]
SH0221		hypothetical protein, similar to aspartate aminotransferase	41.0	Aminotransferase, class I and II	E=5.0E-63	ref|NP_810311.1| putative aminotransferase B [Bacteroides thetaiotaomicron]
					E=2.0E-60	gb|EAA24745.1| Aspartate aminotransferase [Fusobacterium nucleatum]
					E=4.0E-60	ref|NP_603522.1| Aspartate aminotransferase [Fusobacterium nucleatum]
					E=1.0E-59	ref|NP_391022.1| aminotransferase [Bacillus subtilis]
					E=9.0E-59	emb|CAD30137.1| putative class-II aminotransferase [Bacillus thuringiensis serovarisraelensis]
SH0459	argD	hypothetical protein, similar to pyridoxal-phosphate dependent aminotransferase	48.9	Aminotransferase class-III Aminotransferase class I and II	E=0.0	ref|NP_373128.1| hypothetical protein [S. aureus]
					E=0.0	ref|NP_647340.1| hypothetical protein, similar to PLP dependent aminotransferase [S. aureus]
					E=0.0	ref|NP_765703.1| hypothetical 4-aminobutyrate aminotransferase [S. epidermidis]

					E=1.0E-123 ref|ZP_00070586.1| hypothetical protein [Oenococcus oeni] E=1.0E-118 ref|NP_785297.1| aminotransferase [Lactobacillus plantarum]
SH0492		hypothetical protein, similar to aspartate aminotransferase	43.0	Aminotransferase, class I and II Aminotransferases class-I pyridoxal-phosphate-binding site Allinase Beta-eliminating lyase	E=0.0 ref|NP_765677.1| N-succinyldiaminopimelate aminotransferase [S. epidermidis] E=0.0 ref|NP_373084.1| hypothetical protein [S. aureus] E=0.0 ref|NP_647298.1| hypothetical protein, similar to aspartateaminotransferase [S. aureus] E=8.0E-82 ref|NP_463817.1| similar to aminotransferase [Listeria monocytogenes] E=9.0E-81 ref|NP_469659.1| similar to aminotransferase [Listeria innocua]
SH0654	narG	respiratory nitrate reductase alpha chain	139.7	Nitrate reductase, alpha subunit Molybdopterin oxidoreductase Molydopterin dinucleotide binding domain Prokaryotic molybdopterin oxidoreductase Aminotransferase, class-II	E=0.0 ref|NP_765530.1| respiratory nitrate reductase alpha chain [S. epidermidis] E=0.0 ref|NP_372921.1| respiratory nitrate reductase alpha chain [S. aureus] E=0.0 ref|NP_647136.1| respiratory nitrate reductase alpha chain [S. aureus] E=0.0 ref|NP_785105.1| nitrate reductase, alpha chain [Lactobacillus plantarum] E=0.0 gb|AAC82542.1| NarG [S. carnosus]
SH0880	glmS	glucosamine-fructose-6-phosphate aminotransferase	65.9		E=0.0 ref|NP_372678.1| glucosamine-fructose-6-phosphate aminotransferase [S. aureus] E=0.0 ref|NP_765306.1| glucosamine-fructose-6-phosphate aminotransferase [S. epidermidis] E=0.0 ref|NP_646897.1| glucosamine-fructose-6-phosphate aminotransferase [S. aureus] E=0.0 ref|NP_830058.1| Glucosamine--fructose-

					E=0.0	6-phosphate aminotransferase [isomerizing][Bacillus cereus] ref|NP_654100.1| GATase_2, Glutamine amidotransferases class-II [Bacillus anthracis]
SH1097	gsaB	glutamate-1-semialdehyde aminotransferase	43.0	Aminotransferase class-III pyridoxal-phosphate attachment site Aminotransferase class-III Aminotransferase class I and II	E=0.0 E=0.0 E=0.0 E=1.0E-166 E=1.0E-166	ref|NP_765103.1| glutamate-1-semialdehyde aminotransferase [S. epidermidis] ref|NP_372388.1| glutamate-1-semialdehyde aminotransferase [S. aureus] ref|NP_646621.1| glutamate-1-semialdehyde aminotransferase [S. aureus] ref|NP_388751.1| glutamate-1-semialdehyde aminotransferase [Bacillus subtilis] sp|P71084|GSAB_BACSU Glutamate-1-semialdehyde aminotransferase
SH1172	dat	D-amino acid aminotransferase	31.8	D-amino acid aminotransferase Aminotransferase, class IV	E=1.0E-159 E=1.0E-133 E=1.0E-130 E=1.0E-130 E=1.0E-130	sp|P54694|DAAA_STAHA D-alanine aminotransferase (D-aspartate aminotransferase) (D-aminoacid aminotransferase) (D-amino acid transaminase)(DAAT) ref|NP_764978.1| D-alanine aminotransferase [S. epidermidis] emb|CAB82475.1| D-alanine aminotransferase [S. aureus] ref|NP_646510.1| D-alanine aminotransferase [S. aureus] ref|NP_372274.1| D-alanine aminotransferase [S. aureus]
SH1201		hypothetical protein, similar to transaminase	43.1	Aminotransferase, class V pyridoxal-phosphate attachment site	E=1.0E-155	ref|NP_646482.1| ORFID:MW1665~hypothetical protein,

				Aminotransferase, class V		similar to soluble hydrogenase42 kD subunit [S. aureus]
					E=1.0E-154	ref|NP_764955.1| conserved hypothetical protein [S. epidermidis]
					E=1.0E-153	ref|NP_372247.1| hypothetical protein [S. aureus]
					E=2.0E-78	ref|NP_693546.1| transaminase [Oceanobacillus iheyensis HTE831]
					E=3.0E-69	ref|ZP_00072921.1| hypothetical protein [Trichodesmium erythraeum IMS101]
SH1209		hypothetical protein, similar to L-cysteine sulfurtransferase	42.1	Aminotransferase, class V Aminotransferase class I and II BTG family Pyridoxal-dependent decarboxylase DegT/DnrJ/EryC1/StrS aminotransferase Beta-eliminating lyase	E=1.0E-144	ref|NP_764947.1| iron-sulfur cofactor synthesis protein nifZ [St epidermidis]
					E=1.0E-135	ref|NP_372240.1| hypothetical protein [S. aureus]
					E=1.0E-135	ref|NP_646476.1| ORFID:MW1659~hypothetical protein, similar to iron-sulfur cofactorsynthesis protein nifZ [S. aureus]
					E=6.0E-80	ref|NP_244070.1| L-cysteine sulfurtransferase (iron-sulfur cofactor synthesis) [Bacillus halodurans]
					E=3.0E-76	ref|NP_658680.1| aminotran_5, Aminotransferase class-V [Bacillus anthracis A2012]
SH1260	hemL	glutamate-1-semialdehyde 2,1-aminomutase	46.3	Aminotransferases class-III pyridoxal-phosphate attachment site Glutamate-1-semialdehyde-2,1-aminomutase Aminotransferase class-III Aminotransferase class I and II Beta-eliminating lyase	E=0.0	ref|NP_764897.1| glutamate-1-semialdehyde 2,1-aminomutase [S. epidermidis]
					E=0.0	ref|NP_372191.1| glutamate-1-semialdehyde 2,1-aminomutase [S. aureus]
					E=0.0	sp|O34092|GSA_STAAU Glutamate-1-semialdehyde 2,1-aminomutase (GSA)(Glutamate-1-semialdehyde aminotransferase) (GSA-AT)
					E=0.0	ref|NP_646428.1| glutamate-1-semialdehyde 2,1-aminomutase [S. aureus]

					E=0.0	gb|AAC45836.1| GSA-1-aminotransferase [S. aureus]
SH1297		hypothetical protein, similar to aminotransferase class-V	42.4	Aminotransferase, class V DegT/DnrJ/EryC1/StrS aminotransferase Aminotransferase class I and II Beta-eliminating lyase Pyridoxal-dependent decarboxylase	E=0.0 E=1.0E-173 E=1.0E-173 E=3.0E-98 E=4.0E-98	ref|NP_764860.1| iron-sulfur cofactor synthesis protein-like protein [S. epidermidis] ref|NP_646389.1| ORFID:MW1572~iron-sulfur cofactor synthesis protein homolog [S. aureus] ref|NP_372146.1| iron-sulfur cofactor synthesis protein homolog [S. aureus] ref|NP_658426.1| aminotran_5, Aminotransferase class-V [Bacillus anthracis A2012] ref|NP_846844.1| aminotransferase, class V [Bacillus anthracis str. Ames]
SH1916	hisC	HisC romatic amino acid aminotransferase HisC homolog	44.0	Aminotransferases class-I pyridoxal-phosphate attachment site Aminotransferase, class I and II Aminotransferase class-V DegT/DnrJ/EryC1/StrS aminotransferase e Cys/Met metabolism PLP-dependent enzyme SUA5 domain	E=0.0 E=1.0E-177 E=1.0E-100 E=8.0E-99 E=6.0E-98	ref|NP_764302.1| HisC protein [Staphylococcus epidermidis] ref|NP_371573.1| HisC homolog [S. aureus] ref|NP_833728.1| Aromatic amino acid aminotransferase [Bacillus cereus] ref|NP_658046.1| aminotran_1_2, Aminotransferase class I and II [Bacillus anthracis] ref|NP_347015.1| PLP-dependent aminotransferase (gene patA) [Clostridiumacetobutylicum]
SH1993	rocD	ornithine aminotransferase	43.4	Aminotransferases class-III pyridoxal-phosphate attachment site Aminotransferase class-III Aminotransferase class I and II Beta-eliminating lyase DegT/DnrJ/EryC1/StrS	E=0.0 E=0.0 E=1.0E-164 E=1.0E-156	ref|NP_371481.1| ornithine aminotransferase [S.aureus] ref|NP_764208.1| ornithine aminotransferase [S. epidermidis] ref|NP_391914.1| ornithine aminotransferase [Bacillus subtilis] ref|NP_244811.1| ornithine

				aminotransferase Aminotransferase class-V Cys/Met metabolism PLP-dependent enzy		aminotransferase [Bacillus halodurans] ref|NP_693208.1| ornithine aminotransferase [Oceanobacillus iheyensis]
SH2037		aminotransferase NifS homologue	45.9	Aminotransferase, class V Beta-eliminating lyase Cys/Met metabolism PLP-dependent enzy DegT/DnrJ/EryC1/StrS aminotransferase Aminotransferase class I and II Pyridoxal-dependent decarboxylase con Peptidase M15	E=0.0 E=0.0 E=0.0 E=1.0E-163 E=1.0E-159	ref|NP_371368.1| aminotransferase NifS homologue [S. aureus] ref|NP_764163.1| aminotransferase NifS-like protein [S. epidermidis] ref|NP_645614.1| aminotransferase NifS homologue [S. aureus] sp|Q9K7A0|CSD_BACHD Probable cysteine desulfurase ref|NP_834652.1| Cysteine desulfhydrase [Bacillus cereus ATCC 14579]
SH2169		hypothetical protein, similar to histidinol phosphate aminotransferase	39.6	Aminotransferases class-II pyridoxal-phosphate attachment site Amidohydrolase Aminotransferase, class I and II Aminotransferase, class-V Beta-eliminating lyase	E=1.0E-167 E=1.0E-166 E=1.0E-160 E=4.0E-86 E=4.0E-84	ref|NP_371248.1| hypothetical protein [S. aureus] ref|NP_645503.1| hypothetical protein, similar to histidinol-phosphateaminotransferase [S. aureus] ref|NP_764059.1| putative histidinol-phosphate aminotransferase [St epidermidis] ref|NP_832687.1| Histidinol-phosphate aminotransferase [Bacillus cereus] ref|NP_656824.1| aminotran_1_2, Aminotransferase class I and II [Bacillus anthracis]
SH2449	ilvE	branched-chain amino acid aminotransferase/4-	40.2	Branched-chain amino acid aminotransferase II	E=0.0	ref|NP_763873.1| Branched-chain amino acid aminotroansferase-like protein[S. epidermidis]

		amino-4-deoxychorismate lyase		Aminotransferase, class IV	E=1.0E-179 E=1.0E-173 E=1.0E-119 E=1.0E-113	ref|NP_371078.1| branched-chain amino acid aminotroansferase homologue [S. aureus] emb|CAC12788.1| branched-chain amino acid aminotransferase [S.carnosus] ref|NP_388121.1| similar to branched-chain amino acid aminotransferase [Bacillus subtilis] ref|NP_391734.1| alternate gene name: ipa-0r~similar to branched-chain amino acidaminotransferase [Bacillus subtilis]
SH2457		2-amino-3-ketobutyrate CoA ligase homolog (glycine acetyl transferase)	43.0	Aminotransferase, class I and II Cys/Met metabolism PLP-dependent enzyme Aminotransferase class-III Serine hydroxymethyltransferase Aminotransferase class-V DegT/DnrJ/EryC1/StrS aminotransferase	E=0.0 E=0.0 E=1.0E-175 E=1.0E-168 E=1.0E-168	ref|NP_645322.1| hypothetical protein, similar to glycineC-acetyltransferase [S. aureus] ref|NP_371074.1| hypothetical protein [S. aureus] ref|NP_693976.1| glycine C-acetyltransferase [Oceanobacillus iheyensis] ref|NP_830437.1| 2-amino-3-ketobutyrate coenzyme A ligase [Bacillus cereus] ref|NP_654563.1| aminotran_1_2, Aminotransferase class I and II [Bacillus anthracis]

Table A4: Genes from *S. haemolyticus* JCSC1435 with aminotransferases, transaminases or β-eliminating pattern.

*Database of the genomes analyzed at Nite (National Institute of Technology and Evaluation): http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=sha.
**Search for Pfam domains (E.value < 1.10^{-4}) and Prosite patterns: http://motif.genome.jp, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

b) *MalY-type C-S lyases*

Recently, two MalY-type C-S β-lyases have been characterized from the axillary bacterial strains, *C. striatum* and *C. jeikeium* [7][8]. MalY represents a bifunctional pyridoxal 5′-phosphate-dependent enzyme acting as a β-cystathionase and as a repressor of the maltose regulon. Each subunit of the MalY dimer is composed of a large pyridoxal 5′-phosphate-binding domain and a small domain similar to aminotransferases. An homology search has been carried out with the amino-acid sequences of these enzymes and the *S. haemolyticus* published genome (*Table A5*). Four enzymes, SH0221, SH1916, SH0492, SH2169, which belong to the PLP dependent aminotransferase superfamily, and with homologies to *mal*Y, have been isolated (*Table A5*) The four enzymes contain both an aminotransferase and a β-eliminating lyase pattern. Percentage identities with *C. striatum* and *C. jeikeium* protein sequences were between 20 and 28% over a sequence up to 100 amino-acids. The percentage identity measures how many amino-acids are identical in an alignement of two sequences. A percent-identity score up to 25% over a sequence of 100 amino-acids was considered as a good score. The percentage similarity is defined as the ratio between the alignment score (using some substitution matrix and gap costs) and the worse of the two self-alignment scores for the two sequences. High degree of similarity between two sequences (> 30%) usually implies similar function and/or structure.

Table A5: Homology search between *S. haemolyticus* JCSC1435 genome and published C-S lyase from *C. striatum* and *C. jeikeium*[5].

		C. striatum C-S lyase*		***C. jeikeium C-S lyase*****	
Protein Name	**Function**	**Identity (%)**	**Similarity (%)**	**Identity (%)**	**Similarity (%)**
SH0221	hypothetical protein, similar to aspartate aminotransferase	28	47	28	43
SH1916	HisC aromatic amino acid aminotransferase HisC homolog	20	42	19	40
SH0492	hypothetical protein, similar to aspartate aminotransferase	22	40	18	36
SH2169	hypothetical protein, similar to histidinol phosphate aminotransferase	25	43	20	40

*[7], amino acid sequence of the *C. striatum* C-S lyase contains an aminotransferase class I and II (Pfam Aminotran_1_2) and a β-eliminating lyase (Pfam Beta-elim-lyase) pattern.
**[8], amino acid sequence of the *C. jeikeium* C-S lyase contains an aminotransferase class I and II (Pfam Aminotran_1_2) and an orthopoxvirus A36R protein (Pfam Orthopox_A36R) pattern.

[5] http://wishart. Biology.ualberta.ca/BacMap/index_1.html ; http://cmr.jcvi.org/cgi-bin/CMR

c) *Dipeptidase and carboxylesterase*

Whereas the release of 3-methyl-3-sulfanylhexan-1-ol may be the result of a single enzyme, it has been shown that the sequential action of a dipeptidase and a β-lyase from corynebacteria was necessary to transform the Cys-Gly-*S*-conjugate into this volatile thiol in the axillary region [9]. Similarly to what happens in the gluthathion detoxification pathway or in domestic cats sulfur volatile production [10], a dipeptidase or a carboxylestarase is first required to transform the cysteine-glycine S-conjugate into a cysteine-S conjugate and then a β-lyase is needed to release the volatile thiol (see *Chapter1, Part 1.2.3*). An homology search using protein public databases with *S. haemolyticus* genome, corynebacteria dipeptidase and cat carboxylesterase, results in nine enzymes having similarities with corynebacteria dipeptidase (see *Table A6*), and four enzymes having similarities with the Cauxin, carbolylesterase from cat (see *Table A*7) (Percentage similarity up to 30%).

None of these enzymes has been found to have homologies with enzymes from the aminotransferase superfamily, and most of them are present in *S. epidermidis* and *S. aureus*. From these results we conclude that the addition of *S. epidermidis* or *S. aureus*, having dipeptidase activity, to corynebacteria, having Cysteine S-lyase activity can increase the Cys-Gly-*S*-conjugate conversion yield. However, this effect has not been proved *in vitro* (see *Appendix 7.2*).

Table A6. Homology search (blastp search) between *S. haemolyticus* genome and the *C. striatum* Ax20 thiol precursor dipeptidase[6] amino-acid sequence (tpdA gene; accession number: EU311559) [9].

			C. striatum* dipeptidase	
Protein Name	**Function**	**Motifs[7]**	**Identity (%)**	**Similarity (%)**
SH0586	argE acetylornithine deacetylase homolog	Peptidase family M20/M25/M40 Peptidase dimerisation domain	23	38
SH2265	hypothetical protein, similar to succinyl-diaminopimelate desuccinylase	Peptidase family M20/M25/M40 Peptidase dimerisation domain Peptidase family M28 ArgE / dapE / ACY1 / CPG2 / yscS family signature 2 ArgE / dapE / ACY1 / CPG2 / yscS family signature 1	20	37
SH0132	hypothetical protein, similar to succinyl-diaminopimelate desuccinylase	Peptidase family M20/M25/M40 Peptidase dimerisation domain ArgE / dapE / ACY1 / CPG2 / yscS family signature 2 ArgE / dapE / ACY1 / CPG2 / yscS family signature 1	35	53
SH1171	Xaa-His dipeptidase homolog	Peptidase family M20/M25/M40 Peptidase dimerisation domain	30	42
SH1472	hypothetical protein	YSIRK type signal peptide Gram positive anchor Chitinase A, N-terminal domain	25	37
SH2420	hexulose-6-phosphate synthase homolog	Orotidine 5'-phosphate decarboxylase DeoC/LacD family aldolase HMGL-like – Tryptophan synthase alpha chain Ribulose-phosphate 3 epimerase family	25	41
SH2624	hypothetical protein		33	50
	trxA thioredoxin	Thioredoxin family active site Thioredoxin Redoxin Glutaredoxin	37	68
SH1471	hypothetical protein, similar to hemagglutinin/hemolysin-related protein	YSIRK type signal peptide Gram positive anchor Cna protein B-type domain Alpha-2-macroglobulin family N-termin	32	40

*The *C. striatum* dipeptidase contains the following motifs and domains: ArgE / dapE / ACY1 / CPG2 / yscS family signature 1 (PS00758); Peptidase family M20/M25/M40 (Pfam Peptidase M20) and Peptidase dimerisation domain (Pfam : M20 dimer).

[6] http://wishart.biology.ualberta.ca/BacMap/index_1.html; http://cmr.jcvi.org/cgi-bin/CMR
[7] http://motif.genome.jp; http://www.bio.nite.go.jp/dogan

Table A7. Homology search (blastp search) between *S. haemolyticus* genome and Cauxin amino-acid sequence, a carboxylesterase-like protein from domestic cat urine[8] [10].

			Cauxin*	
Protein Name	**Function**	**Motifs[9]**	**Identity (%)**	**Similarityy (%)**
SH0602	hypothetical protein, similar to para-nitrobenzyl esterase chain A	Carboxylesterases type-B serine active site Carboxylesterase 118 alpha/beta hydrolase fold Prolyl oligopeptidase family	35	56
SH2238	hypothetical protein, similar to lipase LipA	alpha/beta hydrolase fold Prolyl oligopeptidase family	25	40
SH0452	hypothetical protein	haloacid dehalogenase-like hydrolase Polynucleotide kinase 3 phosphatase	37	64
	ahpF alkyl hydroperoxide reductase subunit F	Pyridine nucleotide-disulphide oxidoreductases class-II active site Pyridine nucleotide-disulphide oxidor Glutaredoxin 3 HI0933-like protein ThiF family FAD dependent oxidoreductase CobB/CobQ-like glutamine amidotransfe Glucose inhibited division protein A	35	45

*Cauxin contains the following motifs and domains: Carboxylesterase (Pfam CO esterase,); alpha/beta hydrolase fold (Pfam Abhydrolase); Carboxylesterases type-B serine active site (PS00122); Carboxylesterases type-B signature 2 (PS00941).

d) *Conclusions*

From these results we can conclude that **SH0221** and **SH2169**, which are not present in *S. epidermidis* and *S. aureus* or have low levels of similarities with the *S. epidermidis* or *S. aureus* enzymes amino-acids sequences (see *Table A4*), may be good candidates for Carbon-Sulfur β-lyase activity in the axillary isolate *S. haemolyticus Ax4.* An homology search with the published genome sequence of *Bacillus licheniformis ATCC14580,* an axillary bacteria having Carbon-Sulfur β-lyase activity on Cys-Gly-*S*-conjugate (see *Appendix 7.2*), confirmed high identities between *S. haemolyticus* and *B. licheniformis* enzymes: 35 % identity (56% similarity) was found between SH0221 and a probable aminotransferase B [H] (Bli 03326) from *B. licheniformis* and 46% identity (66% similarity) between SH2169 and a histidinol-phosphate aminotransferase (Bli02397). However, the role of *S. haemolyticus* enzymes in the formation of volatile thiols in the axillary region has to be directely proved by either gene knockout or heterologous gene expression in *E. coli* in order to confirm these hypotheses and to determine whether or not the formation of the volatile thiols is due to the activity of one gene or is mutligenic, as shown for wine fermentation (see *Chapter 1, Part 1.2.3*) [11].

[8] http://wishart.biology.ualberta.ca/BacMap/index_1.html; http://cmr.jcvi.org/cgi-bin/CMR
[9] http://motif.genome.jp; http://www.bio.nite.go.jp/dogan

7.5 Patent: Inhibition of sweat malodor

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(54) Title: INHIBITION OF SWEAT MALODOUR

(57) Abstract: The present invention relates to a method for screening compounds having the ability to prevent, treat or reduce malodour development on body surfaces. In particular, the method allows to efficiently screen for compound having the ability of preventing sweat malodour development caused by volatile sulfur compounds (VSCs). The present invention is based on the finding of the direct precursor of naturally VSCs, which is present in human sweat and which will be metabolised by Staphylococci to VSCs.

Inhibition of Sweat Malodour

Technical Field

The present invention relates to a method for screening compounds having the ability to prevent, treat or reduce malodour development on body surfaces. The present invention also relates to a compound of formula (I), which is a precursor of malodorous volatile sulfur compounds. In addition, the present invention relates to methods for preventing malodour and to methods for preparing products having the ability to reduce malodour development.

Background of the Invention and Problem to be Solved

The prevention of axillary malodour is a constant objective of scientific endeavour. It has been recognized for some time that sweat itself, as it is excreted from apocrine sweat gland abundantly present in the underarm skin, is generally odourless. Axillary malodour essentially develops upon the metabolic activity of certain strains of bacteria that have evolved to live in this niche environment and which are well adapted to growing on the peculiar cocktail of odourless precursors found in apocrine sweat.

Several classes of malodorous substances have been isolated so far, one class is from steroidic origin, another class encompasses bacterial degradation compounds: short chain fatty acids like (E/Z)-3-methyl-2-hexenoic acid, described as predominant olfactory contributor of the sweat malodour.

A third class of sulfur compounds has only very recently been discovered and has been disclosed independently in WO 200403766, in *Troccaz et al.*, "3-Methyl-3-sulfanylhexan-1-ol as a Major Descriptor for the Human Axilla-Sweat Odour Profile" Chemistry & Biodiversity, Vol. 1 (2004); and in *Natsch et al.*, "Identification of Odoriferous Sulfanylalkanols in Human Axilla Secretions and Their Formation through Cleavage of Cysteine Precursors by a C-S Lyase Isolated from Axilla Bacteria", Chemistry & Biodiversity, Vol. 1 (2004).

One particularly malodourous specimen of the class of sulfur compounds was found to be 3-methyl-3-sulfanylhexan-1-ol (*Troccaz et al.*, followed by *Natsch et al.* and *Hasegawa et al.* "Identification of New Odoriferous Compounds in Human Axillary Sweat" Chemistry and Biodiversity Vol. 1 (2004) 2042-50). The same compound is also disclosed in US 6,610,346, where it is used as a flavour in foods and beverages for providing cooked vegetable (onion) and meaty notes to food products.

Presently, malodour development has been tackled in different ways, for example by applying anti-bacterial substances to the axillary skin, by providing perfume compositions

capable of masking malodour, by trapping malodorous molecules, for example by applying cyclodextrin, for inhibiting β-lyases, for example.

With the objective to prevent the formation of volatile sulfur compounds (VSCs) as, for example, S-3-methyl-3-sulfanylhexan-1-ol it becomes indispensable to elucidate its metabolic pathway, and, in particular, its direct precursor. Such insight would allow designing screening for compounds having the ability of intervening in the pathway and thus inhibit the formation of the VSC.

Accordingly, *Natsch et al.* have speculated that the cysteine conjugate of VSCs (Cys-S-3-methyl-3-sulfanylhexan-1-ol), was the direct precursor for VSCs and that, cleavage of the precursor by a C-S β-lyase present in *Corynebacterium spp.* would directly yield the VSC.

Similarly, *Lyon et al.* (US 5,213,791), disclosing amino acid β-lyase inhibitors as deodorants, considered that the Cys-S-conjugate was the most relevant precursor on col. 2, line 5-6. These findings corresponds to the well-reported pathway of thiols and thiolmethyl metabolites starting with glutathione conjugates, which undergoes sequential enzymatic hydrolysis to yield the thioether of cysteine.

A further objective of the present invention is to identify the bacterial species or strains, which are able to convert non-odoriferous precursors of human sweat into malodorous VSCs, allowing to more precisely targeting the origin of malodour development.

Accordingly, *Natsch et al.* concluded that non-odoriferous precursors of axillary secretions are transformed into volatile substances by bacterial enzymes present only in *Corynebacterium spp.* and not in staphylococci.

In view of the prior art, the objective of the present invention is to find other, direct precursors of volatile sulfur compounds responsible for axillary malodour. It is a further objective to identify further bacterial strains responsible of producing volatile sulfur compounds. The knowledge on precursors and bacterial strains at the origin of malodorous compounds may then be used to more effectively combat axillary malodour development in humans, for example by providing effective screening methods for compounds inhibiting the formation of VSCs. Therefore, it is a further objective of the present invention to provide new methods or ways for preventing development of malodour.

Summary of the Invention

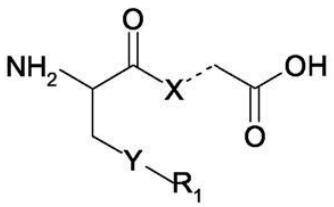
The inventors of the present invention surprisingly found that the direct precursor of volatile sulfur compounds (VSCs) is the S-conjugate of Cysteine-Glycine, and that the Cysteine conjugate reported from the prior art was a clearly less efficient precursor of VSCs. In further contrast to current prior art teaching, strains of *Staphylococcus*

haemolyticus are able to convert the Cys-Gly conjugate into VSCs with higher efficiency than *Corynebacterium* and *St. epidermidis* strains, all of which found in human axillae.

Accordingly, the present invention provides, in a first aspect, a method for screening compounds having the ability to prevent, treat or reduce malodour development on body surfaces, the method comprising the steps of

- providing a medium comprising a compound to be screened,
- adding to the medium at least one precursor compound of the formula (I),
- determining the increase of at least one metabolite of the precursor, and/or the disappearance of the precursor, and,
- concluding, from the ability of the compound to prevent increase of a metabolite of the precursor, or to prevent disappearance of the precursors, the compound's ability to prevent or treat malodour development.

In a second aspect, the present invention provides a compound of formula (I)



(I)

in which –Y defines a leaving function and -R_1 represents a C_1-C_{20} residue for an active compound of the formula HY- R_1, or, in which –Y-R_1 is OH or SH, and,

in which the dotted line represents a double bond, in case of which X is selected from CR_4, and N, or,

in which the dotted line represents a single bond, in case of which X is selected from NR_4, CR_4R_5, O and S, with R_4 and R_5 being, independently of each other, selected from H and C_1-C_{10} alkyl, alkenyl or alkynyl residues.

In a third aspect, the present invention provides the use of bacteria of the family Staphylococci in screening methods, assays or research methods of body malodour development, inhibition, and/or occurrence. Correspondingly, the present invention provides a method for elucidating body malodour development, the method comprising the step of applying Staphylococci, or an enzyme derived from it, to a malodour precursor compound.

In a fourth aspect, the present invention provides the micro-organisms *Staphylococcus haemolyticus* and *St. epidermidis* with CNCM deposit numbers I-3357 and I-3356, respectively.

In a fifth aspect, the present invention provides a method for preventing sweat malodour, the method comprising the step of applying to a body surface, preferably to the axillary skin, a compound capable of inhibiting the conversion of compound (I) with X = NH, the dotted line being a single bond, Y = S and R_1 being a C_4-C_{10} alkyl residue carrying a hydroxyl group to a volatile sulfur compound.

In a sixth aspect, the present invention provides a method for preparing a product having the ability to reduce malodour development, comprising the step of adding to the product a compound capable of inhibiting the conversion of compound (I) as defined in above (fifth aspect) to a VSC.

In a further aspect, the present invention provides a method for metabolising a compound of the formula (I) under controlled conditions, the method comprising the step of exposing the compound of formula (I) to a micro-organism and/or to a β-lyase.

In a still further aspect, the present invention provides a compound of formula (III) given below.

In the figures,

Figure 1 shows HPLC-MS mass range (High Performance Liquid Chromatography-Mass Spectrometry Total Ion Chromatograms) obtained from sterile sweat (trace A) and different elution fractions of sweat incubated with *St. haemolyticus* (trace D) and not incubated (traces B and C). This chromatogram shows the disappearance of a putative precursor compound having a molecular mass of 293 follwing incubation.

Figure 2 shows malodorous volatile sulfur compounds (1, 2, 3) and putative precursor compounds (9, 10, 11), compound **9** of which turned out to be the most efficient precursor for compound **1**. Arrows indicate the steps of synthesis of precursors.

Figure 3 shows chromatograms and mass spectres obtained by LC-MS of the precursor found in "zone 2" of natural sterile sweat (A, B), and of the synthesised precursor **9** (CD).

Figure 4 shows the bioconversion of synthetic precursor compounds 9 and 10 of malodorous VSCs by bacterial strains of *St. haemolyticus*, *St. epidermidis* and *C. xerosis*. It can be seen that the precursor of the present invention (9) has been efficiently converted by all strains.

Figure 5 shows the summary of synthetic pathways to a major VSC (1) from sterile sweat precursors. Compound (9) is an embodiment of the precursor to be used in the screening method of the present invention.

Bibliography

[1] C. Vuilleumier, M. van de Waal, H. Fontannaz, I. Cayeux, P. A. Rebetez. *Perfumer & Flavorist*. 2008, 33, 54.

[2] D. J. Bibel, R. J. Smiljanic, D. J. Lovell, *Applied Env. Microbiol*. **1978**, *35*, 1136.

[3] M. M. Bradford, *Anal. Biochem.* **1976,** *72,* 248.

[4] J. W. Farrior, W. E. Kloss, *Microbiology*. **1976,** *22*, 1680.

[5] H. Wakabayashi, M. Wakabayashi, W. Eisenreich, K.H. Engel, *Eur. Food. Res. Technol*. **2002**, *215,* 287.

[6] H. Wakabayashi. 'Enzyme-catalysed transformations of sulfur-containg flavor precursors', Munchen, 2004.

[7] A. Natsch, J. Schmid, F. Flachsmann, *Chem. Biodiversity* **2004**, *1*, 1058.

[8] A. G. James, C. Austin, D. Hyliands, D. Cox, D. Taylor, S. Grimshaw, R. Calvert, Cosm'ing conference, June **2007**.

[9] R. Emter, A. Natsch, *J. Biol. Chem.* **2008,** *283*, 20645.

[10] Miyazaki M, Kamiie K, Soeta S, Taira H, Yamashita T. *Biochem. J.* **2003**, 370, 101-110.

[11] K.S. Howell, 'The contribution of *Saccharomyces* yeasts to aroma formation during wine fermentation' University of New South Wales, Australia, **2005**.

Author Publications

Troccaz M., Starkenmann C., Niclass Y., Van de Waal M., Clark A. J. 3-Methyl-3-sulfanylhexan-1-ol as a Major Descriptor for the Human Axilla-Sweat Odor Profile. *Chem. Biodiv.* **2004**, 1, 1022-1035.

Starkenmann C., Niclass Y., **Troccaz M.,** Clark A. J. Identification of the Precursor of (*S*)-3-Methyl-3-sulfanylhexan-1-ol, the Sulfury Malodor of Human Axilla Sweat. *Chem. Biodiv.* **2005**, 2, 705-715.

Gautier A. E, Starkenmann C., Begnaud F., **Troccaz M**. Painting and Memory in the Discovery of Aroma Chemicals: the Case of Sulfur Containing Odorants and Odorant Precursors in Axillary Sweat Odor in *Food Flavor: Chemistry, Sensory Evaluation, and Biological activity (ACS Symposium Series).* Chap 10. **2008**.

Troccaz M., Benattia F., Borchard G., Clark A. J. Properties of Recombinant *Staphylococcus haemolyticus* Cystathionine *β*-Lyase (*met* C) and Its Potential Role in the Generation of Volatile Thiols in Axillary Malodor. *Chem. Biodiv.* **2008**, 5, 2372-2385.

Starkenmann C., **Troccaz M.**, Howell K. The Role of Cysteine and Cysteine-*S*-Conjugates as odor precursors in the flavor and fragrance industry. *Flavour Fragr. J.* **2008**, 23,369-381.

Starkenmann C., Cayeux I., Le Calve B., Beccucci S., **Troccaz, M.** Olfactory perception of cysteine-S-conjugates from Fruits and vegetables. *J. Agric. Food Chem*. **2008**, 56(20), 9575-80.

Troccaz M., Borchard G., Vuilleumier C., Raviot-Derrien S., Niclass Y., Beccucci S., Starkenmann C. Gender-specific differences between the concentrations of non-volatile (*R*)/(*S*)-3-methyl-3-sulfanylhexan-1-ol and (*R*)/(*S*)-3-hydroxy-3-methyl-hexanoic acid odor precursors in axillary secretions. *Chem. Senses.* **2009**, 34, 203-210.

Troccaz M., Haefliger O. P, Cayeux I., Beccucci S., Jeckelmann N., Barra J., Clark A. J., Schrenzel [J.,] Baehni P. Effects of flavored mouthrinses on oral malodors: a sensorial, analytical, and microbial evaluation. *J. Clin. Periodontol.* **2009.** Submitted.

Author Patents

Stakenmann, C., Clark A.J, **Troccaz, M**, Niclass, Y. Inhibition of sweat malodor. International Patent No. WO 2006079934 to Firmenich SA, **2006**.

Barra, J., Seyfried, M., Tashiro, H., **Troccaz, M**. Antimicrobial flavoring composition. International Patent No. WO 2008068683 to Firmenich SA, **2008.**

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