Blocking AMRE – Chemistry and Biochemistry at a Nasty Enzyme

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Malodour counteragents: AMRE inhibitors

- Sweat – composition and biosynthetic origin
- AMRE inhibitors: intervention strategy
- Synthesis of inhibitors
- Inhibition rates and structures
- Biochemical and clinical studies
The chemistry of human axilla odors: 1. Volatile carboxylic acids

- 3-methyl-2-hexenoic acid long known as body odorant of schizophrenics
- Later found in the general population
  - Along with other carboxylic acids such as goat acid
- 3-hydroxy-3-methyl-hexanoic acid quantitatively most abundant body odorant
2. Sulfur volatiles

- Three research groups* reported in 2004 sulfanylalkanols as a further structural class
- 3-sulfanyl-3-methyl-hexanol is the most abundant compound

* Hasegawa et al., Starkenmann et al., Natsch et al.
The biochemical formation of axilla odors

- First observations, Shelley et al., 1953 stated:
  
  ‘No odor could be detected in apocrine sweat after collection’
  ‘Bacterial action is necessary for the production of odor from apocrine sweat’

- Leyden et al:
  
  ‘High level of body odor is associated with large population of Corynebacteria in the axilla’

CONCLUSION:

⇒ Sweat contains a non-odoriferous ‘precursor molecule’
⇒ Skin inhabiting Corynebacteria have enzymes which transform the precursor into the odorant
Biochemistry: two key questions

I. Chemical structure of the secreted odor-precursors?

II. Which bacterial enzymes cleave the precursors?
Precursors for acids

- The malodor acids in fresh sweat are covalently linked to a glutamine residue.
Axillary malodor releasing enzyme in skin bacteria

- Cleavage of malodor precursor

<table>
<thead>
<tr>
<th>strain</th>
<th>species assignment</th>
<th>release of 3M2H from 3M2H-Gln</th>
<th>release of HMHA from HMHA-Gln</th>
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<td>&lt;0.005</td>
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<tr>
<td>Ax19</td>
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<td>0.735</td>
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<td>Ax20</td>
<td>Corynebacterium striatum</td>
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<td>Ax21</td>
<td>Corynebacterium bovis</td>
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<td>0.037</td>
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<td>Staphylococcus capitis</td>
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<td>&lt;0.005</td>
</tr>
<tr>
<td>Ax6</td>
<td>Staphylococcus epidermidis</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Ax9</td>
<td>Micrococcus luteus</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

- Malodor is mainly released by *Corynebacteria*
  - Subjects with high population of *Corynebacteria* have strong body odor
Cloning of the gene coding for AMRE

- Enzyme purified from cellular extracts of *Corynebacterium Ax20* with 4 chromatographic steps
- Partial amino acid sequence determined
- Full length gene cloned by degenerated PCR and chromosomal walking
- Protein expressed in recombinant *E.coli*
- Fluorescent high throughput *in vitro* screening assay developed
Biochemistry of release of sulfur compounds

- Focus of today‘s talk is AMRE (Glutamine-aminoacylase), but we also identified two enzymes involved in sulfur release……

- 3-sulfanyl-3-methylhexanol linked to Cys of Cys-Gly dipeptide*, typical degradation product of a glutathione-adduct

![Chemical structures](image)

*Starkenmann et al., 2005
Intervention strategies for deodorant ingredients

Sweat glands secrete

Nutrients and humidity

Antimicrobial compounds

Bacterial growth

Bacteria produce

Specific enzymes

Malodor precursor

Precursor cleavage

Enzyme inhibition

Alternative substrate

Typical axillary malodor

Masking fragrance

Malodor perception

M. Gautschi, A. Natsch, F. Schröder, Chimia 2007
AMRE: replacement of the malodorant by a fragrance ingredient

- Unique substrate specificity: only $N^\alpha$-substituted Glutamines are recognized, closely related amino acid derivatives not.

- A large range of different hydrophobic $N^\alpha$-substituents is tolerated: amides, carbamates...

- Disadvantage: an excess over the natural substrate is needed.

AMRE inhibitors: replacement of the carbamoyl moiety by a non-cleavable group

A first generation of phosphinic acid analogues was synthesized and found to be promising AMRE inhibitors.

IC<sub>50</sub> = concentration of an inhibitor needed to reduce the enzymatic activity by ½ at a given substrate concentration

Phosphinic acid analogues of $\text{N}^\alpha$-substituted glutamines: modifications and building blocks

- N-Trityl amide = “solid phase”
- Michael addition of alkylphosphinic acids

A. Natsch, F. Schröder, WO 2004043971, Givaudan
Preparation of building blocks 1A-C
(0.5 – 1 kg scale)

\[
\begin{align*}
\text{EtO}_2\text{C} & \text{CO}_2\text{Et} \\
& + \\
\text{CO}_2\text{tBu} & \text{CO}_2\text{Et} \\
& \text{CO}_2\text{tBu} \\
1 \text{ eq} & 78\% \text{ (dist)} \\
& \text{toluene, reflux,} \\
a) \text{K}_2\text{CO}_3, & \text{cat. nBu}_4\text{N(HSO}_4) \\
& 18 \text{ h, 65}\% \\
\end{align*}
\]

b) excess TFA / CH₂Cl₂
d) TritNH₂, NEt₃,
e) KOH, EtOH,
f) excess Et₂NH / H₂CO,

m.p. = 120°C

1A

1B

1C

Givaudan
Preparation of building block 1D


Phosphinoyl-AMRE inhibitors: synthesis

C-P Coupling Steps:

Trityl-group ~ solid phase
Yields quantitative
³¹P-NMR and MS-ESI(-)
Filtration of 5 over RP-syringes
Alkylphosphinic acids: synthesis

benzylic bromides

aliphatic iodides

allylic bromides

terminal alkenes

a) H.An et al. JOC 2001
a,b) S.Deprele, J.-L.Montchamp, JOC 2001

Free radical rearrangement:

A. Natsch, F. Schröder, WO 2004043971, Givaudan
Carbocationic rearrangement during de-tritylation

Allylic precursors:
β-substituents fine, but γ- need prior hydrogenation of the β,γ-double bond

\[ \text{H}^+ \xrightarrow{\text{H}^+} \xrightarrow{\text{H}^+} \text{OH} \xrightarrow{\text{2 equiv (IPr)\text{SiH}, TFA}} \text{CO}_2\text{H} \]

\[ \text{H}^+ \xrightarrow{\text{H}^+} \xrightarrow{\text{dito}} \xrightarrow{\text{dito}} \text{CO}_2\text{H} \]

\[ \text{Hydrolysis} \]

\[ \text{a)} \text{cat. Pd/C EE, EtOH, H}_2 \]

\[ \text{b)} \text{Hydrolysis} \]

\[ \text{c)} \text{Detritylation} \]

\[ \text{IC}_{50} = 800 \]

\[ \text{IC}_{50} = 127 \]

\[ \text{IC}_{50} = 19 \]
Inhibitors: via hydrogenation of allylic phosphinyl precursors

\[
\begin{align*}
5zd & \quad \text{IC}_{50} = 18 \\
5ze & \quad \text{IC}_{50} = 120 \\
5zf & \quad \text{IC}_{50} = 150 \\
5n & \quad \text{IC}_{50} = 19
\end{align*}
\]

ex Farnesol

ex nor-Radjanol

ex Geraniol

A. Natsch, F. Schröder, WO 2004043971, Givaudan
Inhibition: methyl-groups at the backbone  
Comparison with first generation inhibitors

Compounds with methyl-groups at the backbone do not inhibit ($IC_{50} > 40000$) in contrast to inhibitors with unsubstituted glutamine backbones.

$$\text{5A: } R = \text{butyl, } IC_{50} = 451$$
$$\text{5B: } R = \text{benzyl, } IC_{50} = 275$$

$\text{5B}$ and $\text{5C}$ show no inhibition.
Inhibition: reference compounds

Glutamine-type backbone + dialkyl phosphinyl group necessary as well as free CO₂H, free amide
Inhibition: P-allylic analogues:
were good but not better than 1\textsuperscript{th} generation inhibitors, Exception 5z

9 examples: $R = \text{ortho- and para-}tBu, \text{iPr, iBu}$
Inhibition: P-benzylic analogues: some were slightly better than the 1th generation P-Benzyl inhibitor

9 examples: +I and –I substituents (fluorination):
steric rather than electronic effects, planarity
Inhibition: P-Alkyl analogues:
good to very good inhibition

8 examples: flexibility in the $\alpha,\beta$-position, P-ethylene bridge
Inhibition: 2th generation

5 best examples: IC$_{50}$ = 11 - 19
Inhibition: 2th generation

- Second generation: IC$_{50}$ ~ 10 – 20 (versus 200 – 400, 1th generation)
Inhibition results: elaboration of the key

- Glutamine backbone
- Substituents at the backbone detrimental
- Requirements: dialkyl phosphinyl group, free CO$_2$H, free amide
- Alkyl phosphoryl side chain
  - P-ethylene bridge (exception β-alkyl
  - Allylic DB needs prior hydrogenation
  - P-alkyl > P-allyl > P-benzyl
Lead compound 5n

IC$_{50}$ = 19
mp 135°-145°C (iPrOH, EE 1:2)
> 98% purity (NMR, ESI-MS)

- 75g prepared in 11 steps starting from 1 kg malonate and acryl ester.
- Optimization: Volume yields, steps combined, RP-filtration of the final product, crystallization.
- Toxicological Testing: AMES (mutagenicity), Acute Toxicity, LLNA → no toxicological effects
Cristall structure of AMRE with bound inhibitor

- The recombinant enzyme was co-cristallised with the most potent enzyme inhibitor
- Crystal structure resolved to 1.75 Å
- Enzyme tetramer with two zinc atoms in each active site

Zinc atoms and ligand in active site
AMRE / Inhibitor Complex: LB / HBs and Zn-interactions at the active Site

- Absolute stereochemistry
- Replacement of functional groups
Unusual binding mode of phosphinic inhibitors

- Phosphinic inhibitors are thought to be classical transition state analogues
- Typically, these are competitive inhibitors
- IC50 is dependent on substrate concentrations (Increasing substrate concentration in test raises IC50)

- This is not the case for the phosphinic inhibitors of AMRE!
- Inhibitors give kinetic plots as suicide inhibitors
- The phosphinic inhibitors catalyze stable tetrimerizations of the enzyme
…and the 'clinical' reality

- The 'clinical test' for deodorant ingredients – Sniff test with human volunteers.
- Sequential studies in the same panel with phosphinic lead inhibitor and alternative substrate

GR-85-4027, best alternative substrate

GR-86-3021, best phosphinic inhibitor
**Effect of triclosan and alternative substrate 85-4027**

- The effect of 85-4027 is significant in the phenoxanol-positive-panelists with higher malodor score.

- The effect on the negative panelists is non-significant.

⇒ The product works on those panelists with a real malodor problem.
Effect of the phosphinic inhibitor

- NO significant effect of the inhibitor after 8 h in three consecutive studies despite an *in vitro* efficacy of 19 nM IC50!
The 'clinical' reality

- The simple alternative substrate gives significant deodorant benefits
- Works best on 'high malodor individuals'
- Gives a convincing target validation
- ... But: The most efficient inhibitor failed in the in vivo studies
- Reasons?
  - Poor bioavailability in the axilla?
  - Specific mode of competitive binding (tetramerization of the enzyme?)
Chemistry and biochemistry of axilla odors - A multidisciplinary project

- **Chemical synthesis** of odorants, precursors, inhibitors and alternative substrates: F. Flachsmann, S. Derrer, T. Granier, S. Elmer, O. Wäckerlig, M. Fournie-Zaluski (Université de Paris, PharmaLeads)

- **GC-MS analysis, NMR**: J. Schmid, G. Brunner

- **Protein purification, enzymatic tests and heterologous expression**: A. Natsch, R. Emter, M. Wasescha, W. Stauch

- **Precursor isolation and LC-MS analytics**: H. Gfeller and G. Acuna

- **Protein crystallisation and structure determination**: A. Douangamath, J. Baker (EvoTec)

- **Threshold determinations and sensory data**: H. Koch

- **Molecular Modelling Studies**: A. Borosy, H.-P. Weber
AND NOW – for something completely different

Why Chinese people do not have this problem - or

The effect of a SNP in the ABCC11 gene

- SNP (single nucleotide polymorphism) in ABCC11 gene: *White earwax instead of yellow earwax.*
- White earwax is known to correlate to low /absent axilla odors
- This mutation is present in > 95% of the people in central chinese populations and > 70% frequency in larger Asian populations
Frequency of the ABCC11 negative haplotype

- Black is the functional allele
- In white frequency of the ABCC11 mutation


High frequency of the haplotype with the mutated allele.
Does ABCC11 influence secretion of malodor precursors?

- 25 panelists, genotyped for the SNP based on mouth swab DNA
- All panelists did donate sweat (physical exercise)
- Analysis for sweat precursors (amino-acid conjugates)

- 11 of the panelists have the mutation on both chromosomes
  - AA
- 7 panelists have one mutated gene, one gene still works
  - AG
- 7 panelists have no mutation
  - GG
<table>
<thead>
<tr>
<th>Genotyp ABCC11</th>
<th>Ethnic population</th>
<th>Secreted amino-acid conjugates (µMol / 2 pads)</th>
<th>HMHA-Gln</th>
<th>3M2H-Gln</th>
<th>86-8434</th>
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<tbody>
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<td>1.23</td>
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<td>0.85</td>
<td>0.18</td>
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</table>

No odor precursor in people with mutation on both chromosomes

One intact gene is sufficient to secrete malodor precursor
ABCC11 – scientific conclusions

- Complete loss of malodor precursor secretion in panelists with two mutant genes
- Second ‘proof of principle‘ or target validation – Secretion of the identified malodor substrates correlates to body odors
- High frequency of this evolutionary young mutation: Positive selection pressure!
- Advantage in partner selection for low odorant individuals in ancient Asian cultures with long culture of personal hygiene?