Blocking AMRE – Chemistry and Biochemistry at a Nasty Enzyme

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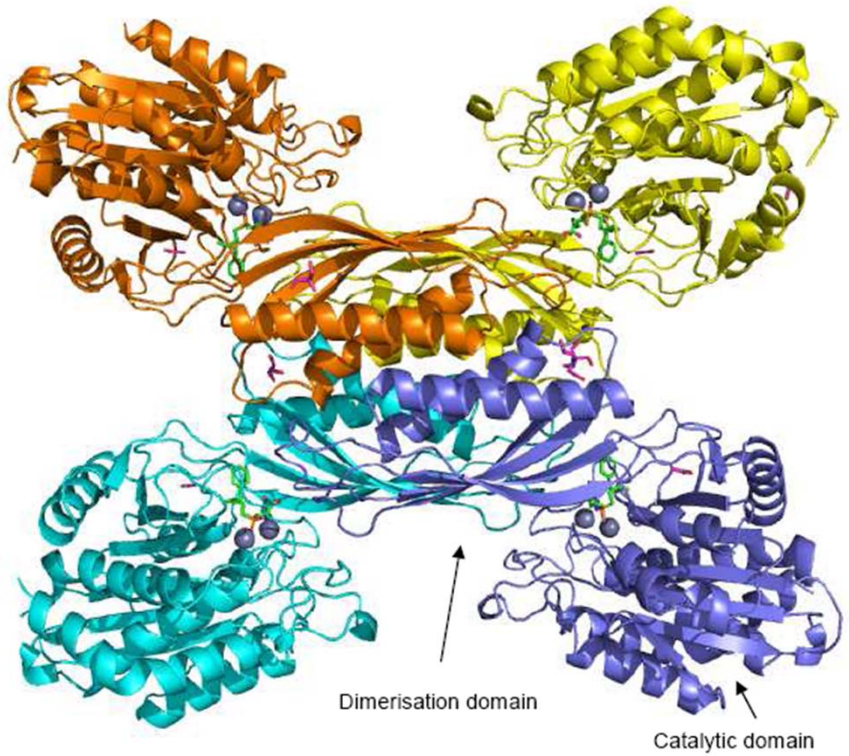
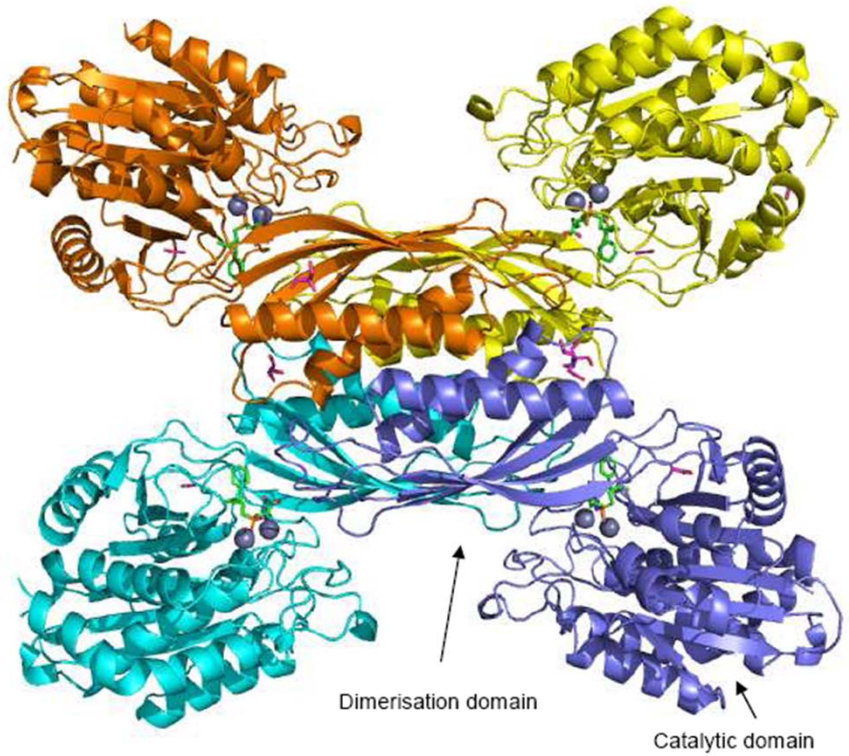


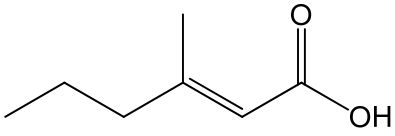
Malodour counteragents: AMRE inhibitors

- **Sweat – composition and biosynthetic origin**
- **AMRE inhibitors: intervention strategy**
- **Synthesis of inhibitors**
- **Inhibition rates and structures**
- **Biochemical and clinical studies**

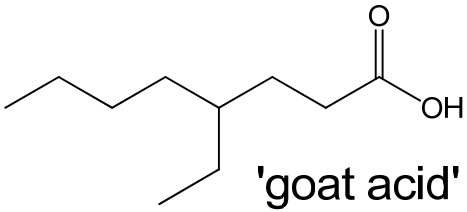


The chemistry of human axilla odors: 1. Volatile carboxylic acids

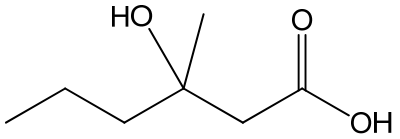
- 3-methyl-2-hexenoic acid long known as body odorant of schizophrenics
- Later found in the general population
  - Along with other carboxylic acids such as goat acid
- 3-hydroxy-3-methyl-hexanoic acid quantitatively most abundant body odorant



3-methyl-
2-hexenoic acid
= 3M2H



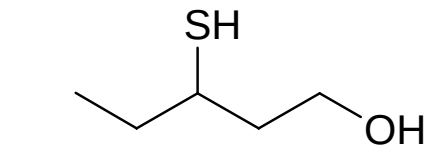
'goat acid'

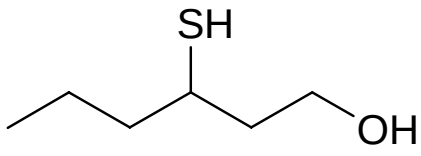


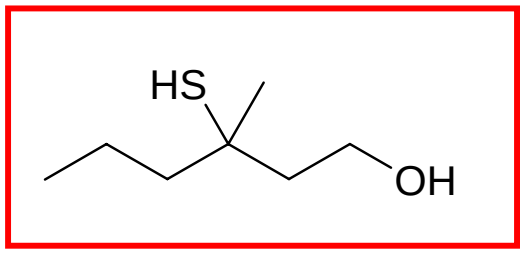
3-hydroxy-3-methyl-hexanoic acid

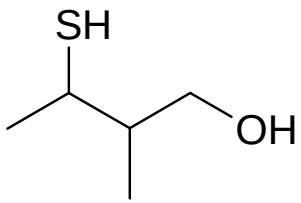
2. Sulfur volatiles

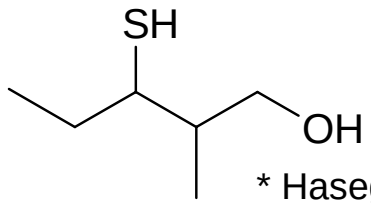
- Three research groups* reported in 2004 sulfanylalkanols as a further structural class
- 3-sulfanyl-3-methyl-hexanol is the most abundant compound











* 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

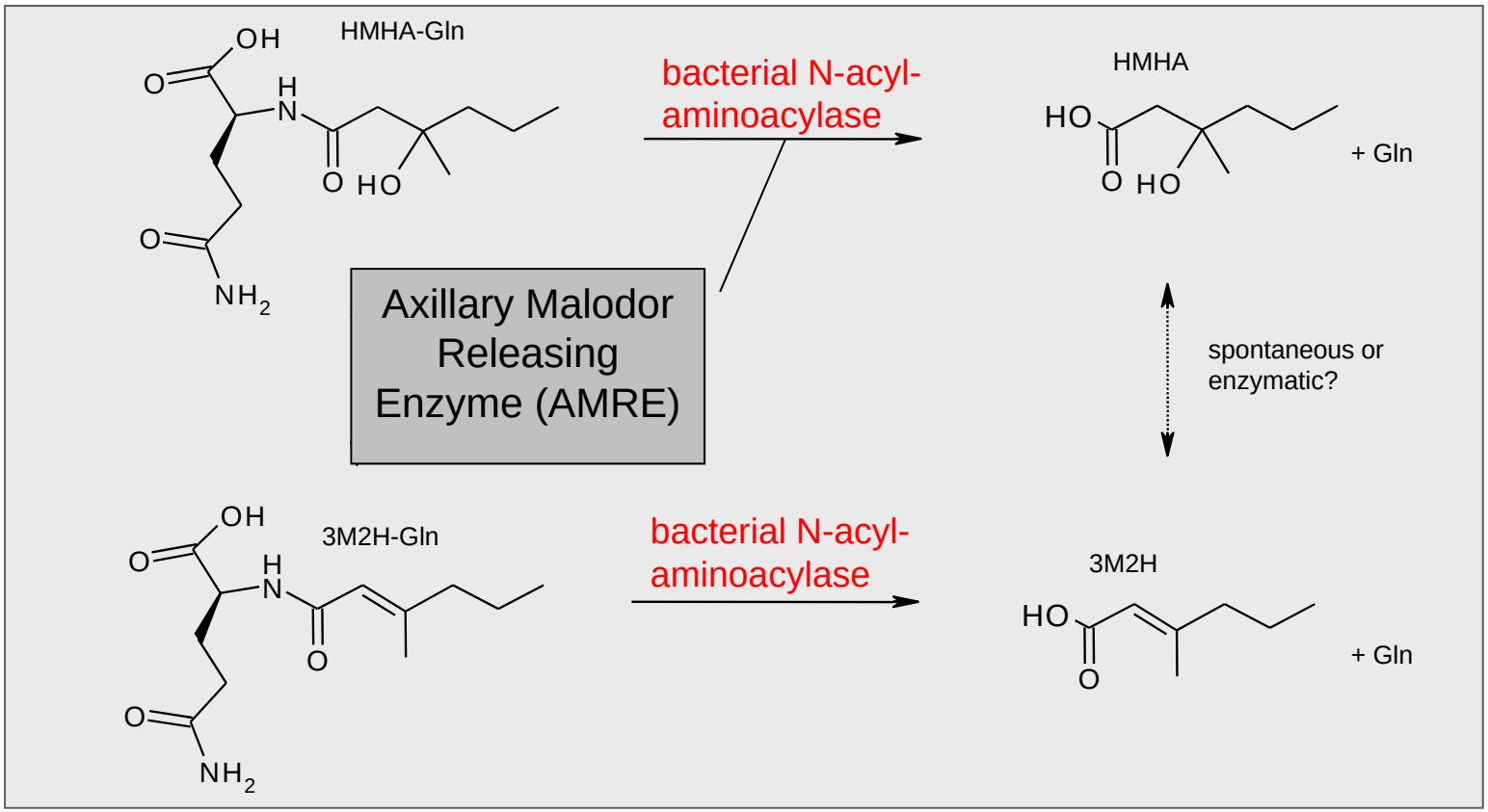
Biochemistry: two key questions

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

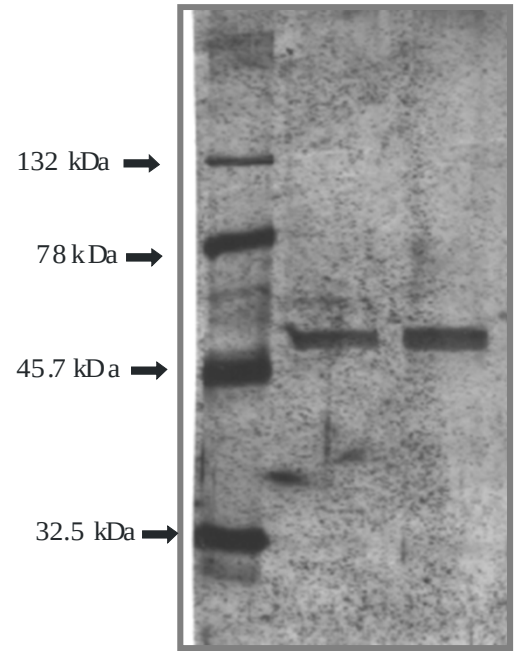
strain	species assignement	release of 3M2H from 3M2H-Gln	release of HMHA from HMHA-Gln
Ax7	*Corynebacterium group G*	<0.005	<0.005
Ax19	***Corynebacterium jeikeium***	1.132	0.735
Ax20	***Corynebacterium striatum***	0.217	0.200
Ax21	***Corynebacterium bovis***	0.449	0.037
Ax1	*Staphylococcus capitis*	<0.005	<0.005
Ax6	*Staphylococcus epidermidis*	<0.005	<0.005
Ax9	*Micrococcus luteus*	<0.005	<0.005

- Malodor is mainly released by *Corynebacteria*

⇨ Subjects with high population of *Corynebacteria* have strong body odor

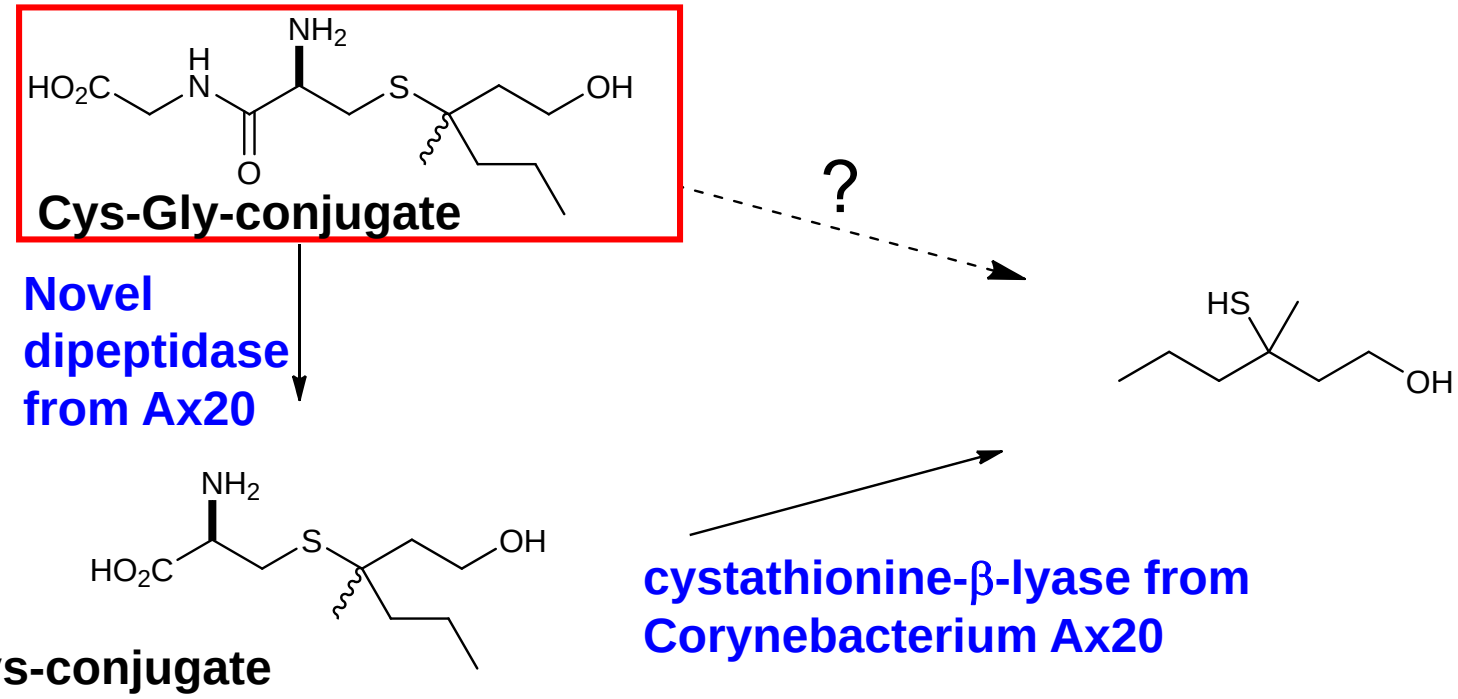
Cloning of the gene coding for AMRE

- 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



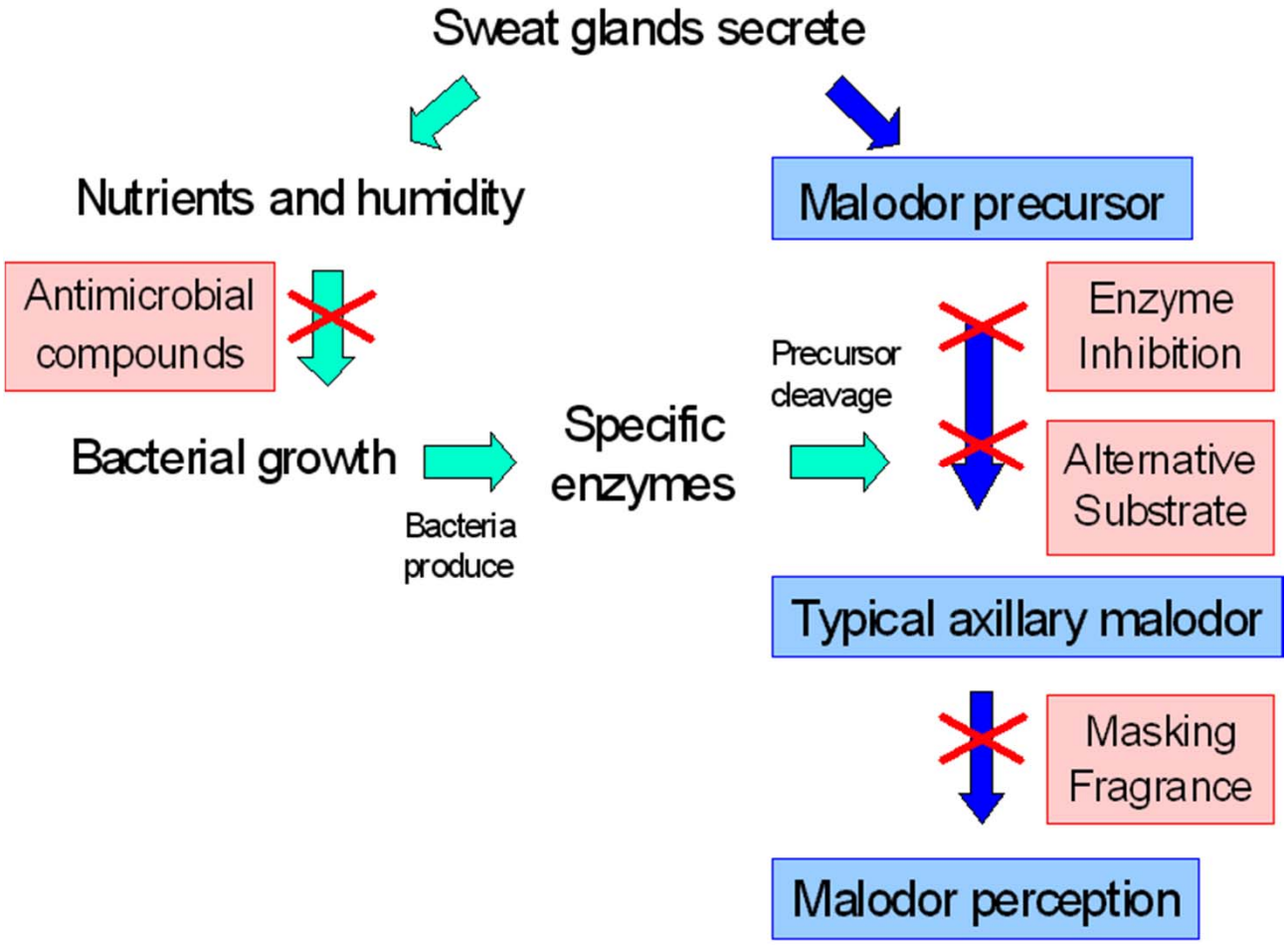
Biochemistry of release of sulfur compounds

- **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



*Starkenmann *et al.*, 2005

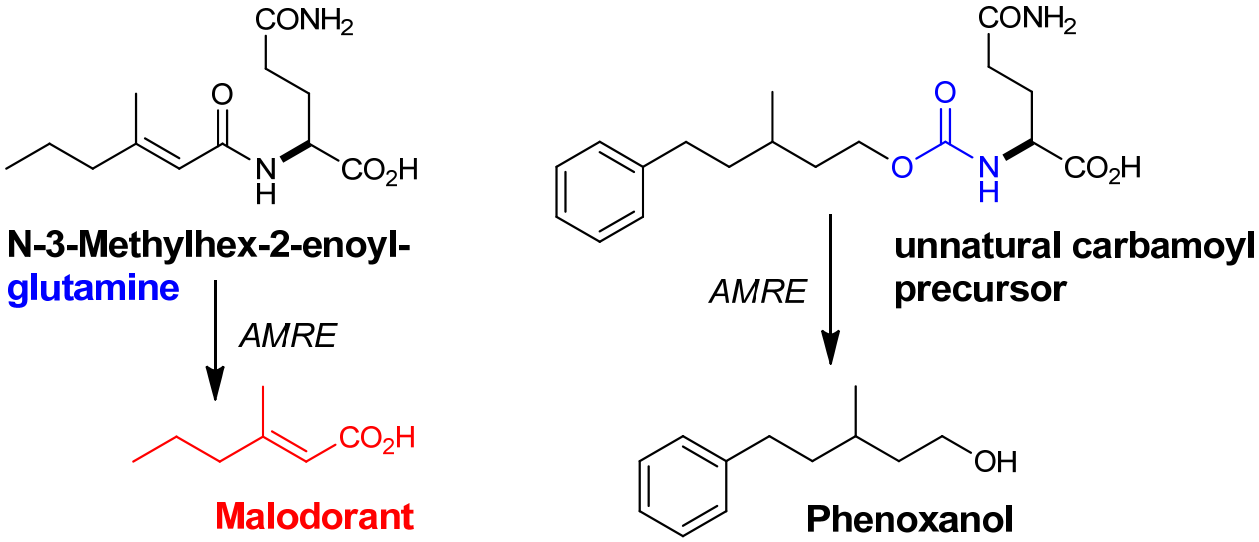
Intervention strategies for deodorant ingredients



M. Gautschi, A. Natsch, F. Schröder, *Chimia* 2007

AMRE: replacement of the malodorant by a fragrance ingredient

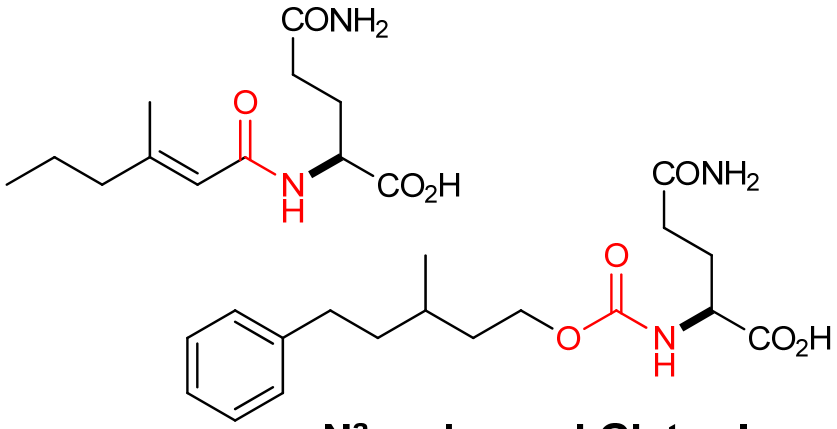
- **Unique substrate specifity: only N$^{\alpha}$-substituted Glutamines are recognized, closely related amino acid derivatives not**
- **A large range of different hydrophobic N$^{\alpha}$-substituents is tolerated: amides, carbamates…**



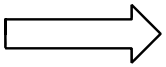
- **Disadvantage: an excess over the natural substrate is needed**

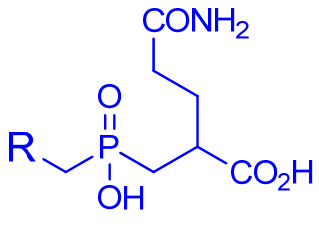
M. Gautschi, A. Natsch, F. Schröder, *Chimia*, 2007

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



N^a-carbamoyl-Glutamine Agonists





5A

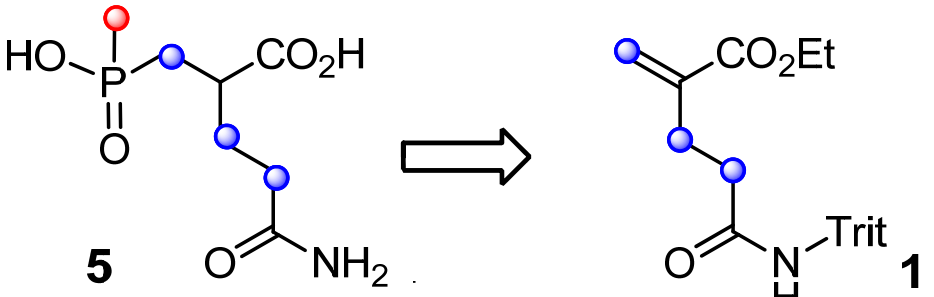
(R = Pr, iPr, Ph,
IC_{50} = 180 - 440 nmol)

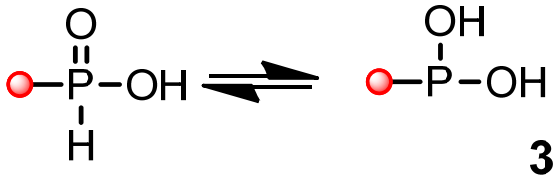
Phosphinic Acid Antagonists

- **A first generation of phosphinic acid analogues was synthesized and found to be promising AMRE inhibitors.**
- **IC_{50} = concentration of an inhibitor needed to reduce the enzymatic activity by ½ at a given substrate concentration**

M. Gautschi, A. Natsch, F. Schröder, *Chimia*, 2007

Phosphinic acid analogues of N^{α}-substituted glutamines: modifications and building blocks



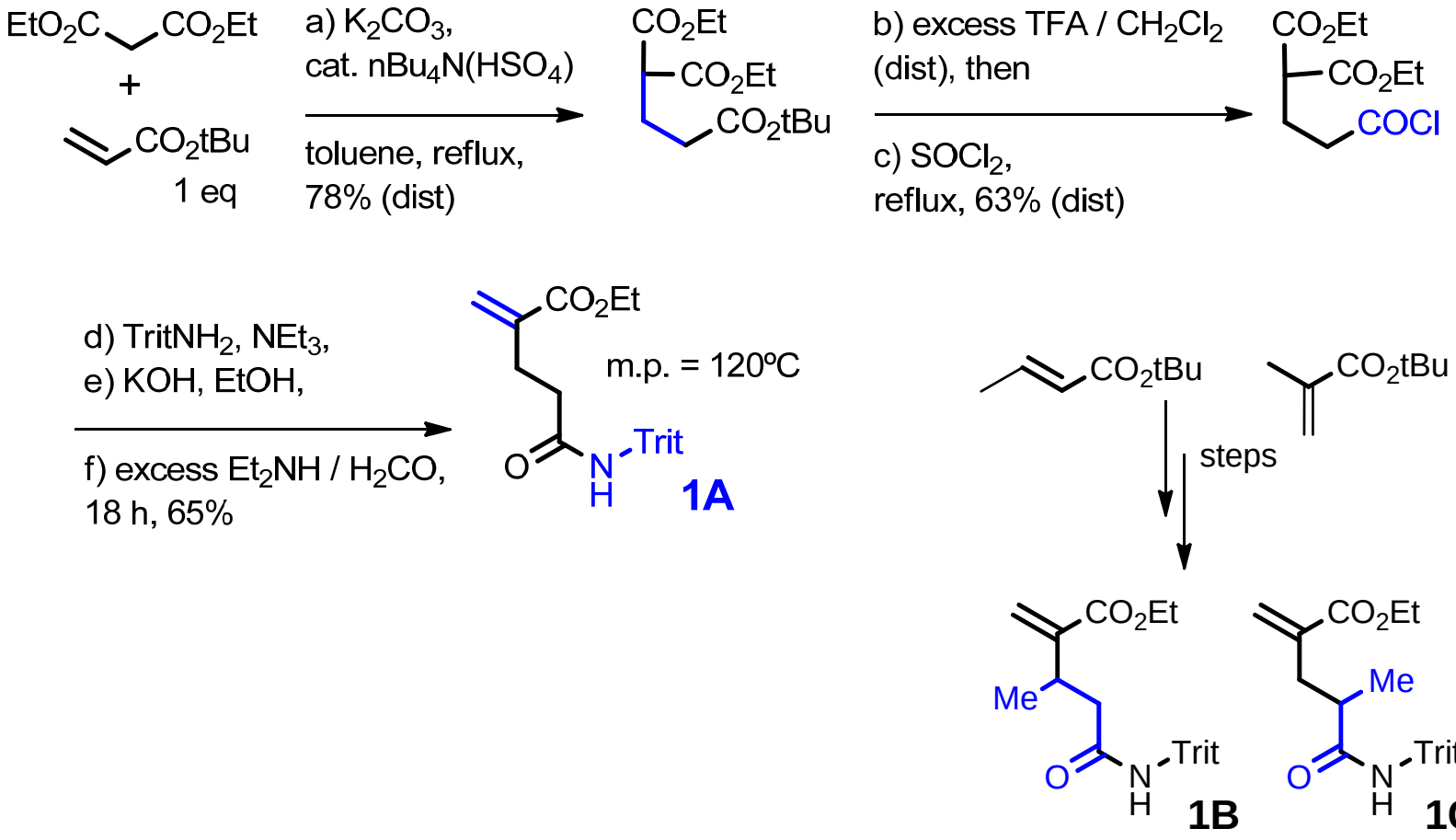


- **N-Trityl amide = “solid phase”**
- **Michael addition of alkylphosphinic acids**

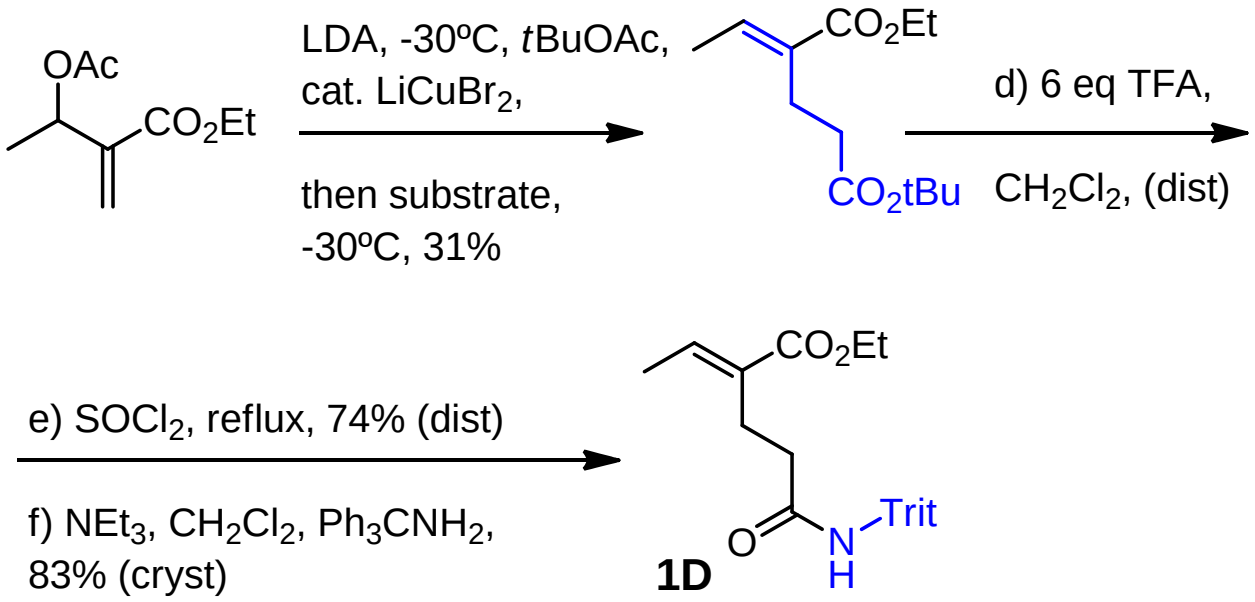
A. Natsch, F. Schröder, WO 2004043971, Givaudan

Preparation of building blocks 1A-C

(0.5 – 1 kg scale)

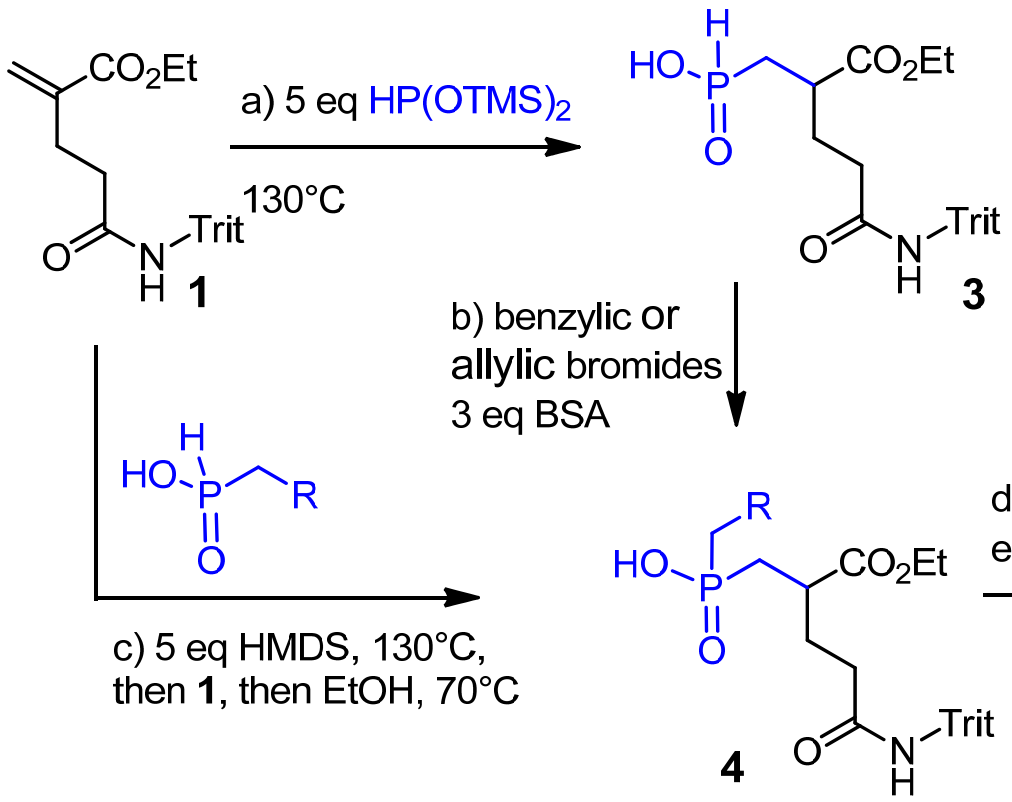


Preparation of building block 1D



Substrate : C. Yu, B. Liu, L. Hu, *J.Org.Chem.* 2001
Allylic substitution: J. Villiéras et al., *J.Organomet.Chem.* 1990

Phosphinoyl-AMRE inhibitors: synthesis



- **Trityl-group ~ solid phase**
- **Yields quantitative**
- **^{31}P-NMR and MS-ESI$^{(-)}$**
- **Filtration of 5 over RP-syringes**

C-P Coupling Steps:

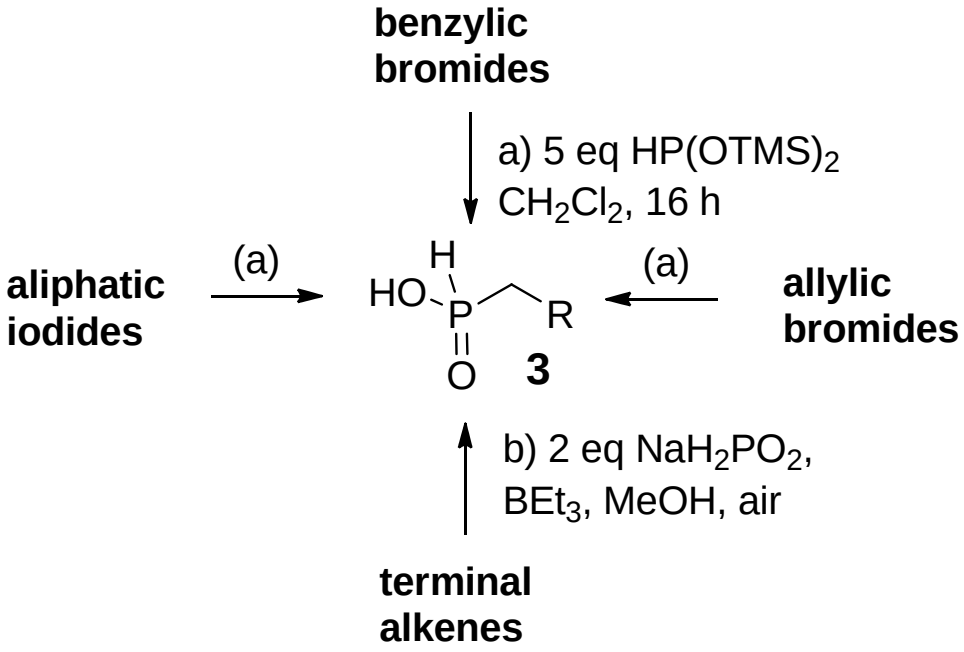
a) Michael addition of $HP(OTMS)_2$: W.P.Malachowski, J.K.Coward *JOC*, 1994

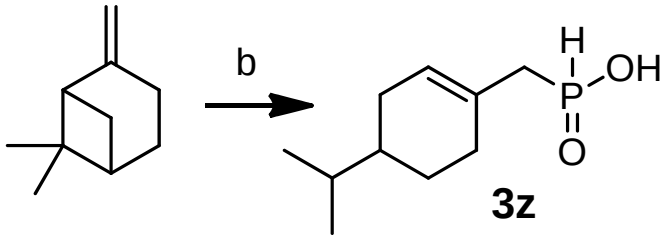
b) Allylation, benzylation: L.A.Reiter, *B.P.Jones, JOC,* 1997

c) Michael addition of alkyl phosphinic acids: D.Georgiadis, A.Yiotakis, *Tetrahedron,* 1999

A. Natsch, F. Schröder, WO 2004043971, Givaudan

Alkylphosphinic acids: synthesis



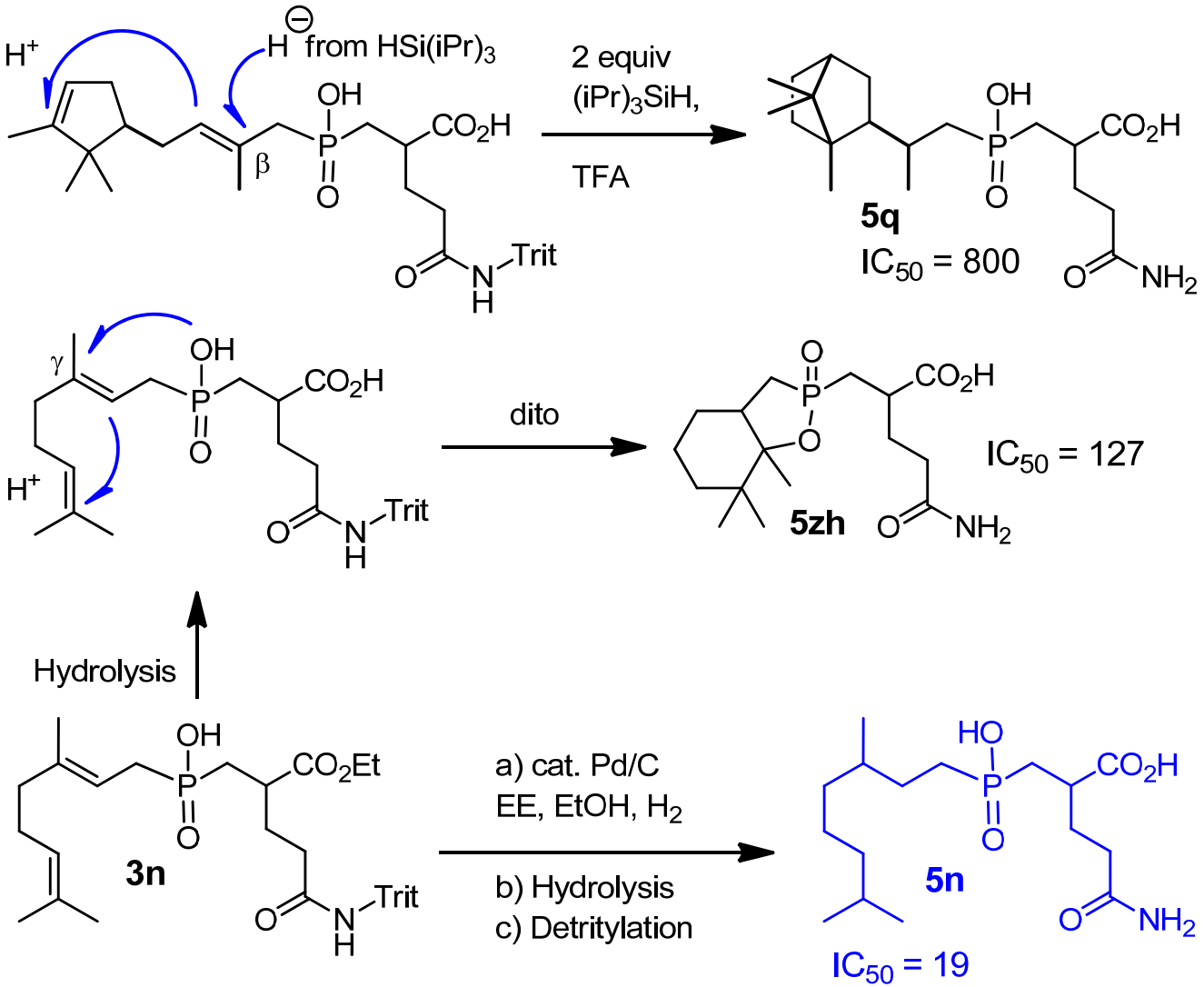


Free radical rearrangement:
D.R.Battiste, D.L.Haseldine, *Synth. Commun*. 1984

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

A. Natsch, F. Schröder, WO 2004043971, Givaudan

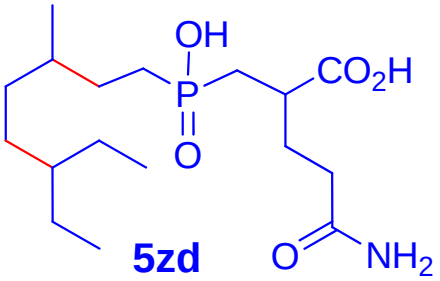
Carbocationic rearrangement during de-tritylation



Allylic precursors:

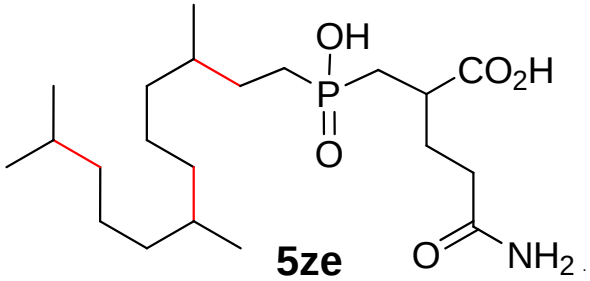
β-substituents fine, but γ- need prior hydrogenation of the β,γ-double bond

Inhibitors: via hydrogenation of allylic phosphinyl precursors



5zd

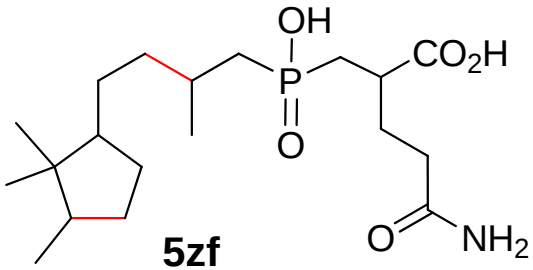
IC_{50} = 18



5ze

IC_{50} = 120

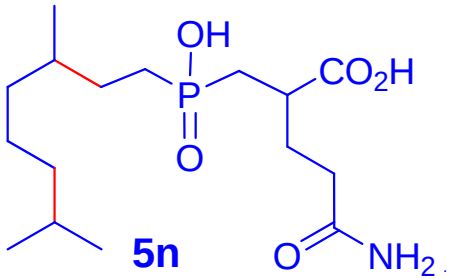
ex Farnesol



5zf

IC_{50} = 150

ex *nor*-Radjanol



5n

IC_{50} = 19

ex Geraniol

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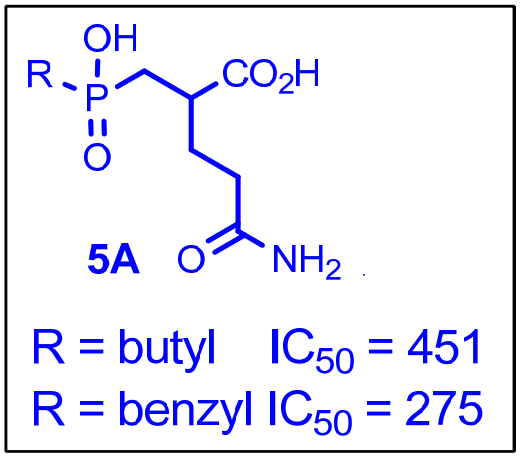
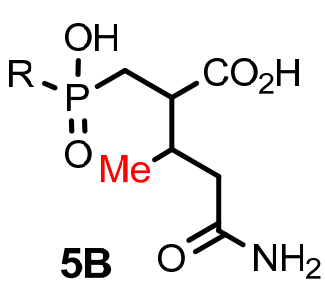
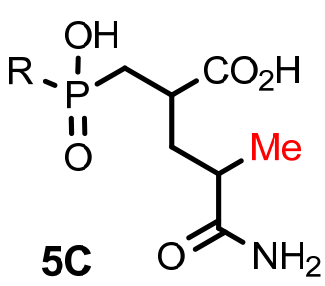
Inhibition: methyl-groups at the backbone

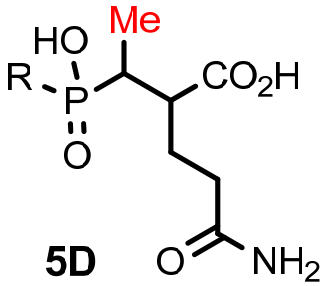
Comparison with first generation inhibitors





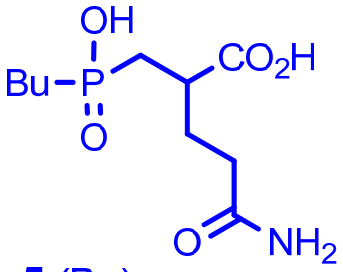
no inhibition





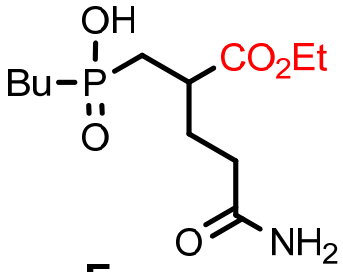
Compounds with methyl-groups at the backbone do not inhibit (IC_{50} > 40000) in contrast to inhibitors with unsubstituted glutamine backbones

Inhibition: reference compounds



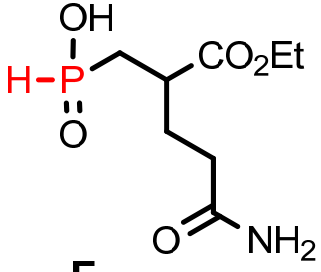
5 (Bu)

IC_{50} = 451



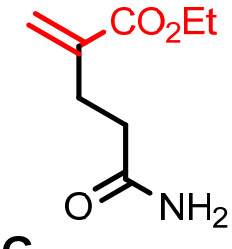
E

IC_{50} = 18000



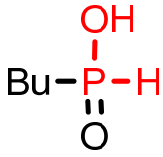
F

none



G

none



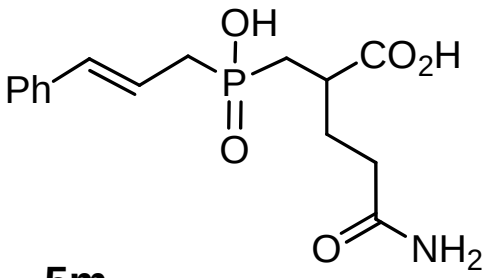
3 (Bu)

none

Glutamine-type backbone + dialkyl phosphinyl group necessary as well as free CO_2H, free amide

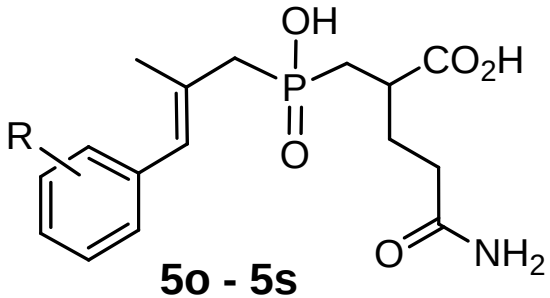
Inhibition: P-allylic analogues:

were good but not better than 1th generation inhibitors, Exception 5z



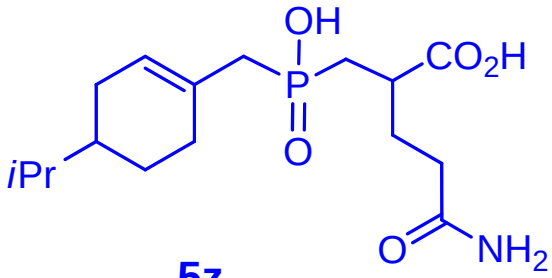
5m

IC_{50} = 1167



5o - 5s

IC_{50} = 460 - 2500



5z

IC_{50} = 32

9 examples: R = *ortho*- and *para*-*t*Bu, *i*Pr, *i*Bu

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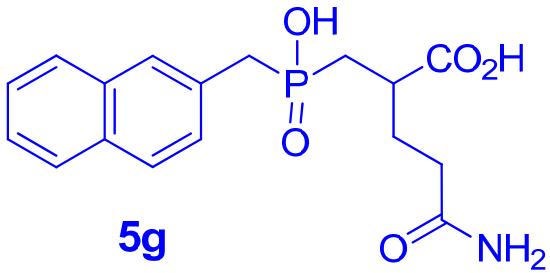
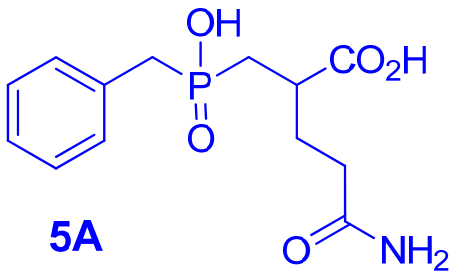
Inhibition: P-benzylic analogues:

some were slightly better than the 1th generation P-Benzyl inhibitor



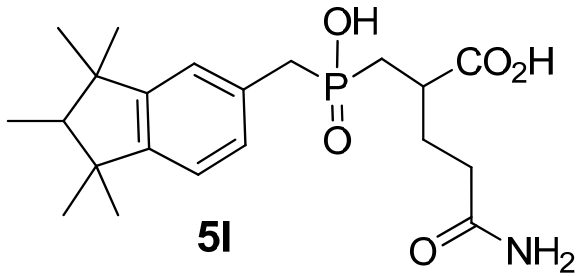
5g

IC_{50} = 138



5A

IC_{50} = 440



5I

IC_{50} > 10000

9 examples: +I and –I substituents (fluorination):

steric rather than electronic effects, planarity

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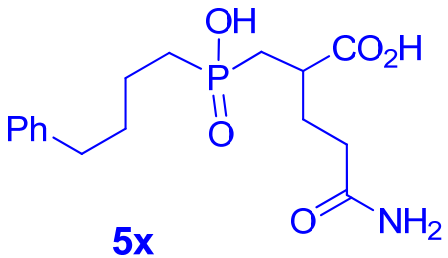
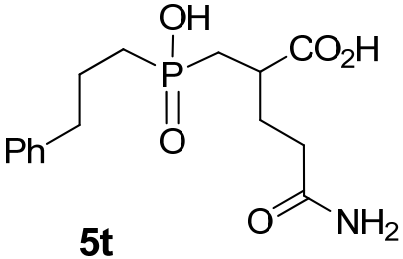
Inhibition: P-Alkyl analogues:

good to very good inhibition



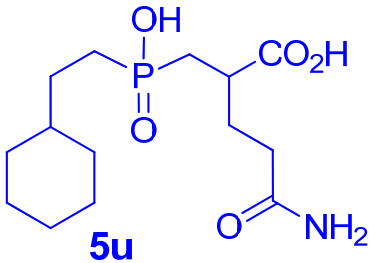
5x

IC_{50} = 40



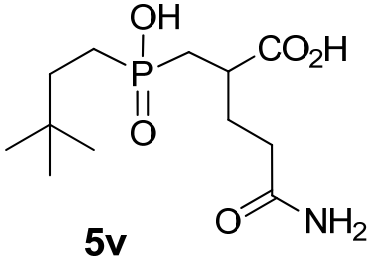
5t

IC_{50} = 125



5u

IC_{50} = 45



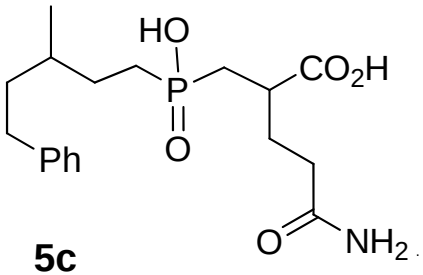
5v

IC_{50} = 100

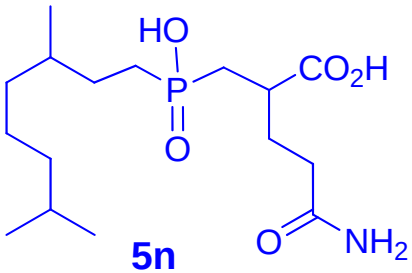
8 examples: flexibility in the α,β-position, P-ethylene bridge

A. Natsch, F. Schröder, WO 2004043971, Givaudan

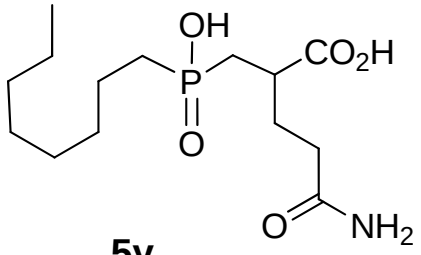
Inhibition: 2th generation



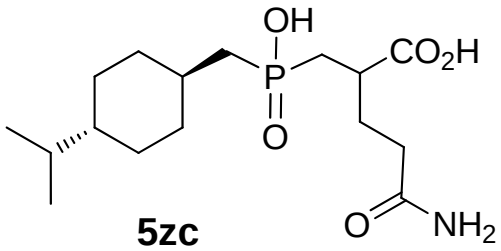
5c
ex Phenoxanol



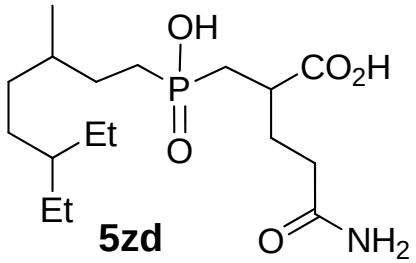
5n
ex Geraniol



5y
ex octyl iodide



5zc
ex Pinene

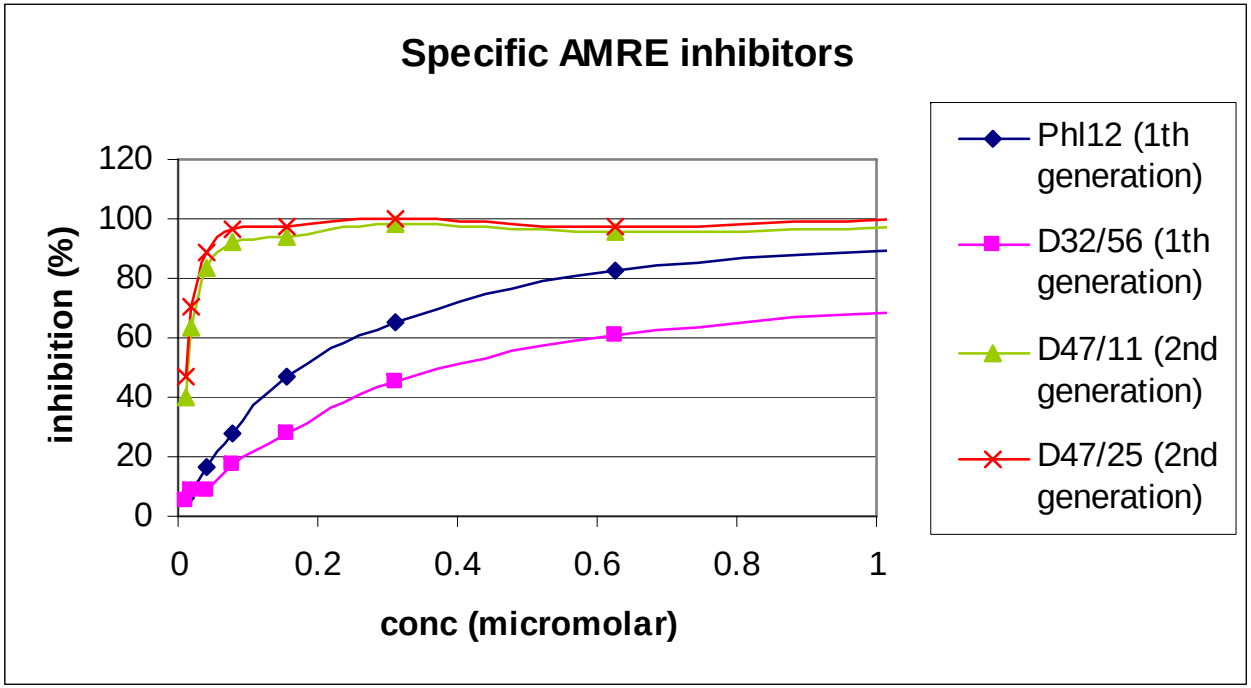


5zd

5 best examples: IC_{50} = 11 - 19

A. Natsch, F. Schröder, WO 2004043971, Givaudan

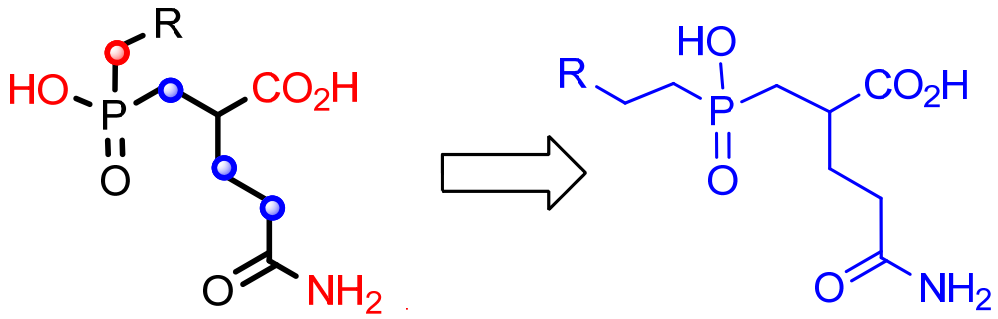
Inhibition: 2th generation



- **Second generation: IC_{50} ~ 10 – 20 (versus 200 – 400, 1th generation)**

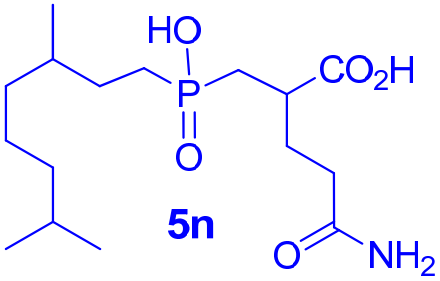
A. Natsch, F. Schröder, WO 2004043971, Givaudan

Inhibition results: elaboration of the key



- **Glutamine backbone**
- **Substituents at the backbone detrimental**
- **Requirements: dialkyl phosphinyl group, free CO_2H, 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



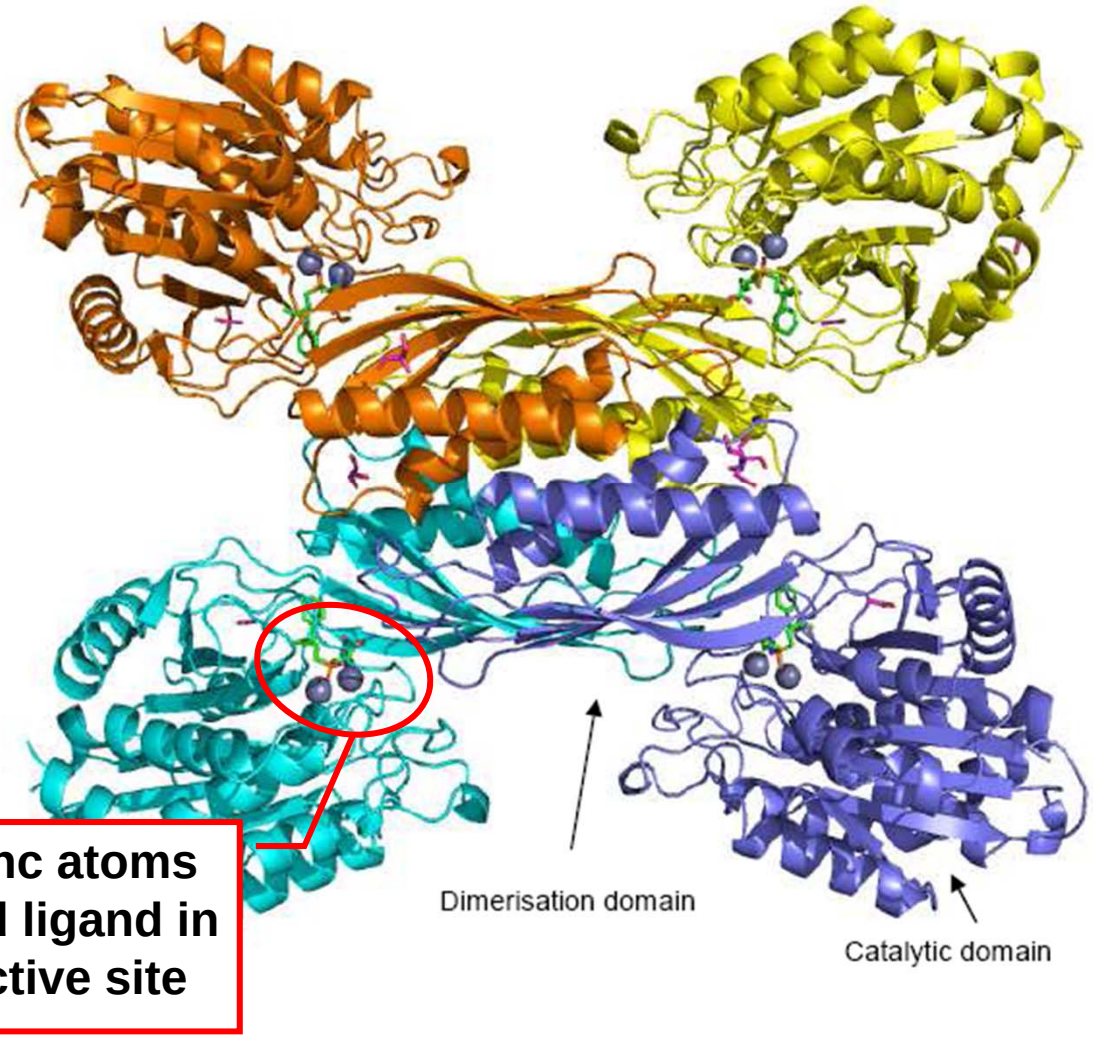
IC_{50} = 19
mp 135°-145°C (iPrOH, EE 1:2)
> 98% purity (NMR, ESI-MS)

- **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

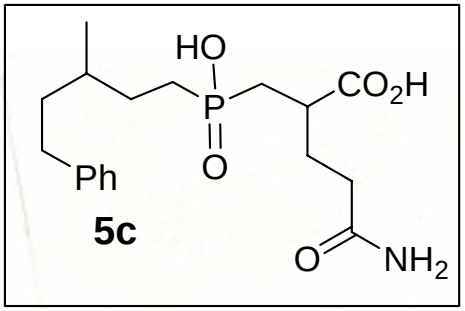
A. Natsch, F. Schröder, WO 2004043971, Givaudan

Cristall structure of AMRE with bound inhibitor

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



AMRE / Inhibitor Complex: LB / HBs and Zn-interactions at the active Site

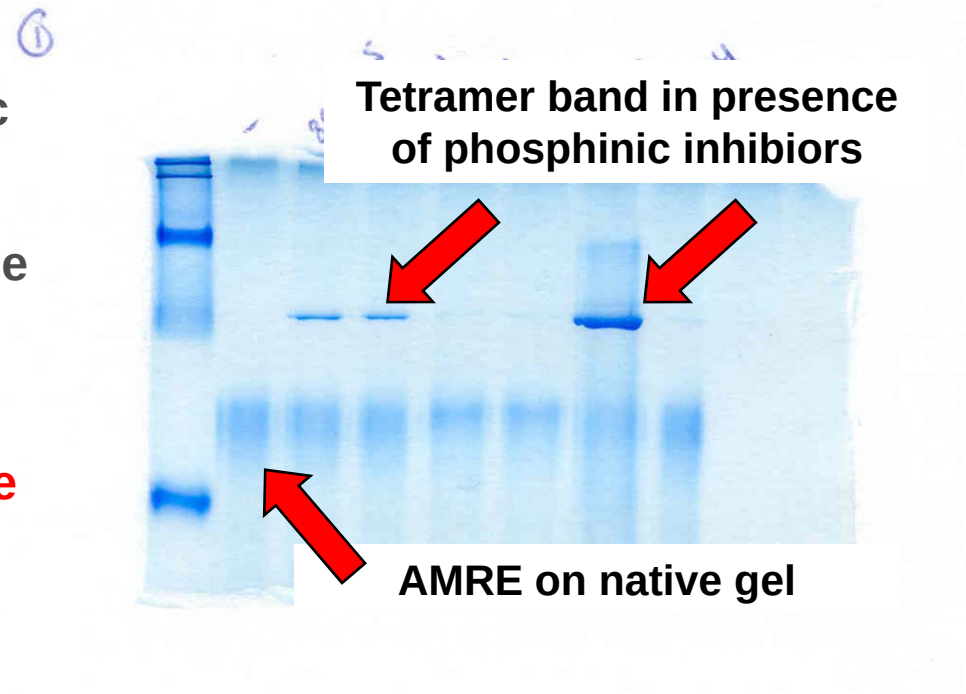


- **Absolute stereochemistry**
- **Replacement of functional groups**

Unusual binding mode of phosphinic inhibitors

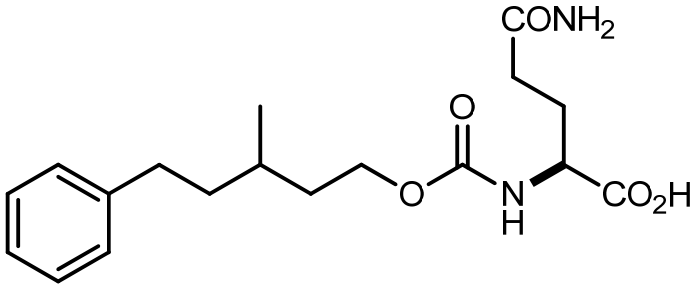
- Phosphinic inhibitors are thought to be classical transition state analogues
- Typically, these are **competitive inhibitors**
- IC50 is dependent on substrate concentrations (Increasing substrate concentration in test raises IC50)

- **This is not the case for the phospinic inhibitors of AMRE!**
- **Inhibitors give kinetic plots as suicide inhibitors**
- **The phosphinic inhibitors catalyze stable tetramerizations of the enzyme**

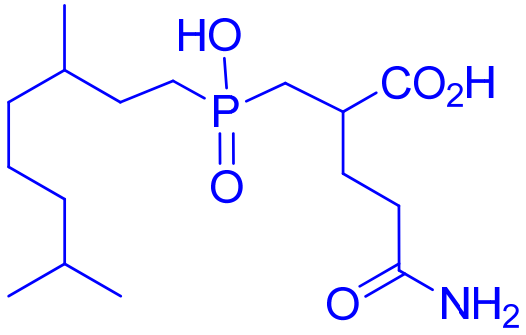


...and the ‚clinical' reality

- 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

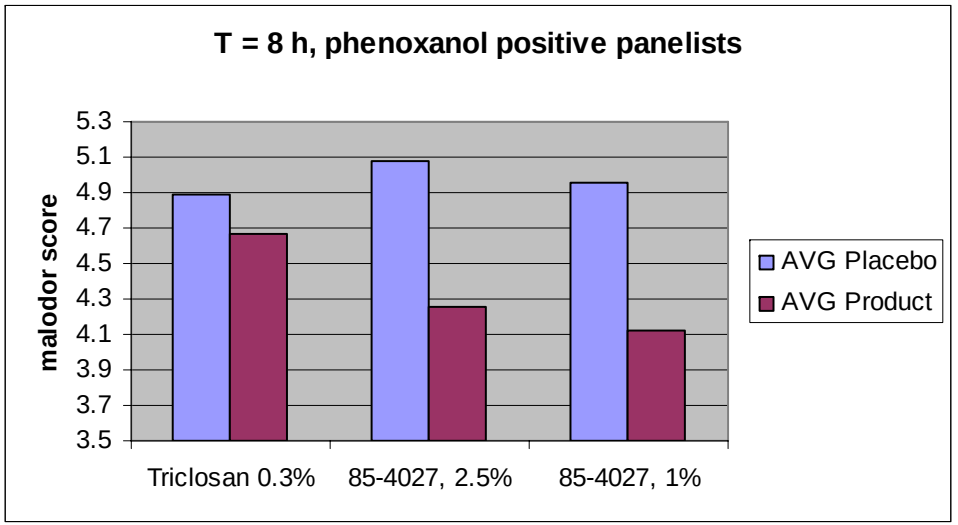


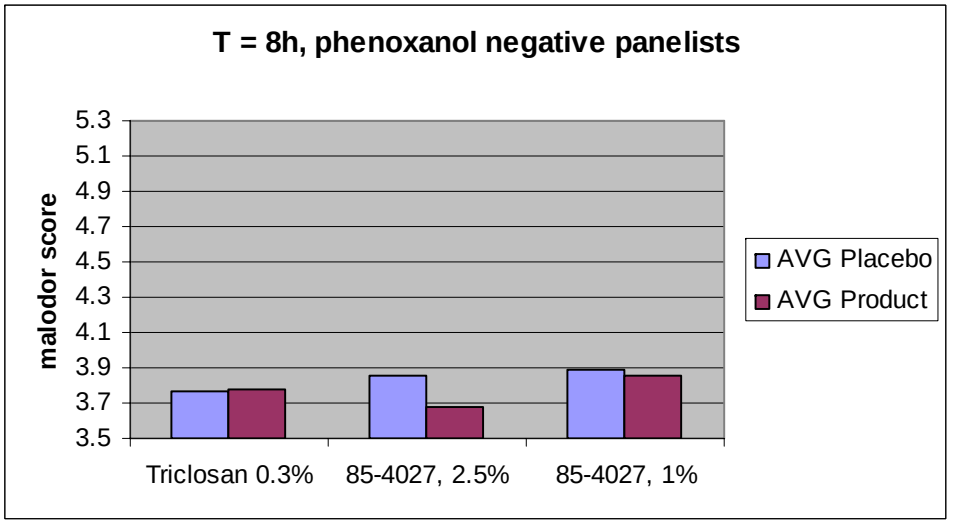
GR-86-3021, best phosphinic inhibitor

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-signifcant

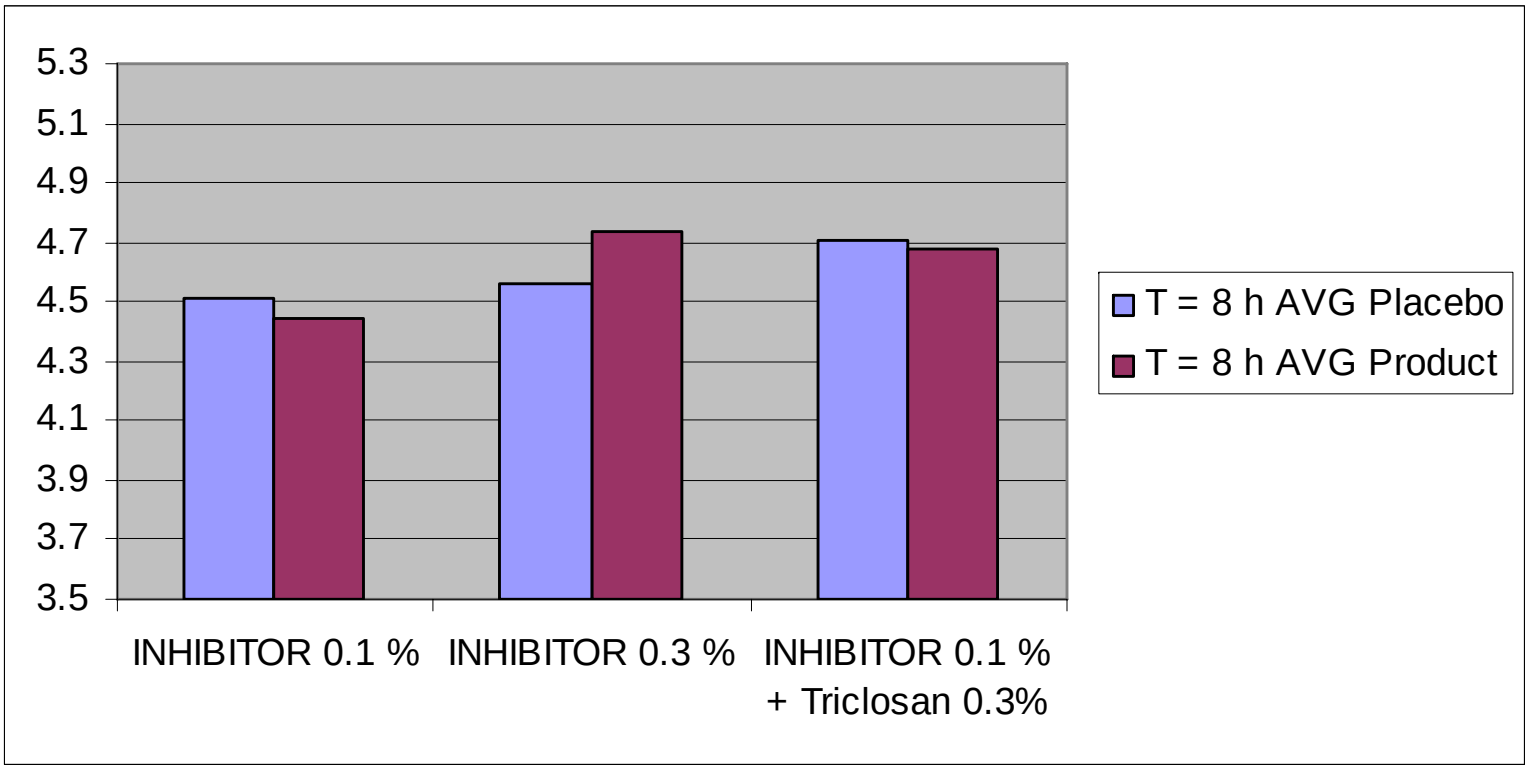
⇨The product works on those panelists with a real malodor problem





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 cristallisation and structure determination**: A. Douangamath, J. Baker (EvoTec)
- **Threshold determinations and sensory data**: H. Koch
- **Molecular Modelling Studies** A.Borosy, H.-P.Weber

Givaudan

AND NOW – for something completely different

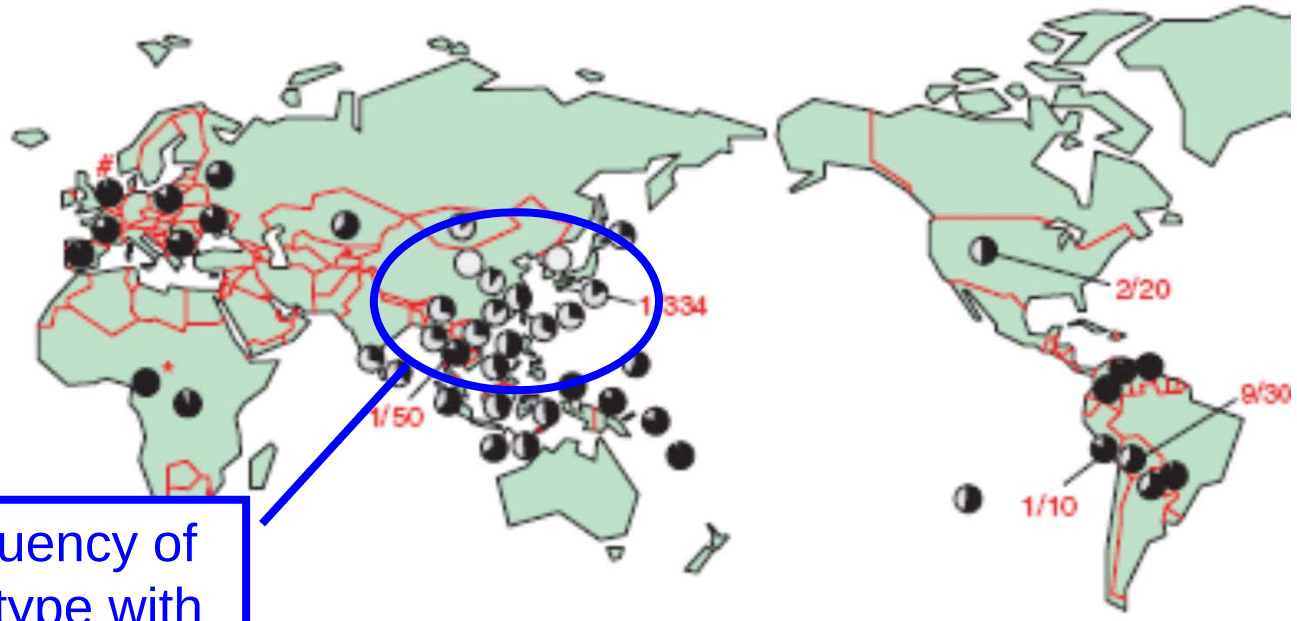
Why Chinese people do not have this problem - or

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 allel
- In white frequency of the ABCC11 mutation



dwide frequency of allele A (open portion in each circle).

High frequency of the haplotype with the mutated allele

Reproduces from Nature genetics,Jan. 2006.

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

Genotyp ABCC11	Ethnic population	Secreted amino-acid conjugates (µMol / 2 pads)		
		HMHA-Gln	3M2H-Gln	86-8434
AA	Philippino	n.d.[1)]	n.d.	n.d.
AA	Chinese	n.d.	n.d.	n.d.
AA	Philippino	n.d.	n.d.	n.d.
AA	Korean	n.d.	n.d.	n.d.
AA	Chinese	n.d.	n.d.	n.d.
AA	Philippino	n.d.	n.d.	n.d.
AA	Philippino	n.d.	n.d.	n.d.
AA	Philippino	n.d.	n.d.	n.d.
AA	Honkong chinese	n.d.	n.d.	n.d.
AA	Philippino	n.d.	n.d.	n.d.
AA	Philippino	n.d.	n.d.	n.d.
AG	Philippino	1.23	0.17	detectable
AG	Philippino	1.58	0.23	0.041
AG	Philippino	0.06	detectable,	n.d.
AG	Philippino	2.71	0.40	detectable,
AG	Thai	0.89	0.14	detectable,
AG	German	1.18	0.18	0.045
AG	German	0.54	0.10	detectable,
GG	Philippino	0.77	0.13	detectable,
GG	Philippino	0.75	0.11	detectable,
GG	German	1.30	0.19	0.041
GG	German	1.12	0.16	0.038
GG	German	2.65	0.43	0.051
GG	German	0.34	0.09	detectable,
GG	Swiss	0.85	0.18	n.d.

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?