

Summer 6-3-2012

Genomics Based Engineering for the Identification and Optimization of Bioactive Microbial Natural Products

Rolf Müller
Saarland University

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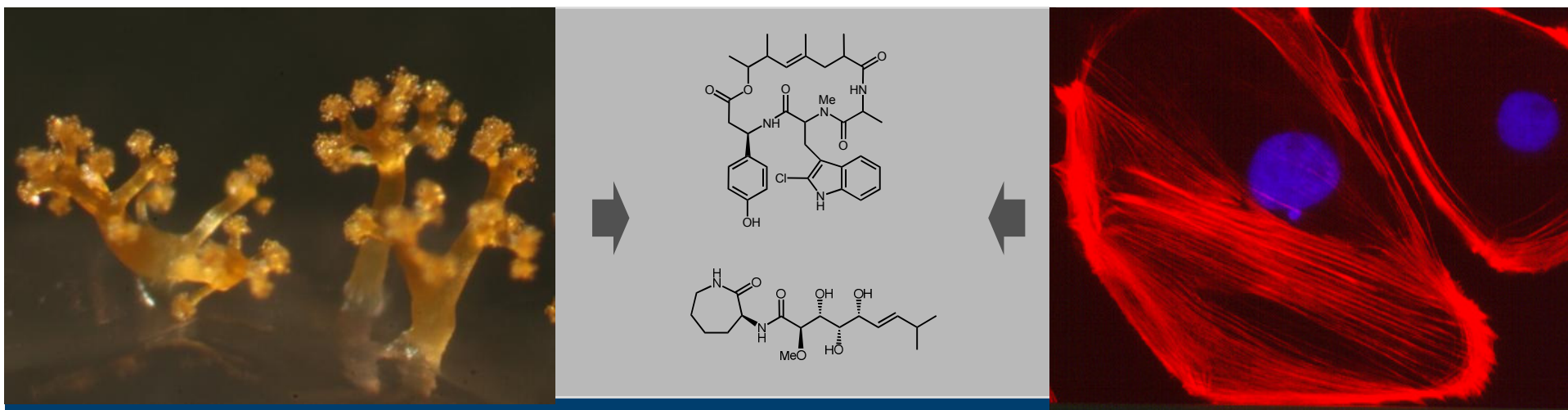
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Genomics based Engineering for the Identification and Optimization of bioactive Microbial Natural Products



Metabolic Engineering IX
Biarritz, June 3-7

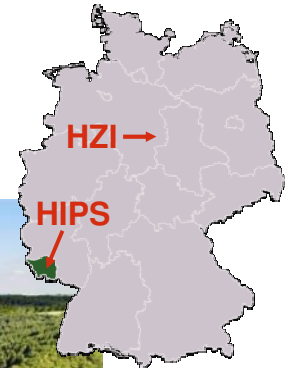
Rolf Müller rom@helmholtz-hzi.de

Helmholtz-Institut für Pharmazeutische Forschung Saarland

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ZENTRUM FÜR
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 UNIVERSITÄT
DES
SAARLANDES

Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS)



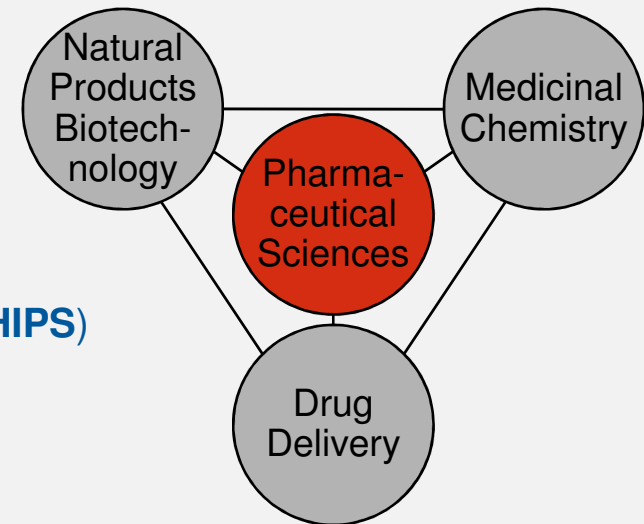
Saarland University Campus



HIPS

- founded Nov. 2009
- part of the Helmholtz-Center for Infection Research

[Helmholtz-Institute for Pharmaceutical Research Saarland \(HIPS\)](#)

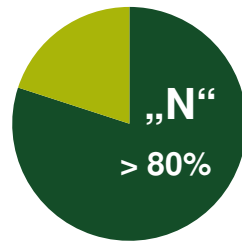
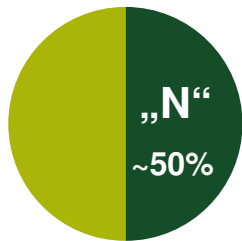


Natural Products Research and Drug Discovery

Role of Natural Products in Drug Discovery 1940-2010:

Anticancer

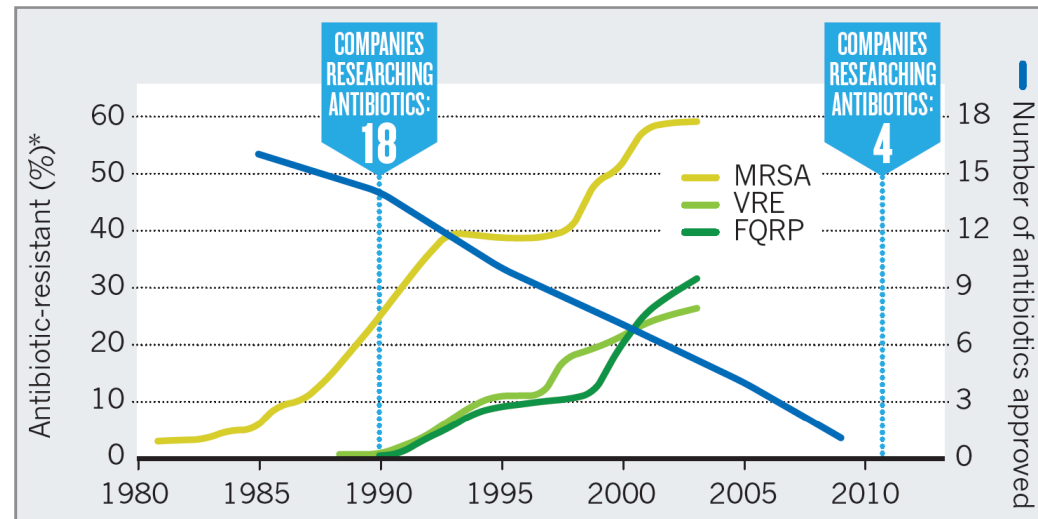
Antibacterial



„N“: NPs or NP-derived Small Molecules

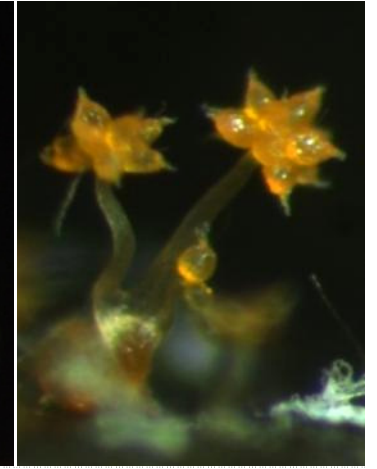
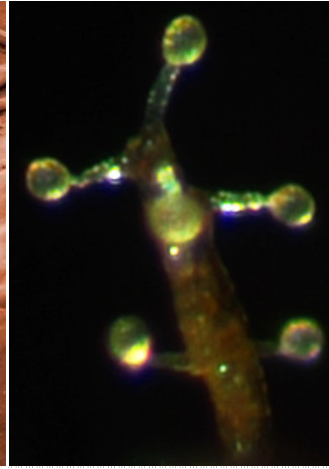
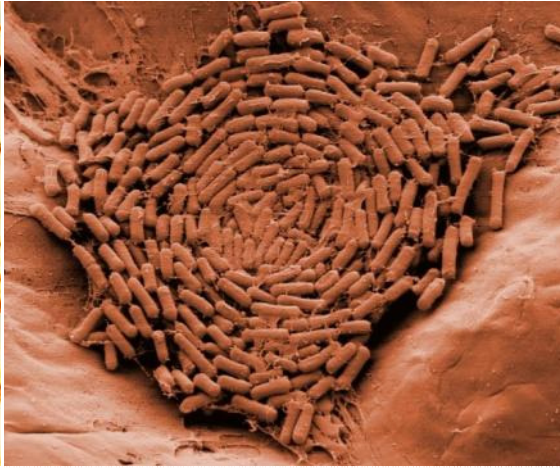
Newman & Cragg, *JNP* 2012

A PERFECT STORM . . .



Cooper & Shlaes, *Nature* 2011

“ ... a significant number of natural product drugs/leads are actually produced by microbes and/or microbial interactions with the “host from whence it was isolated”, and therefore we consider that this area of natural product research should be expanded significantly.”



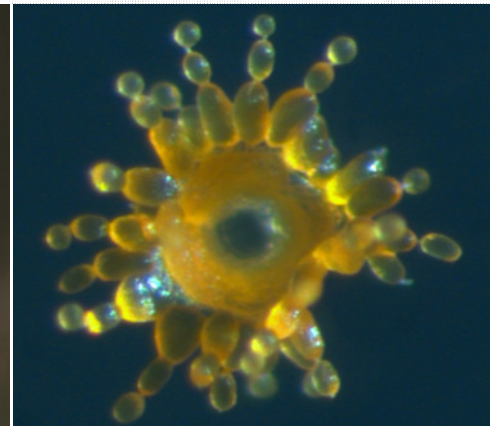
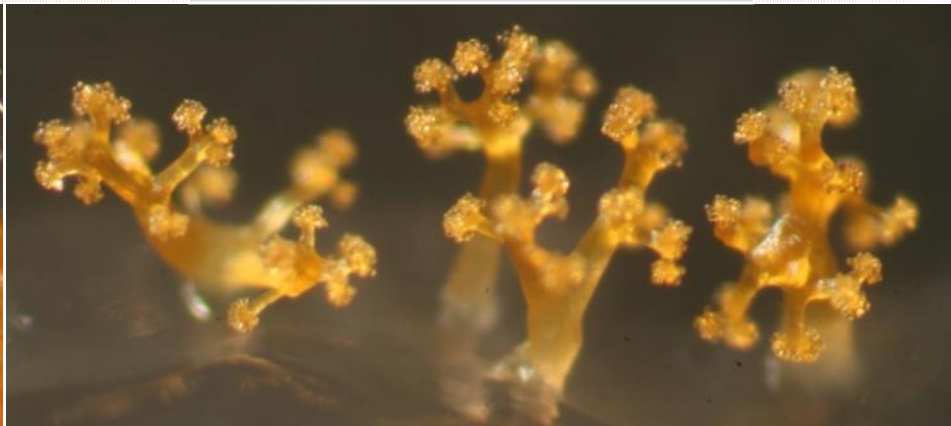
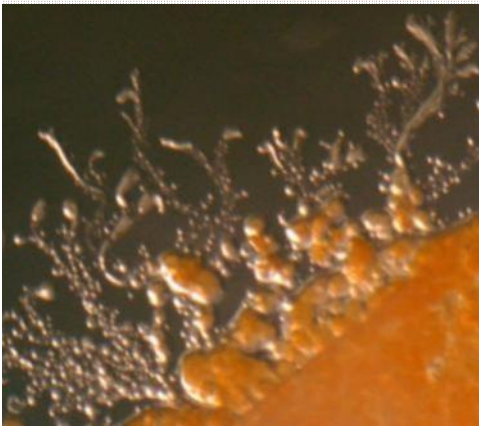
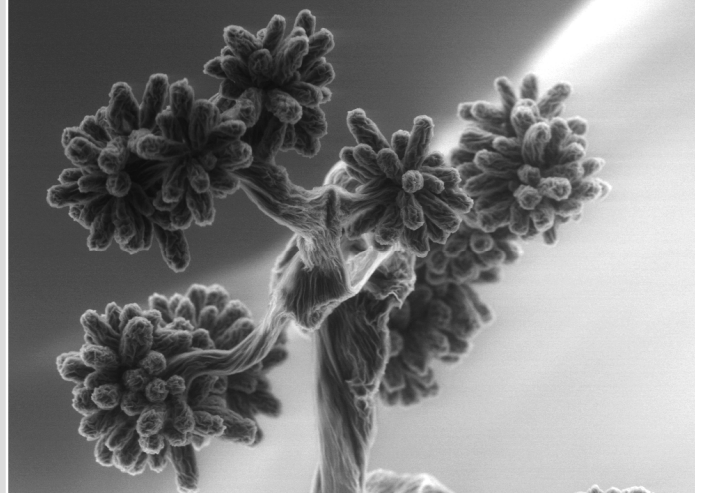
MYXOBACTERIA

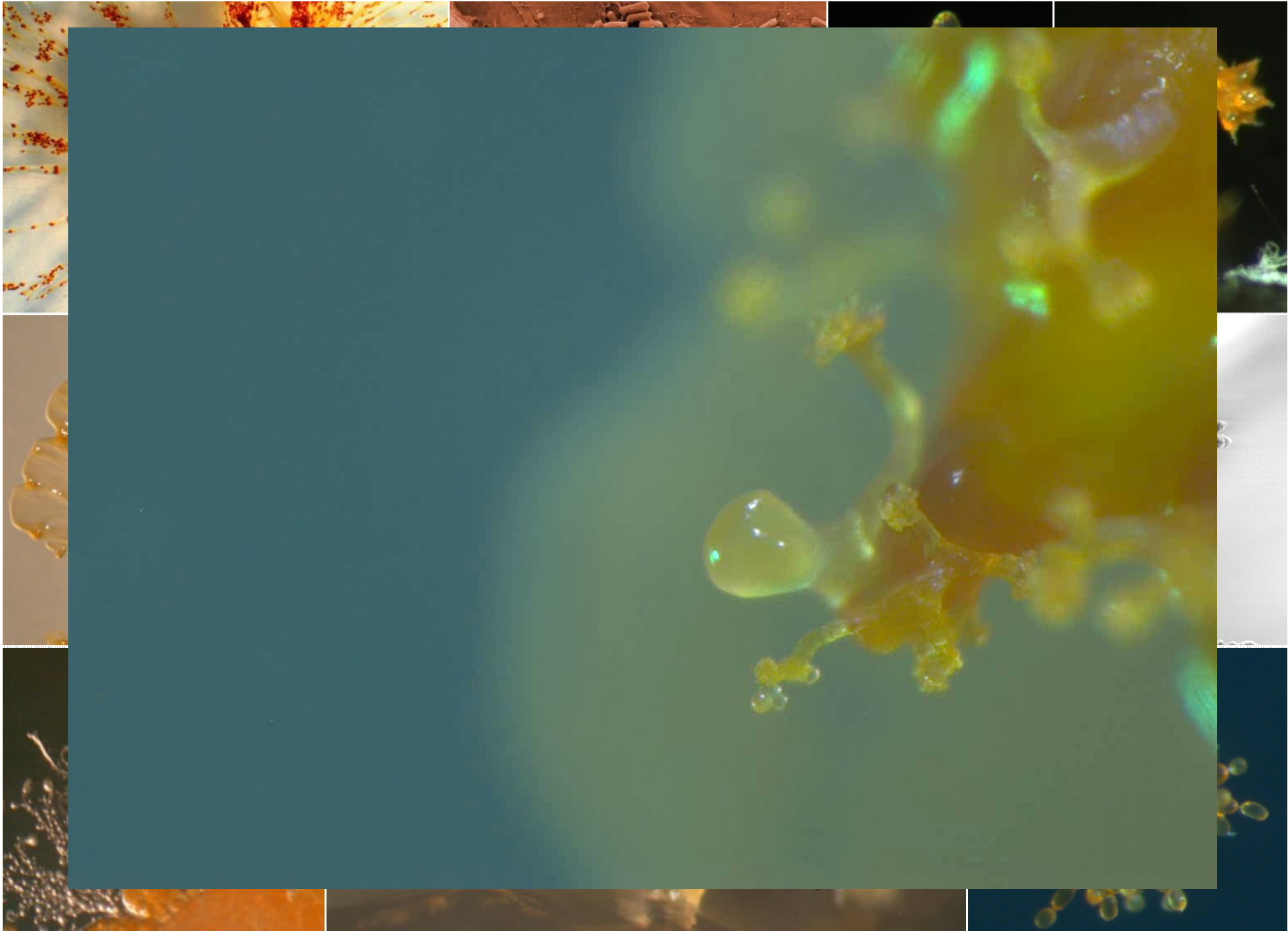
Little studied because of

- slow growth
- difficult isolation procedure
- poorly established genetics

Advantages

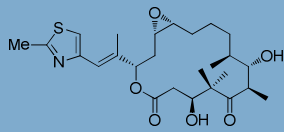
- new compounds
- new modes of action
- low risk of isolating known compounds



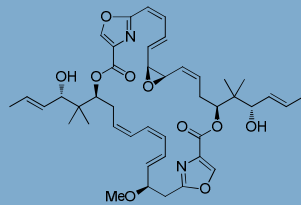


Bioactive Compounds from Myxobacteria

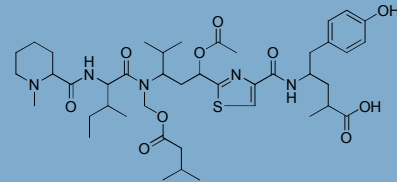
Cytotoxic



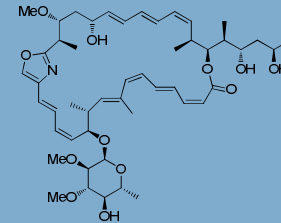
Epothilones



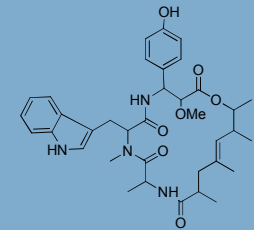
Disorazols



Tubulysins

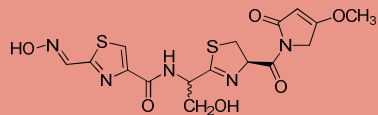


Chivosazols

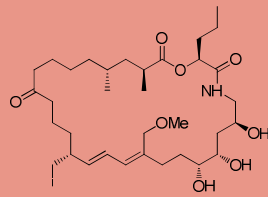


Chondramides

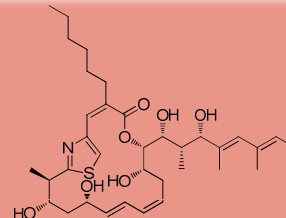
Antibacterial



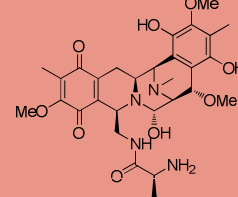
Althiomycin



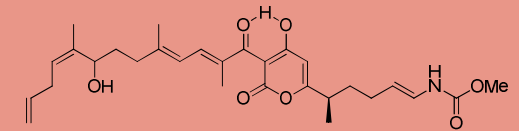
Myxovirescins



Thuggacins

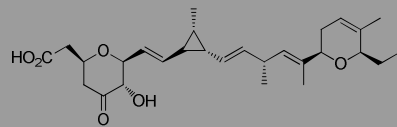


Saframycins

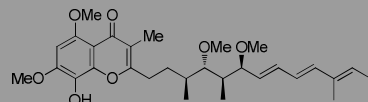


Coralopyronins

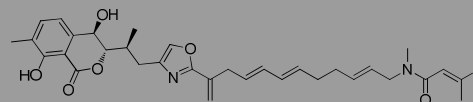
Antifungal



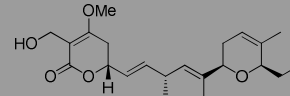
Ambruticin



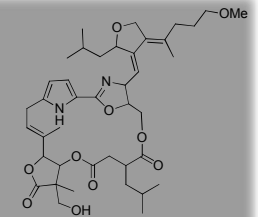
Stigmatellin



Ajudazols

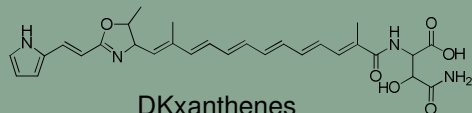


Jerangolids

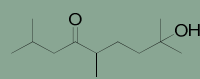


Leupyrrins

Developmental

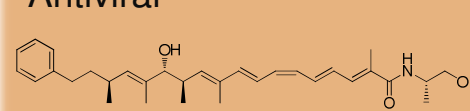


DKxanthenes

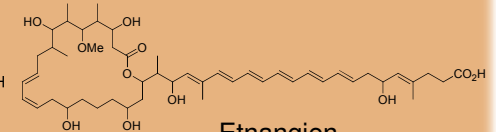


Stigmolone

Antiviral



Phenalamids



Etnangien

Weissman & Müller, *NPR* 2010

Myxobacterial Strain Collection & Screening

> 8000 world-wide isolates

including recently identified novel families, e.g. *Phaselicystidae*

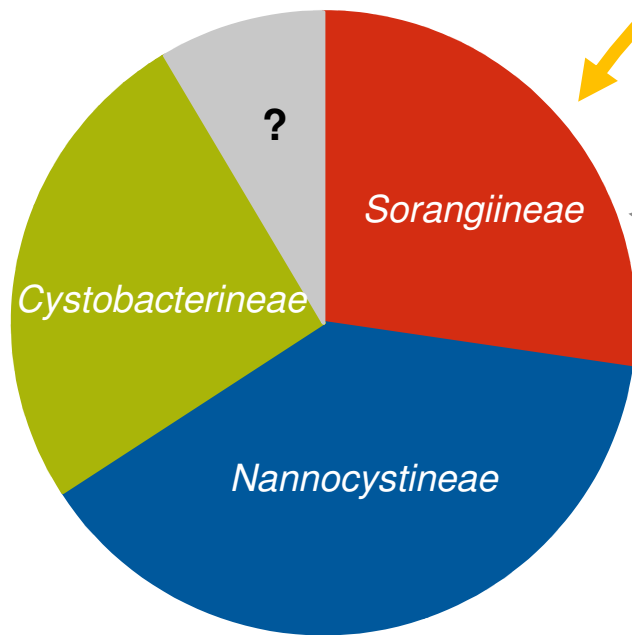
(Garcia et al., *Int. J. Syst. Evolut. Microbiol.*, 2009)



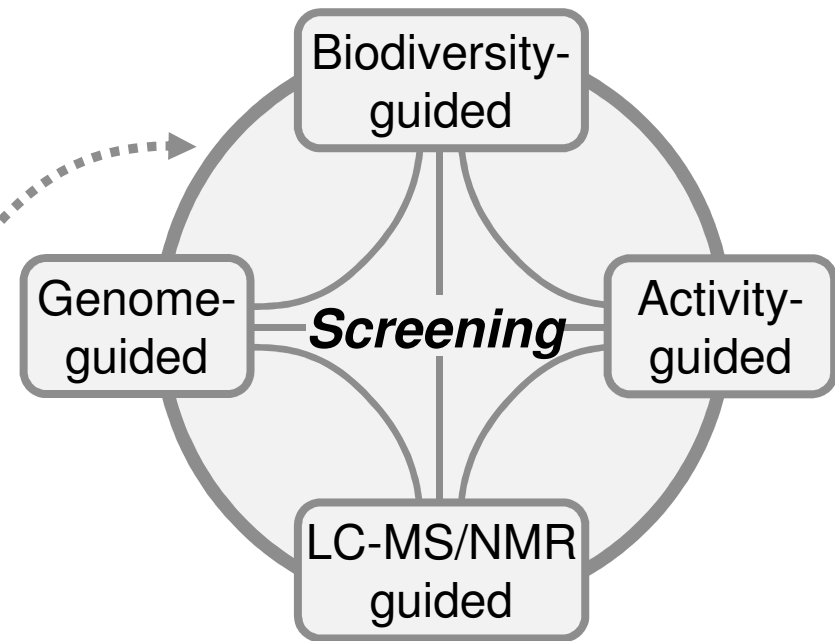
Strain isolation



Soil sample



Myxobase

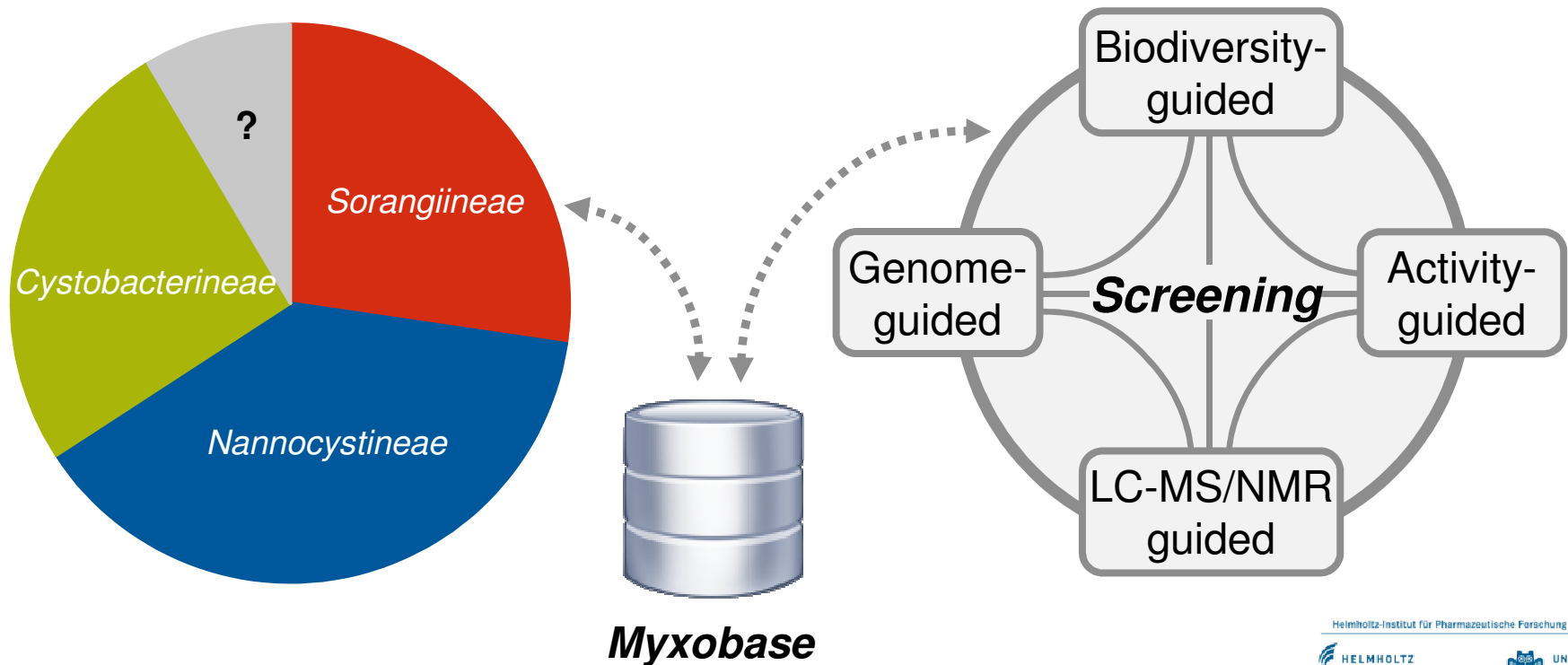
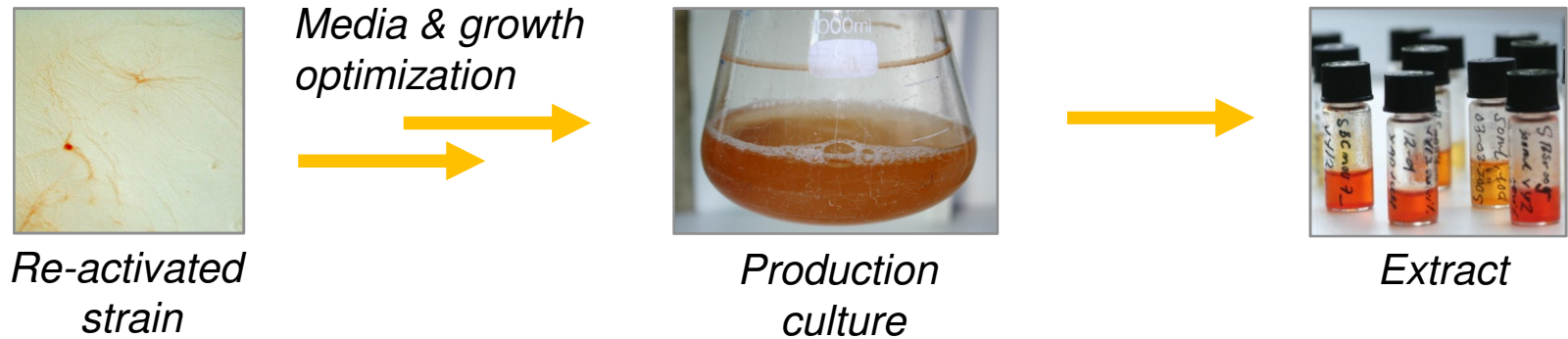


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Myxobacterial Strain Collection & Screening

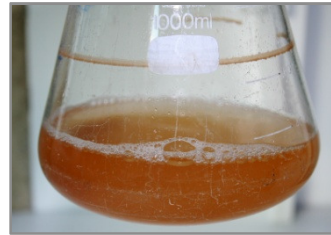


Activity-guided screening



Re-activated strain

Media & growth optimization

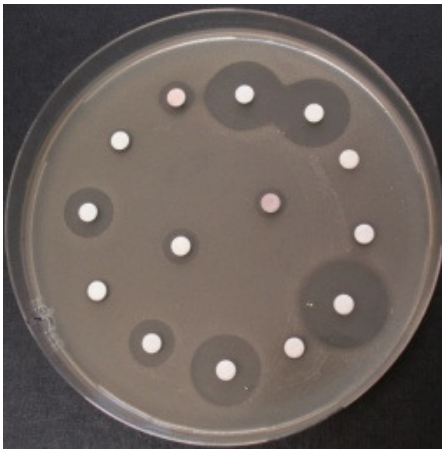


Production culture

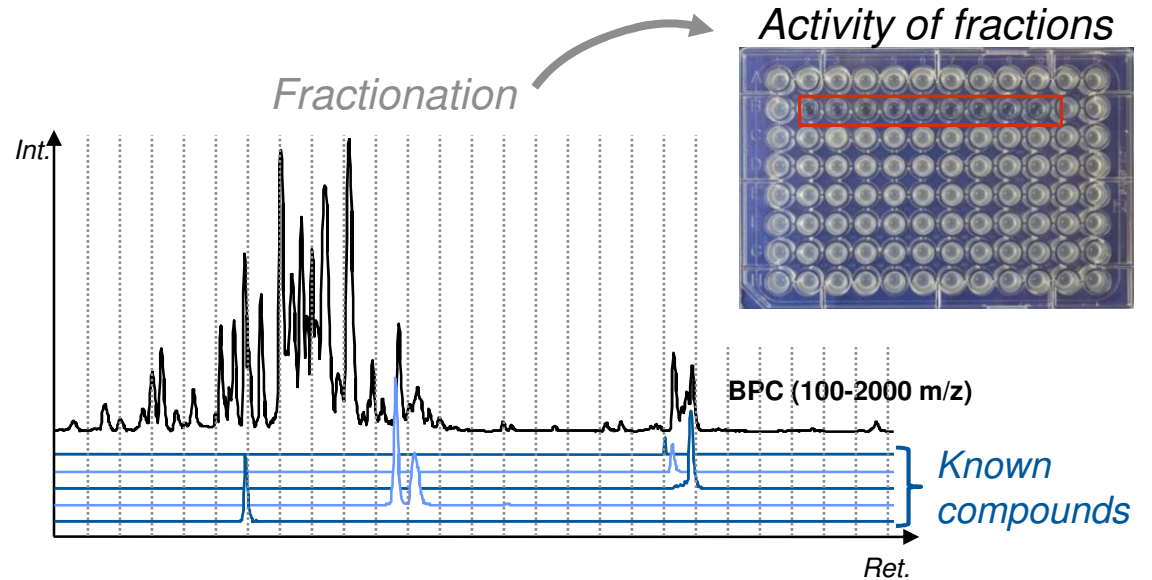


Extract

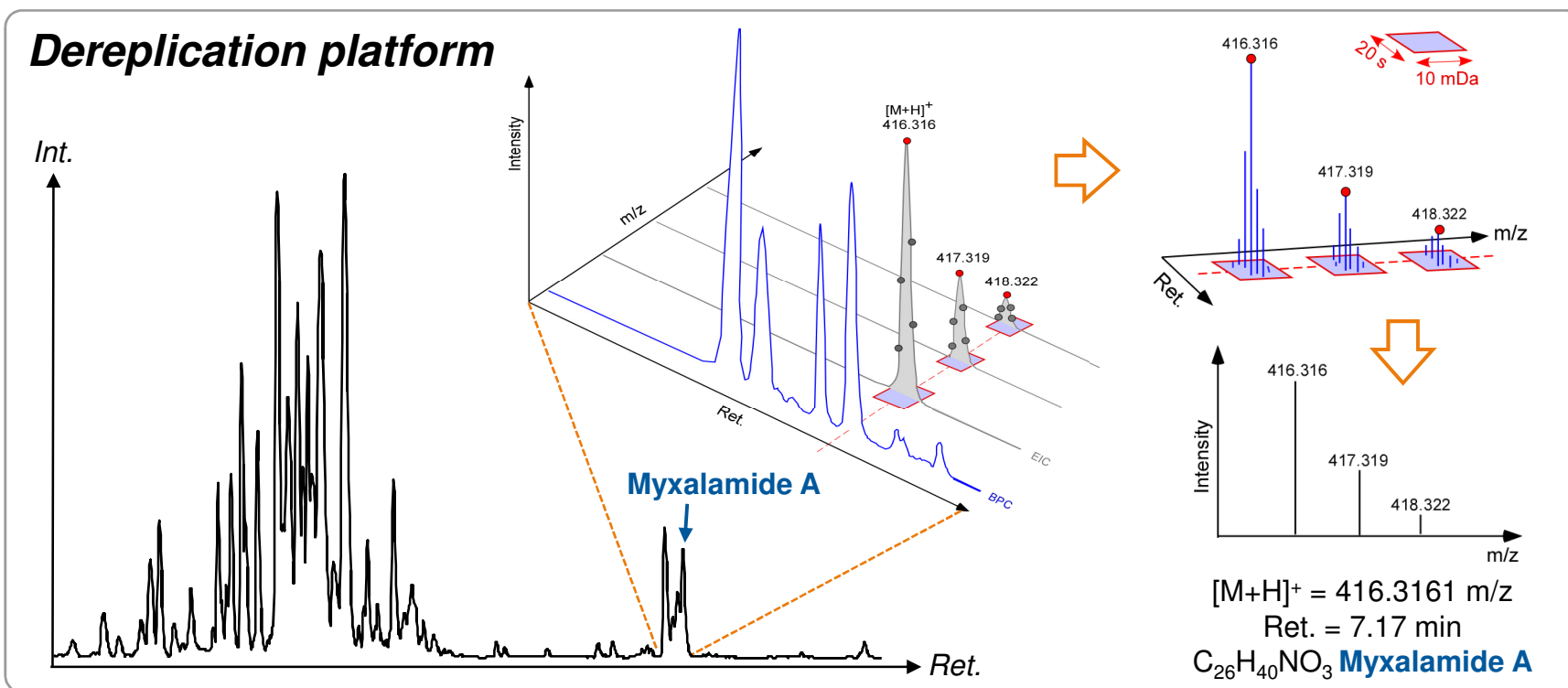
1 'Initial Screen' for activity (crude extract)



2 HPLC fractionation of crude extract coupled to biological assays



How to find something new?



Activity-guided screening – Current status



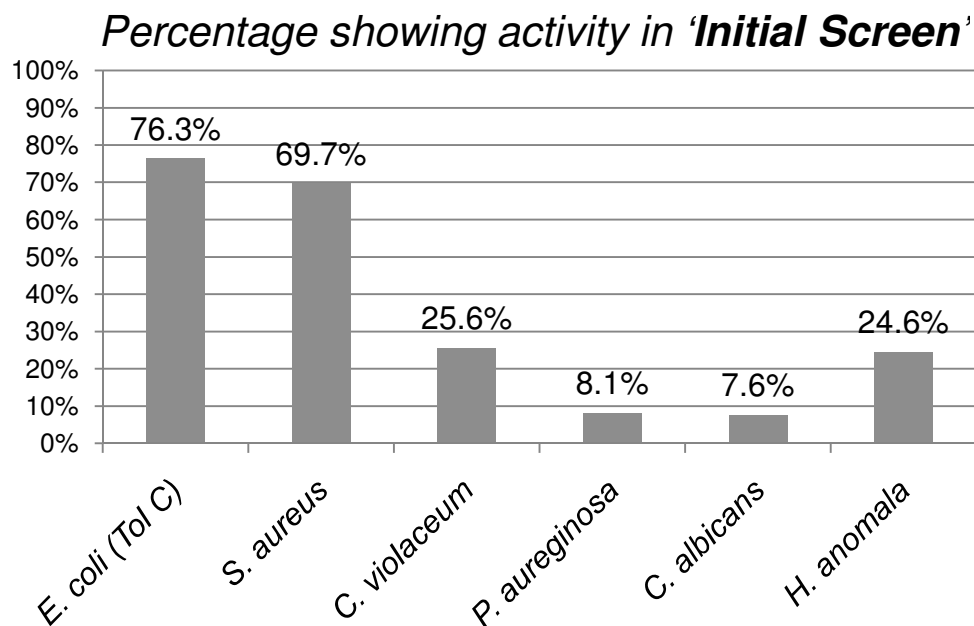
'Myxo strain collection'
~ **8000 strains**

↓

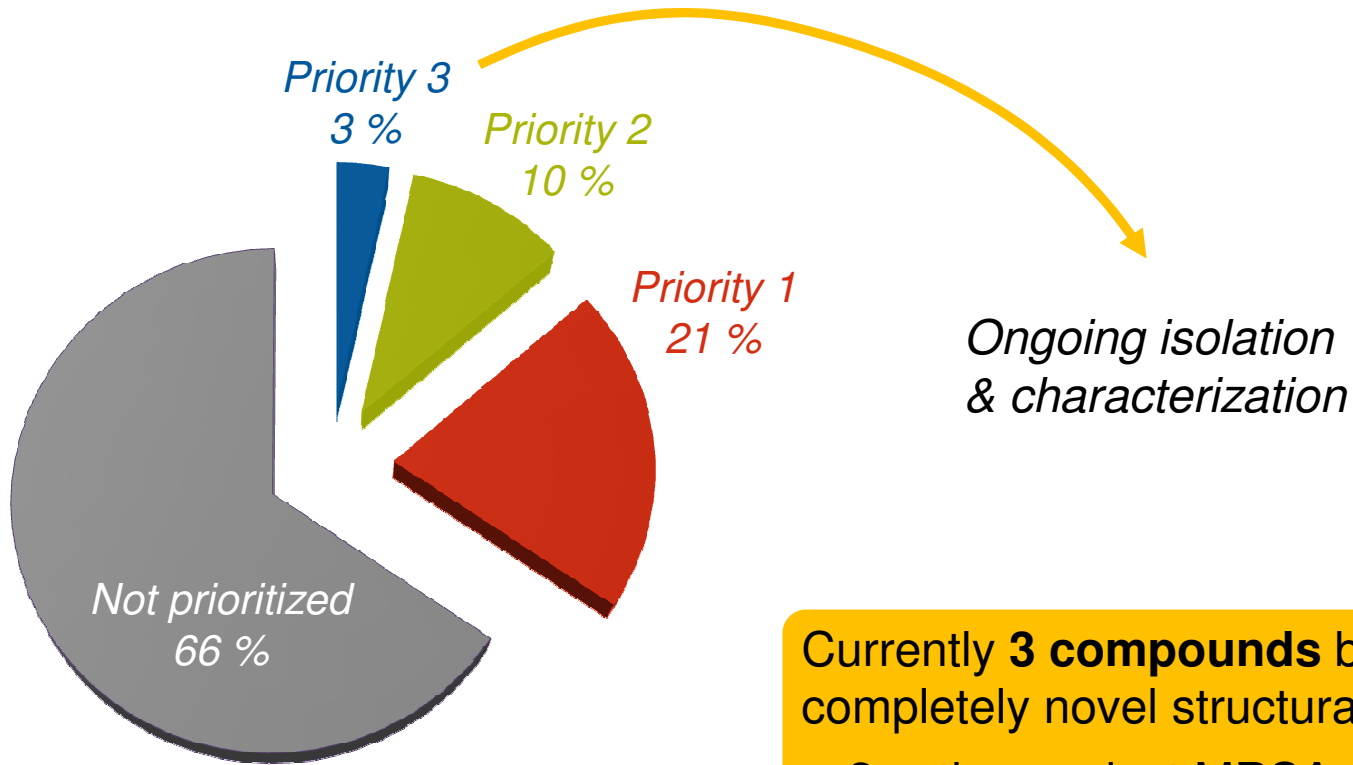
~ **1700 strains** re-activated
& '**Initial Screen**' for activity

↓

~ **330 extracts**
fractionated, de-replicated
and in-depth bioactivity
screening



Re-screening of the Myxobacterial Strain Collection - Prioritization



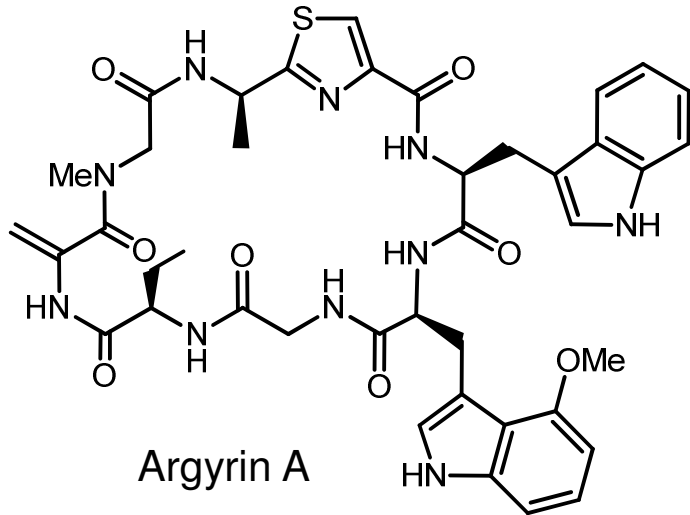
Criteria for further evaluation (done for 330 species):

- Novelty
- Activity
- Yield

Currently **3 compounds** belonging to completely novel structural classes

- 2 active against MRSA
- 1 active against Gram-negatives

Argyrin – Lead structures for antibiotic development?



- Self-resistance (microbial producer)?
- Antibacterial profile/target?
- Development of resistance?

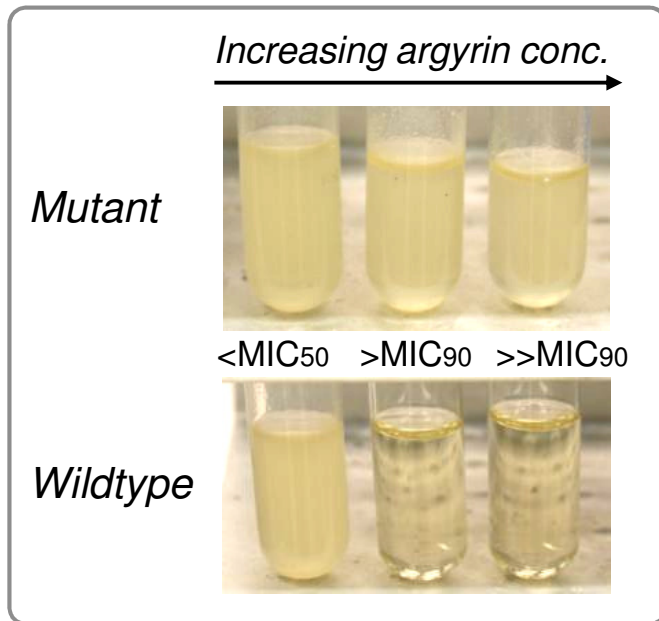
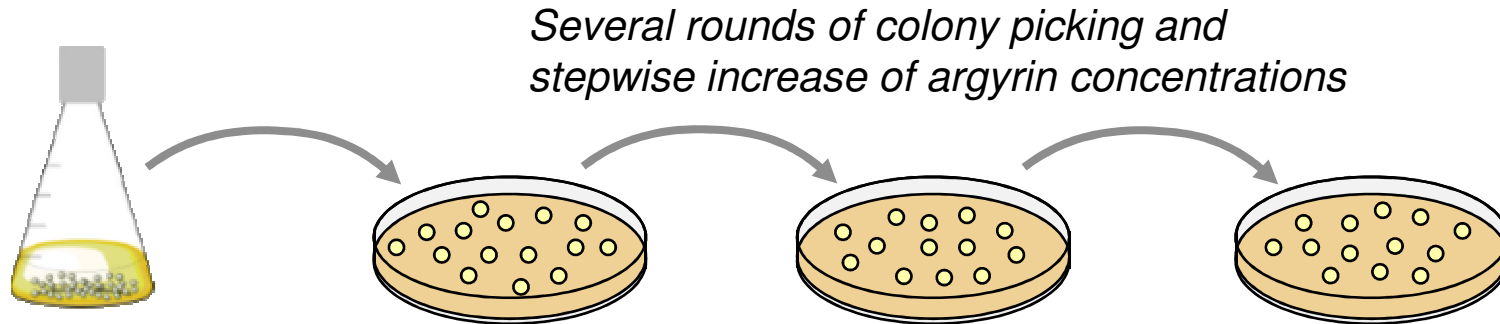
Antimicrobial activity of argyrins A to D

Test organism	Diameter of inhibition zone [mm]			
	A	B	C	D
<i>Bacillus subtilis</i> DSM 10	0	0	0	0
<i>Escherichia coli</i> DSM 498	0	0	0	0
<i>E. coli</i> tolC GBF	9	8	0	0
<i>Micrococcus luteus</i> GBF	0	0	-	7
<i>Mycobacterium phlei</i> GBF	0	0	0	0
<i>Pseudomonas aeruginosa</i> DSM1117	13	15	14	13
<i>P. acidovorans</i> GBF	16	15	15	14
<i>Staphylococcus aureus</i> GBF	7	0	7	0
<i>Candida albicans</i> CBS 1893	0	0	-	-
<i>Hansenula anomala</i> DSM 70263	0	0	0	0
<i>Metschnikowia pulcherrina</i> DSM 70321	0	0	0	0
<i>Botrytis cinerea</i> DSM 877	12	13	11	0
<i>Mucor hiemalis</i> DSM 2655	0	0	-	-
<i>Pythium debaryanum</i> DSM 62946	10	10	0	0

Sasse et al, *J. Antibiot.* 2002

Argyrimin Resistance Mechanism & Bacterial Target

1. Generation of argyrimin-resistant *Pseudomonas aeruginosa* mutants

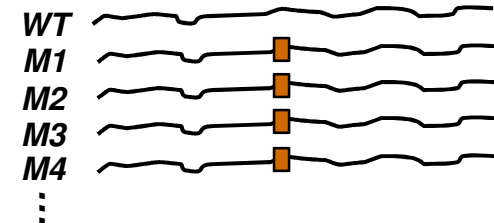
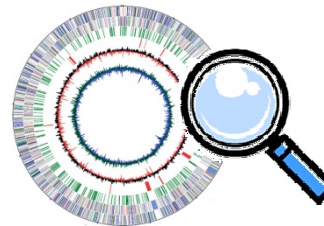


2. Isolation of chromosomal DNA for next-generation genome sequencing

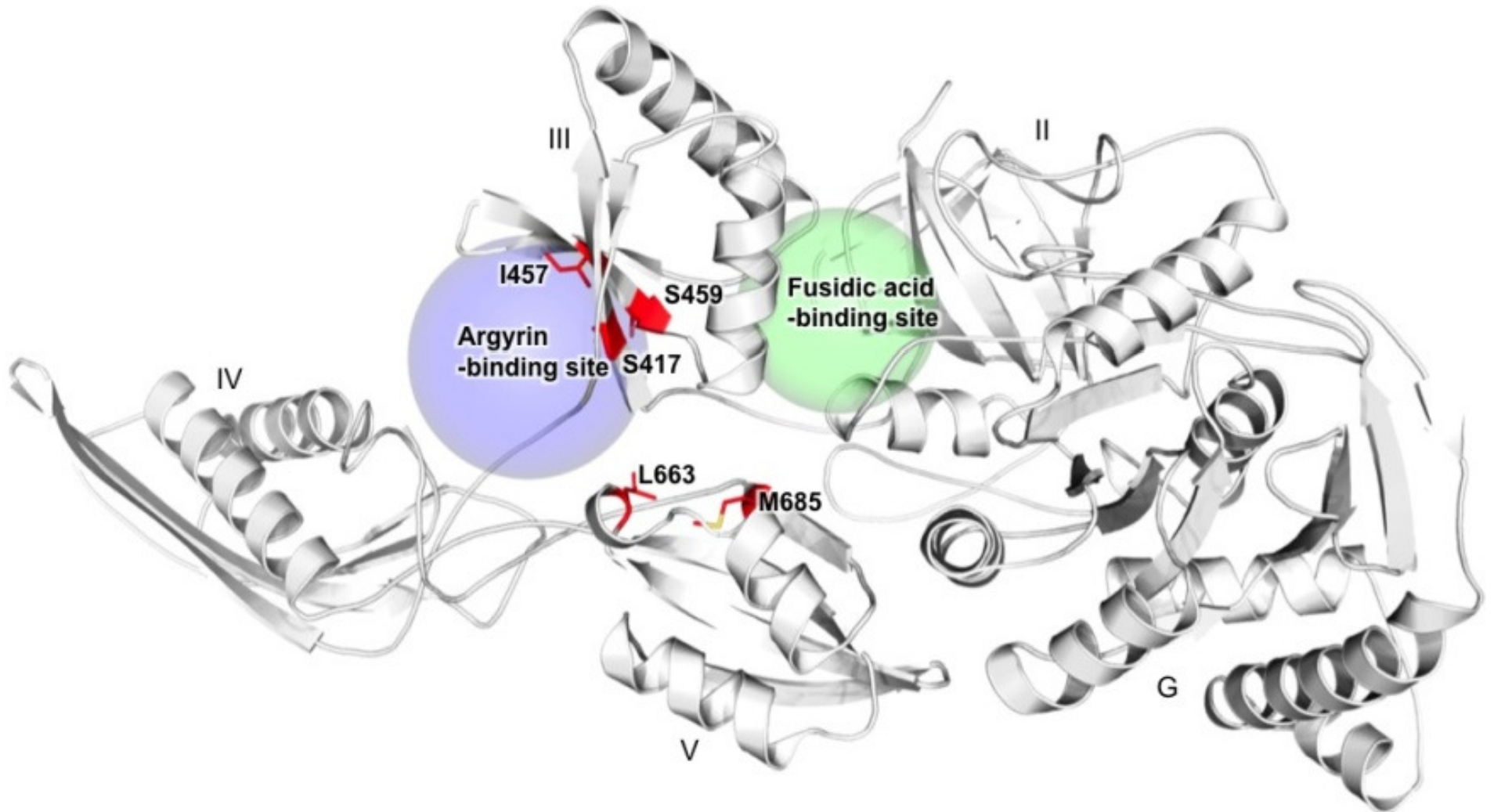
2 x Wildtype DNA

8 x Mutant DNA (independent clones!)

3. *In silico* analysis of the genome data for resistance mutations



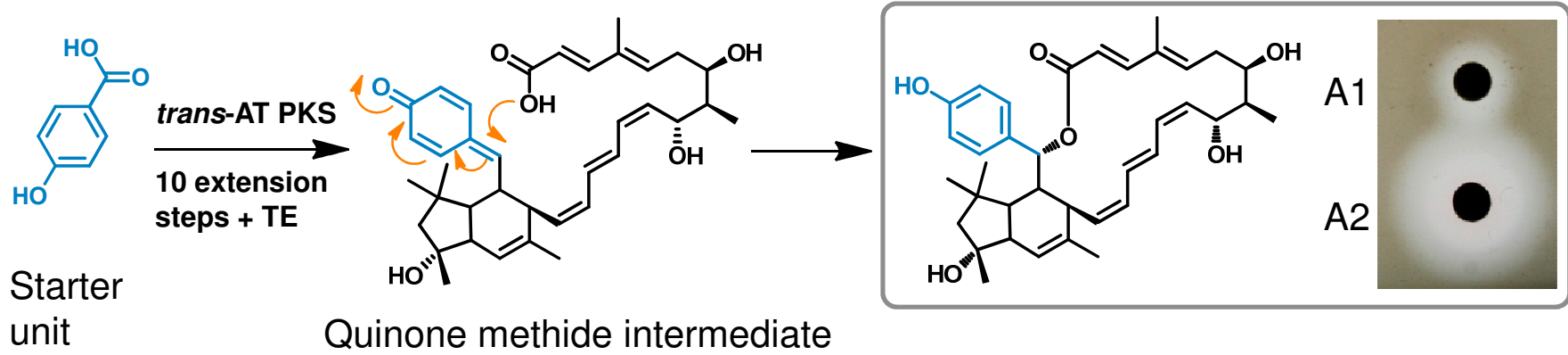
Mutation in Elongation Factor G confers Argyrin Resistance



Bielecki *et al.*, submitted

Elansolids: Novel MRSA actives from *Chitinophaga sancti*

Elansolids A1 and A2: Stable Atropisomers with different activities



➡ Need for stable derivatives

➡ Biosynthetic trickbox



mutasynthesis, precursor directed biosynthesis, domain alteration

+ New target

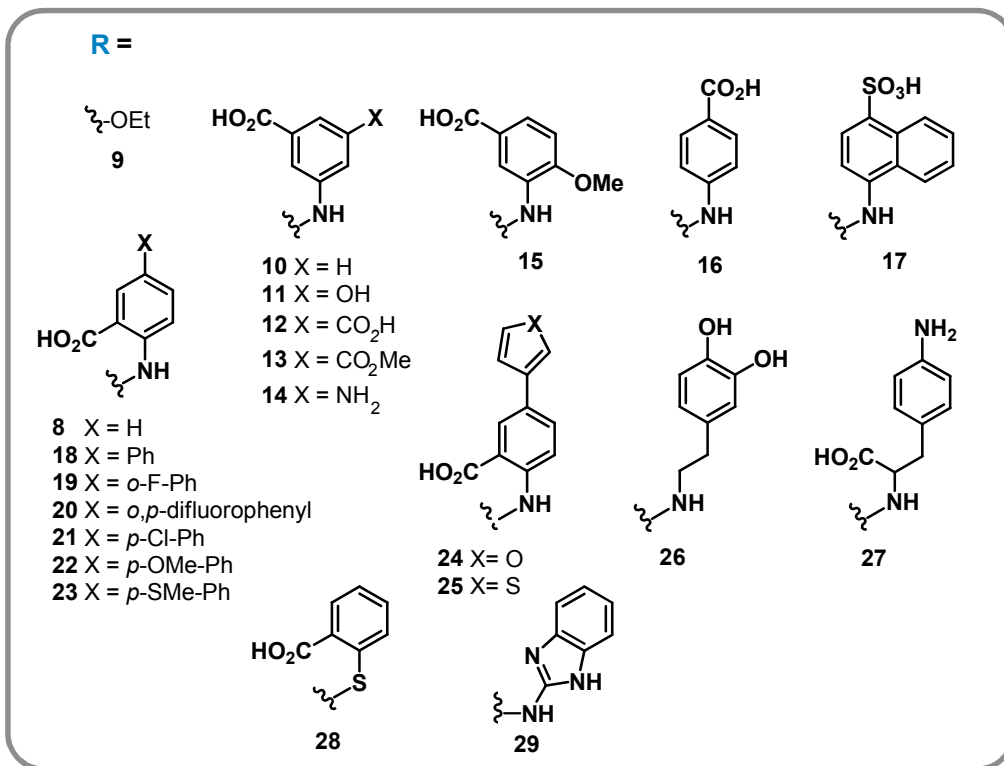
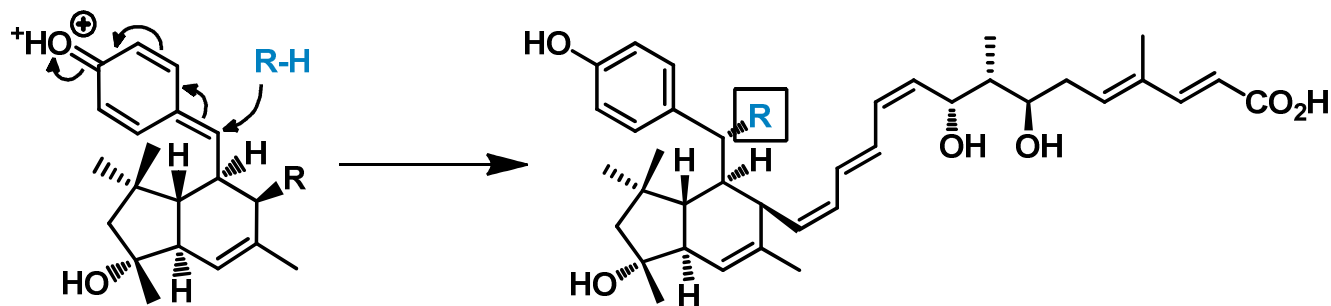
- Very low plasma stability

presumably based on equilibrium:
Lactone/Quinone

Steinmetz et al. (2011) *Angew. Chem. Int. Ed. Engl.* 50, 532.

Dehn et al. (2011) *Angew. Chem. Int. Ed. Engl.* 50, 3882; Jansen et al. (2012) *Chemistry* 17, 7739

Precursor directed Biosynthesis towards novel Elansolids



- Activity slightly decreased

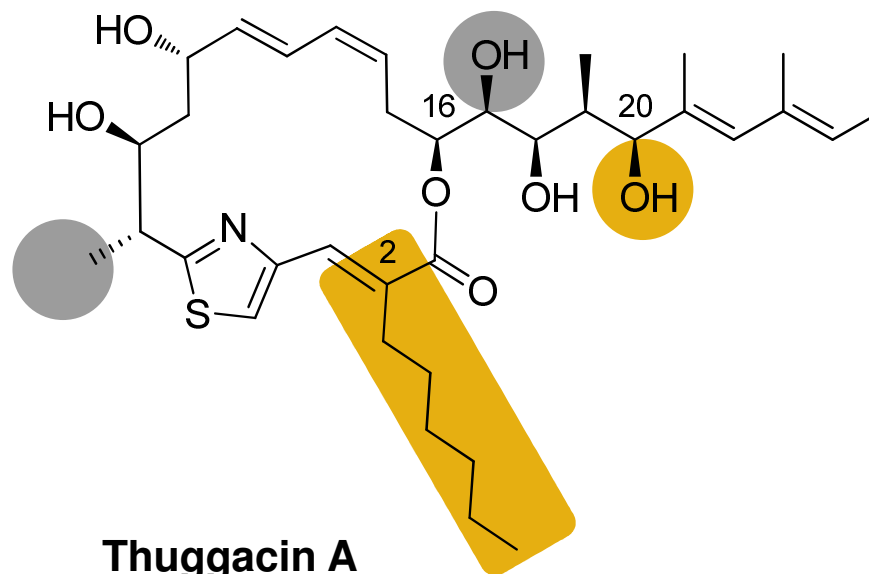
MICs 2 to 8 fold

+ Stability increased

➡ First step towards drugs with optimized/ improved pharmaceutical properties

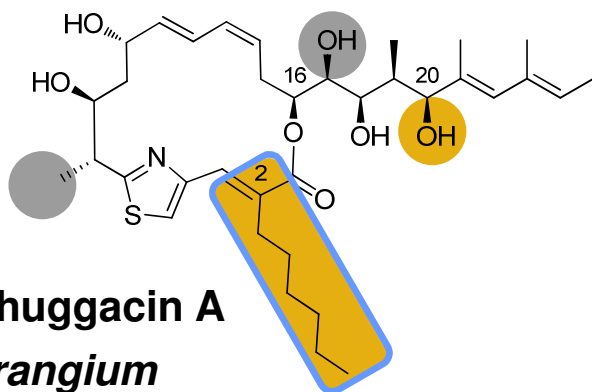
Steinmetz et al. (2012) *ChemBioChem* in press; Jansen et al. (2012) *Chemistry* 17, 7739

Thuggacins from *Sorangium cellulorum* – Novel Anti-Mycobacterial Natural Products

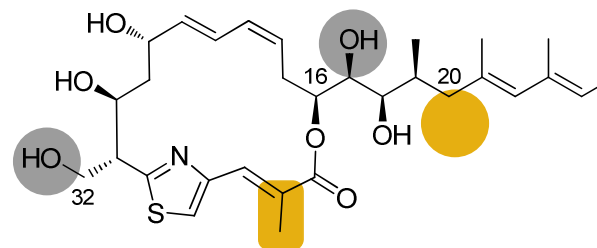


Thuggacin A
(*Sorangium
cellulosum*
So ce895)

Thuggacins – Comparative Cluster Analysis Reveals Basis for Structural Diversity

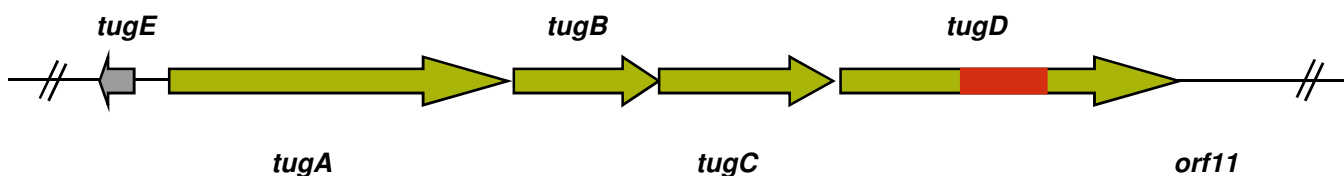


Soce-Thuggacin A
(*Sorangium cellulosum*
So ce895)

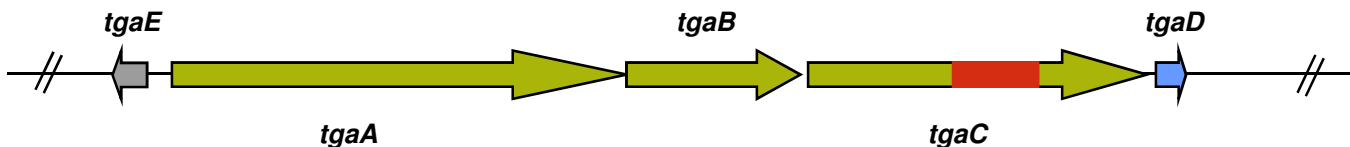


Cmc-Thuggacin A
(*Chondromyces crocatus*
Cm c5)

Cmc gene cluster



Soce Gencluster



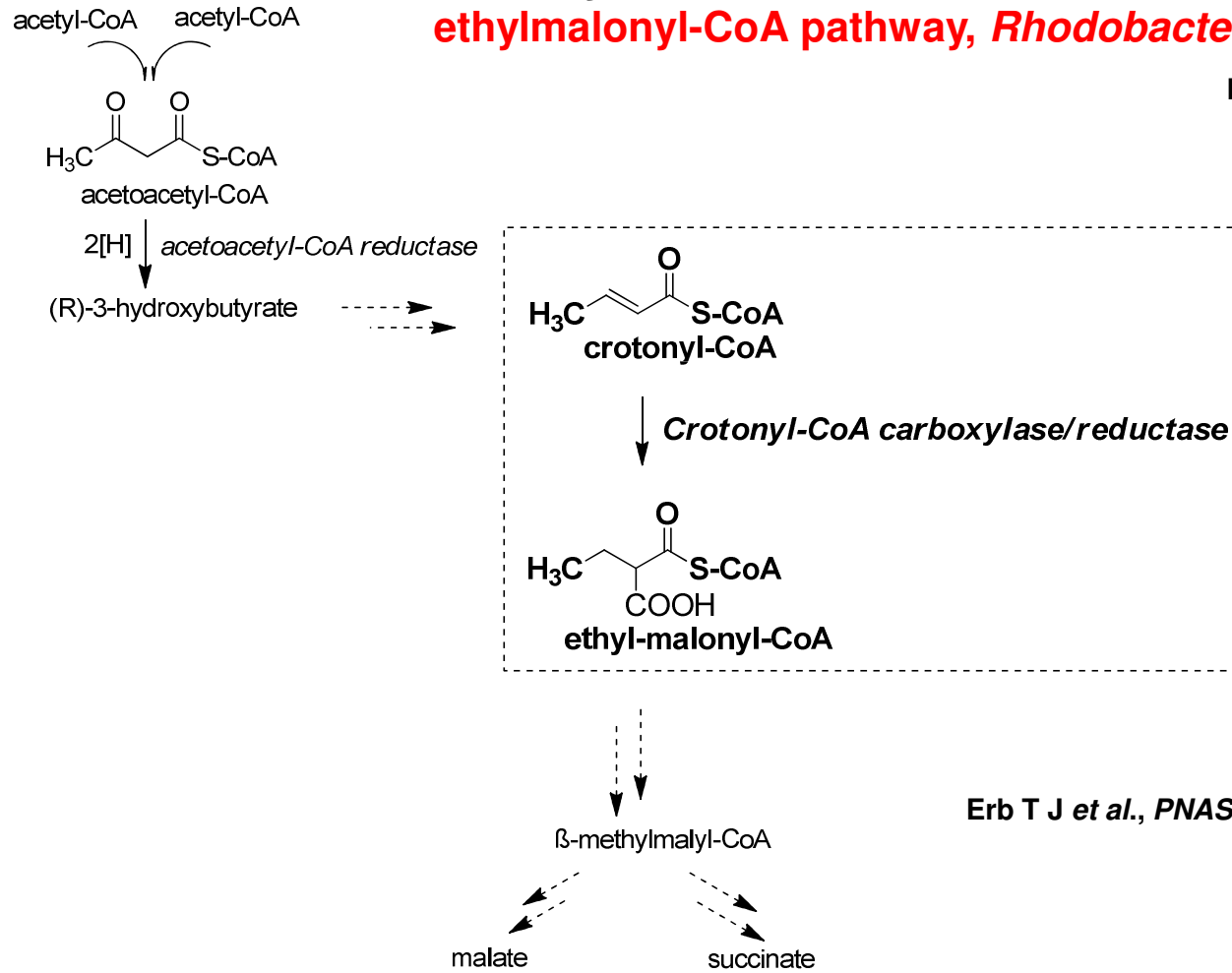
Bock et al., *Angew. Chem. Int. Ed.*, 2008; Buntin et al., *Chem. Biol.* 2010

Crotonyl-CoA Carboxylase/Reductase (Ccr)

Primary metabolism:

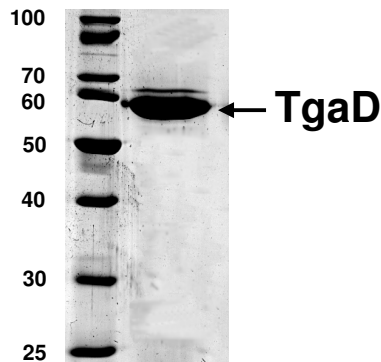
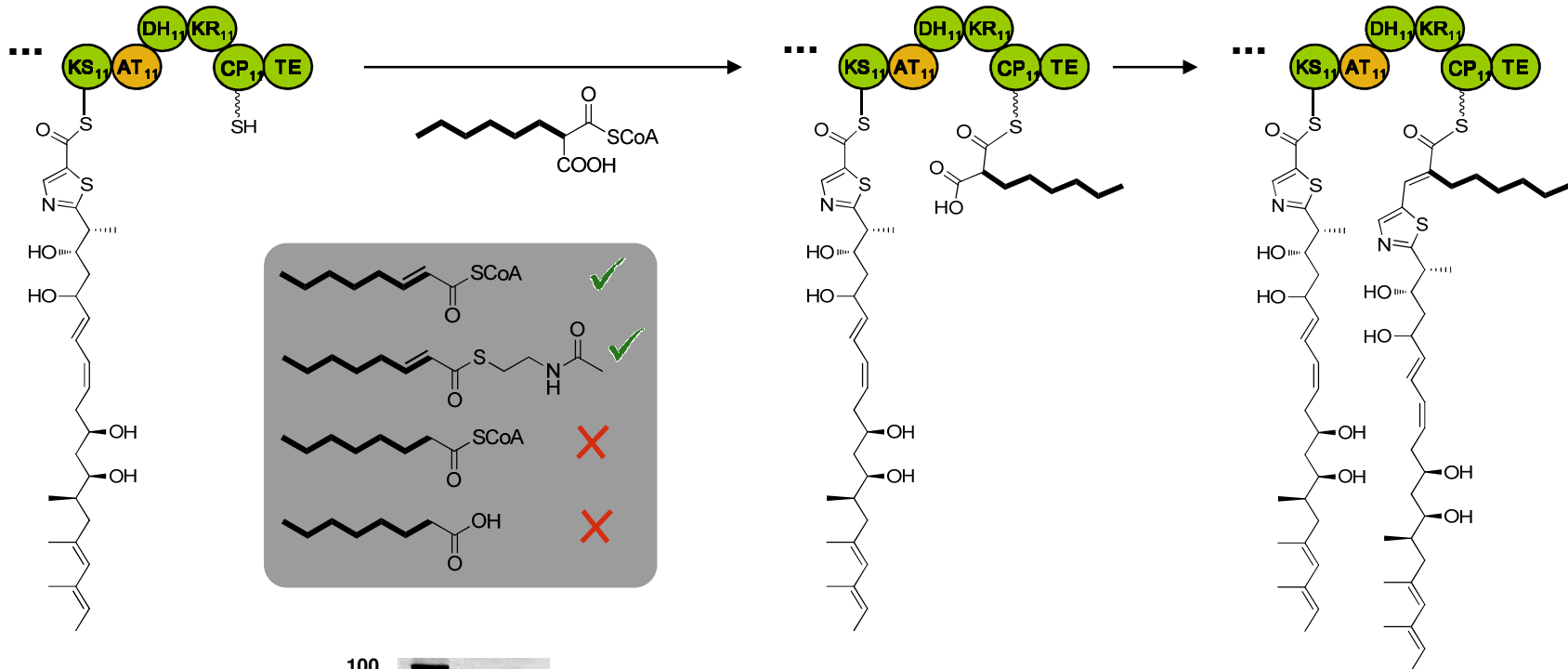
ethylmalonyl-CoA pathway, *Rhodobacter sphaeroides*

Erb T J *et al.*, PNAS. 2007



Erb T J *et al.*, PNAS. 2008

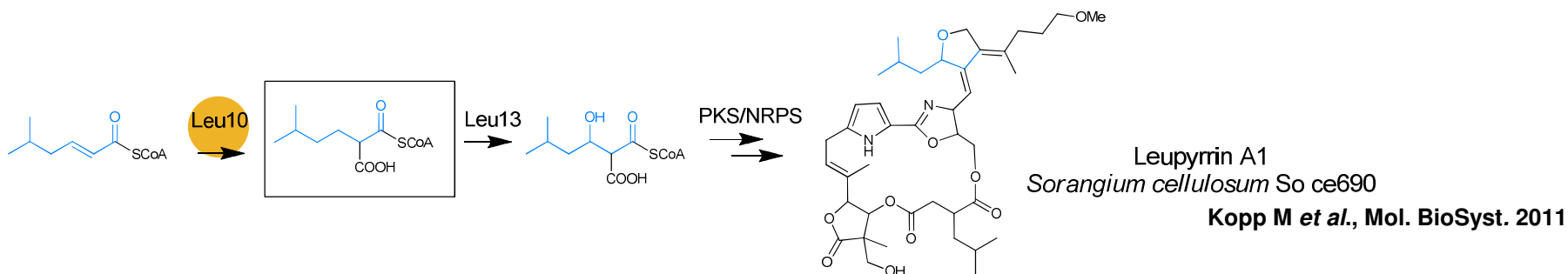
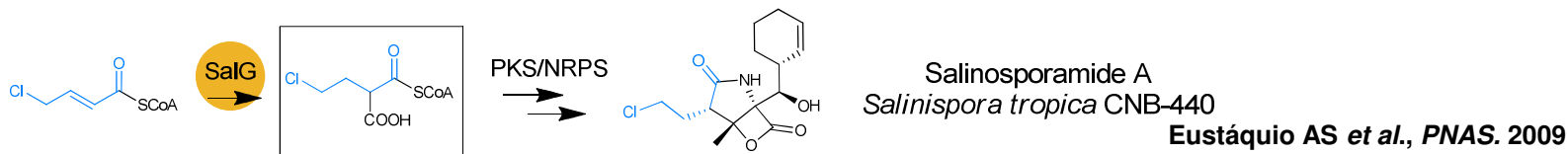
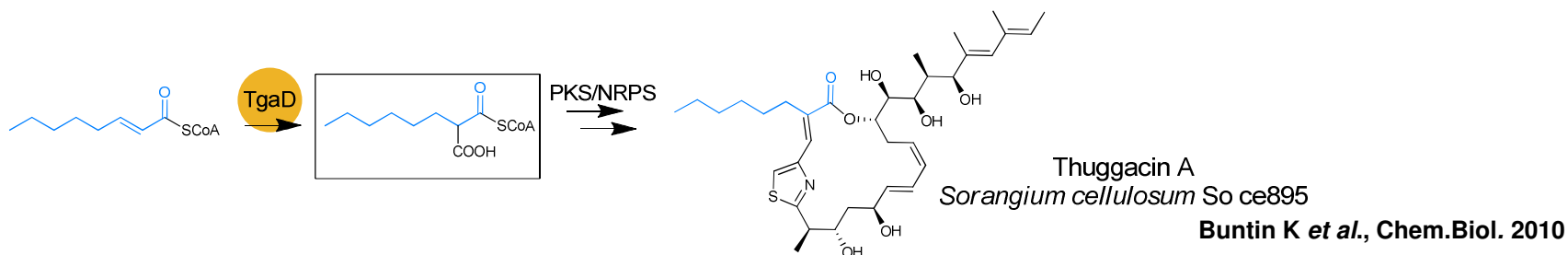
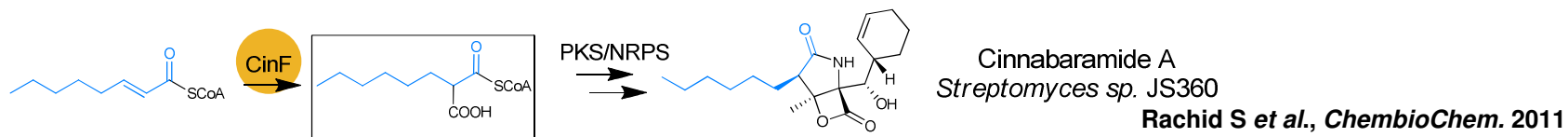
In Vitro Studies on Generation of the unusual Extender Unit: Reductive Carboxylation



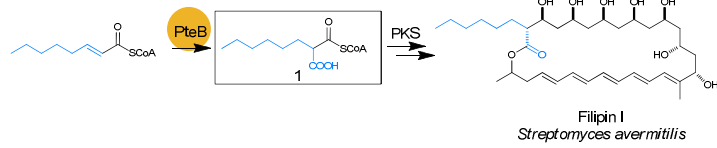
TgaD catalyzes the reductive carboxylation of an octenoyl derivative

Buntin *et al.*, *Chem. Biol.* 2010

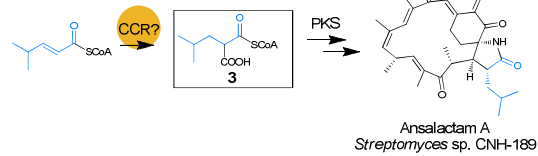
Unusual Alkylmalonyl thioester Building blocks by CCR



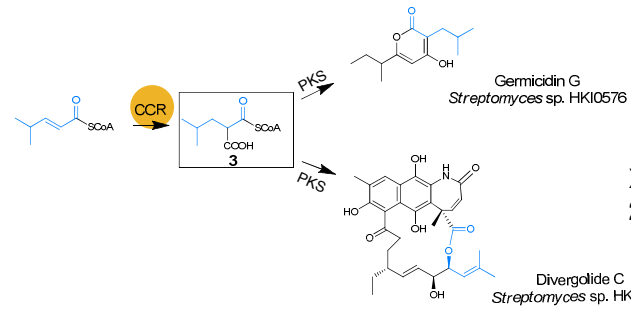
Unusual Alkylmalonyl thioester Building blocks by CCR



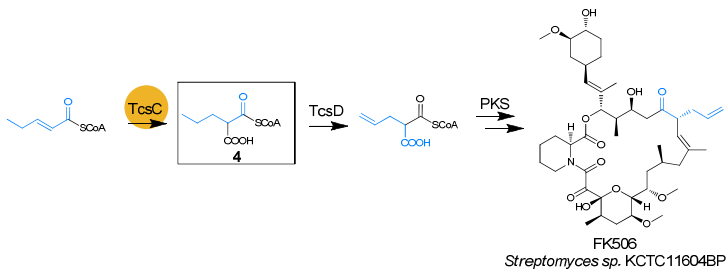
Yoo et al *Biosci Biotechnol Biochem* 2011



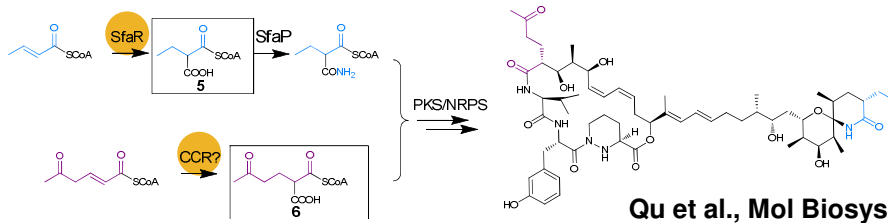
Wilsen et al., *JACS* 2011



Xu et al., *Angewandte Chemie* 2011

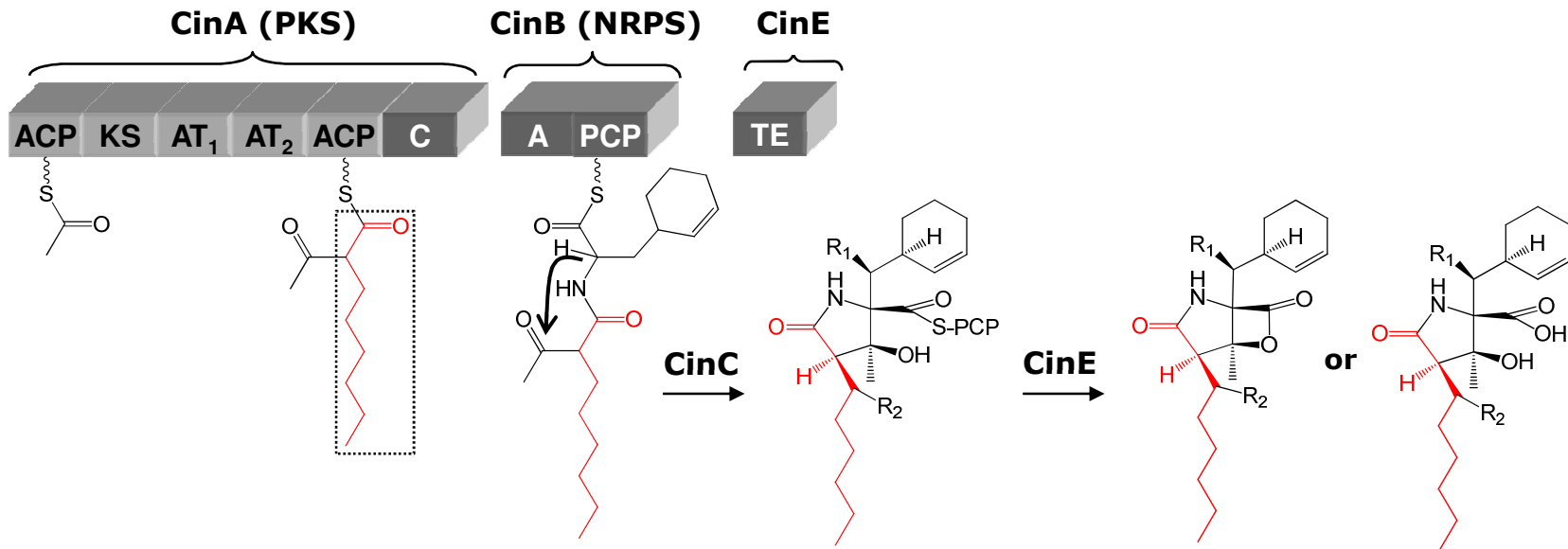


Mo et al., *JACS* 2011

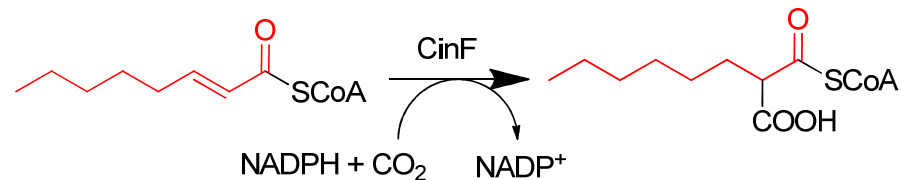


Qu et al., *Mol Biosys* 2011

Cinnabaramides: Potent antifungals

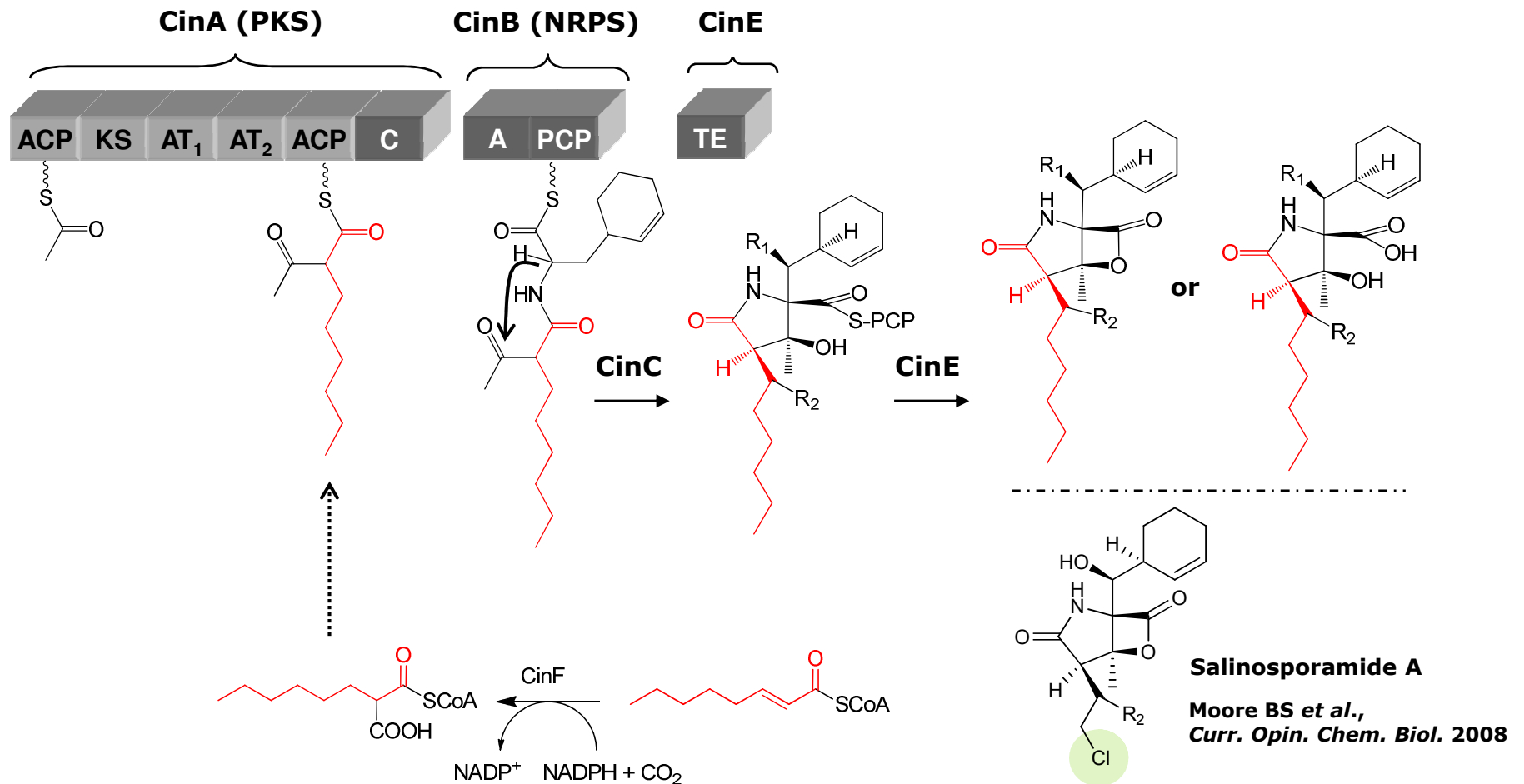


Key to generation of unusal extender unit: hexylmalonyl-CoA:



Rachid *et al.*, *ChemBioChem* 2011

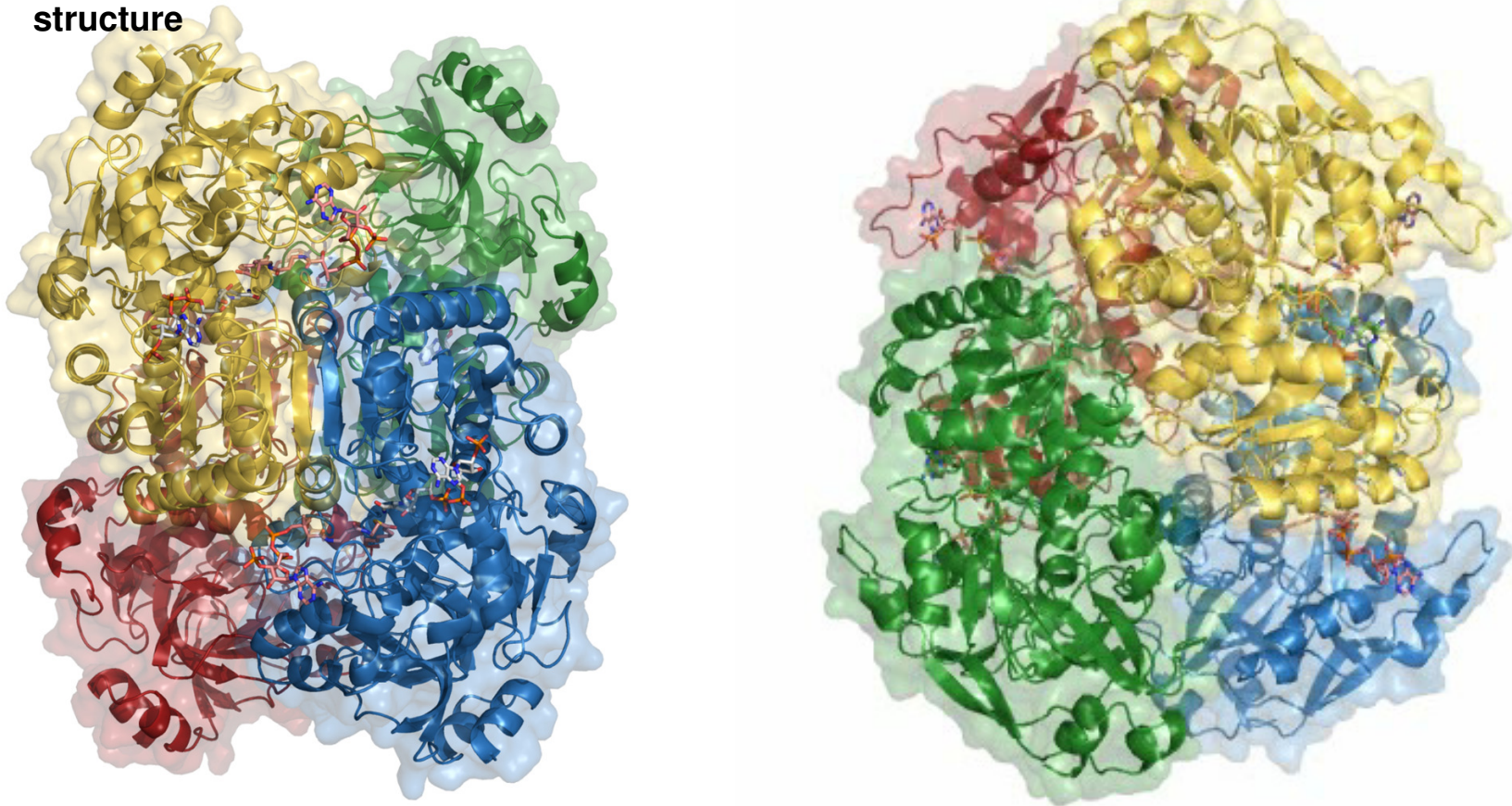
Precursor-directed biosynthesis



**Chlorinated cinnabaramides
exploiting CinF?**

Biosynthetic Studies and Enzyme Engineering: Structure of CinF as 2-octenoyl-CoA carboxylase/reductase

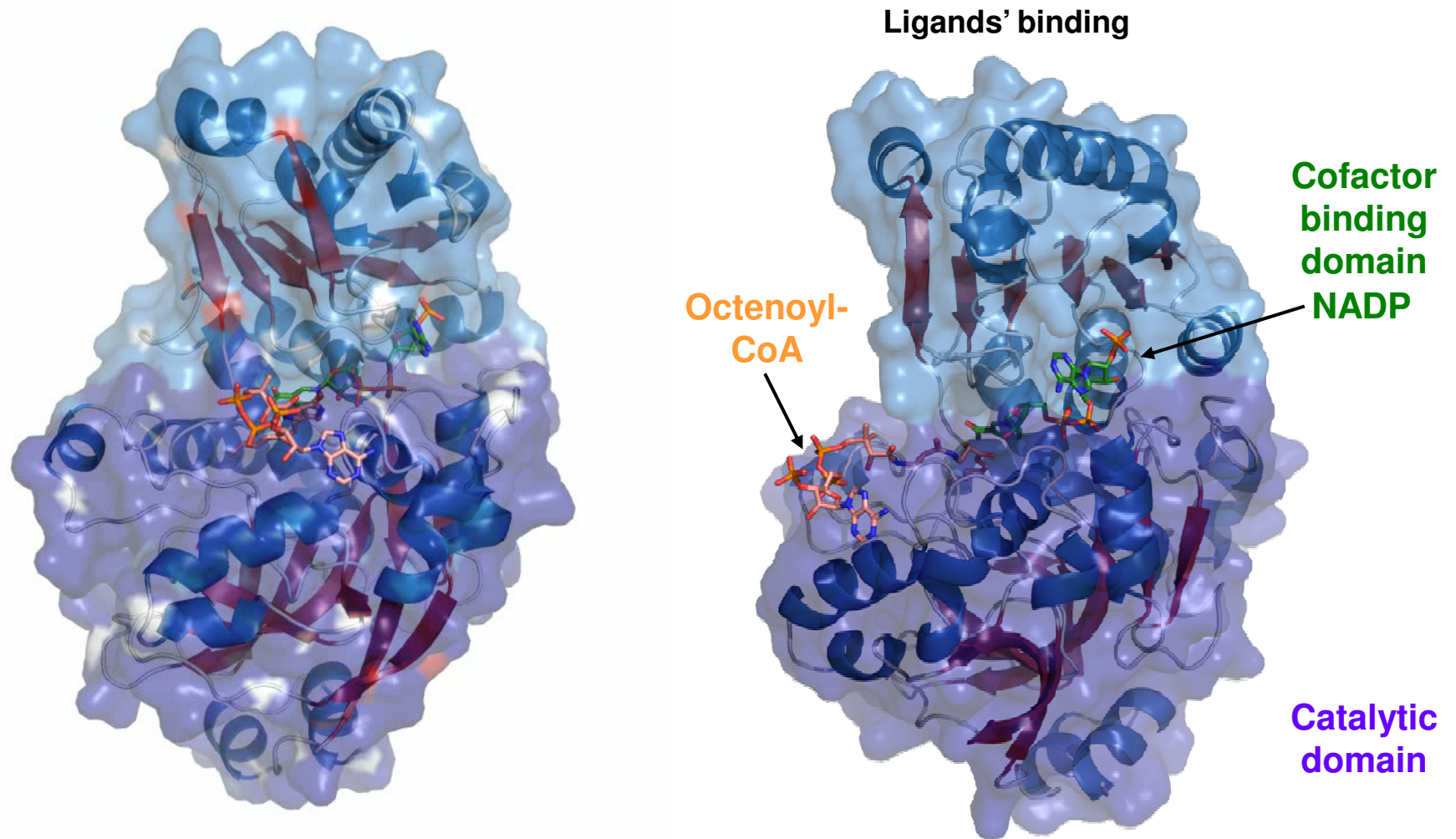
Stereo image of quarternary structure



Asymmetric unit contains four CinF monomers
in form of tetrameric dimer of dimers assembly.

Quade N and Huo L *et al.*, *Nature Chem.Biol.* 2012

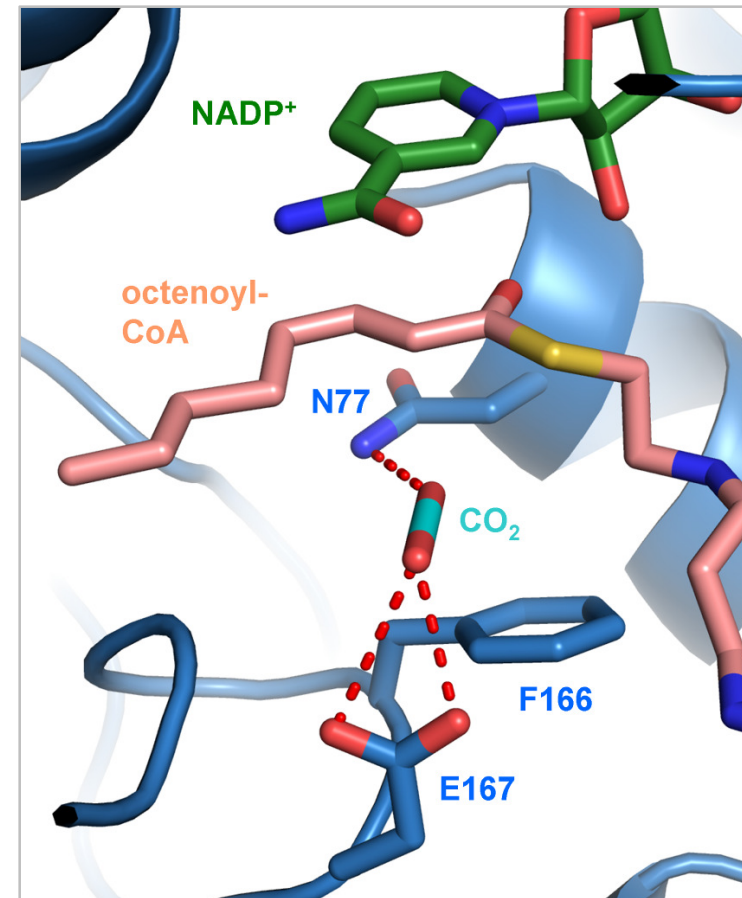
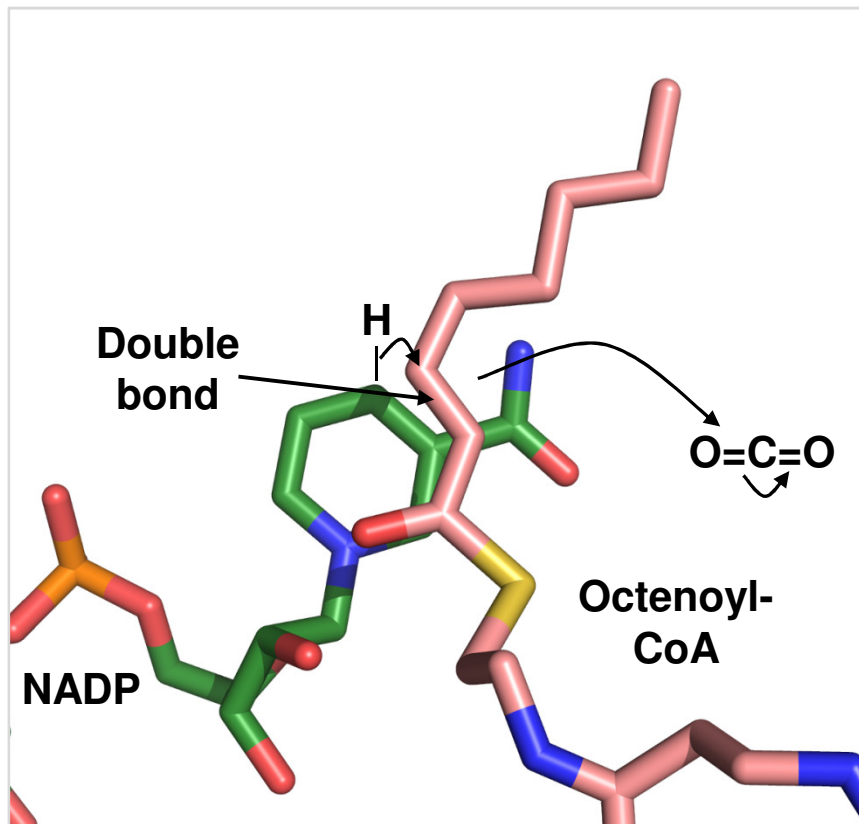
Biosynthetic Studies and Enzyme Engineering: Structure of CinF as 2-octenoyl-CoA carboxylase/reductase



Quade N and Huo L *et al.*, *Nature Chem.Biol.* 2012

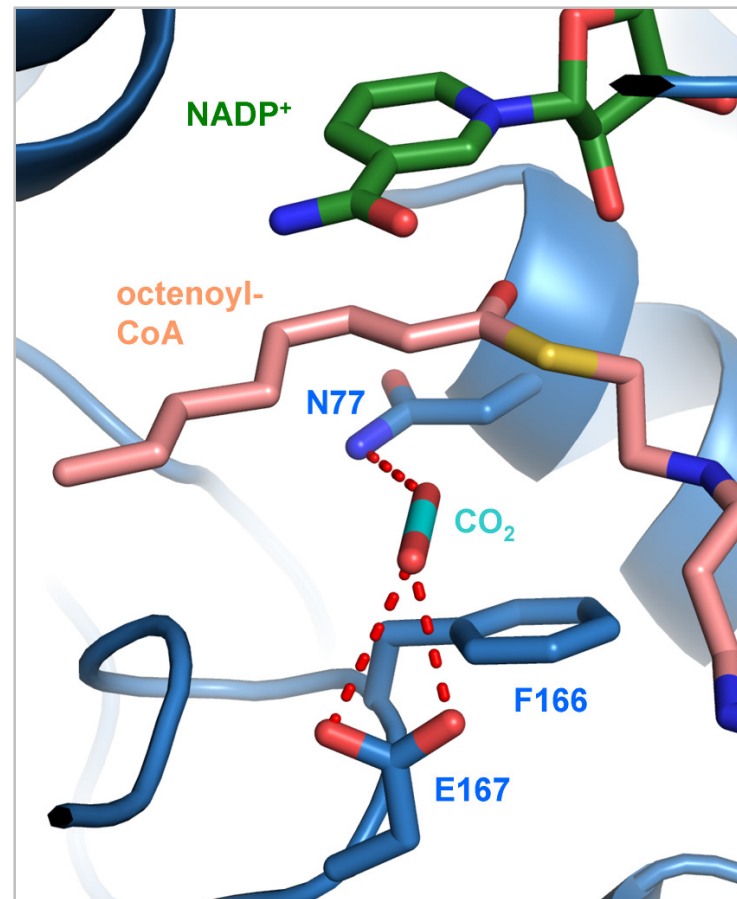
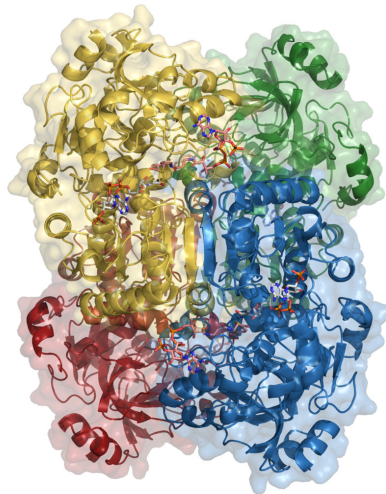
CinF: 2-octenoyl-CoA Carboxylase/Reductase

Model of CO₂ docked into the structure of CinF



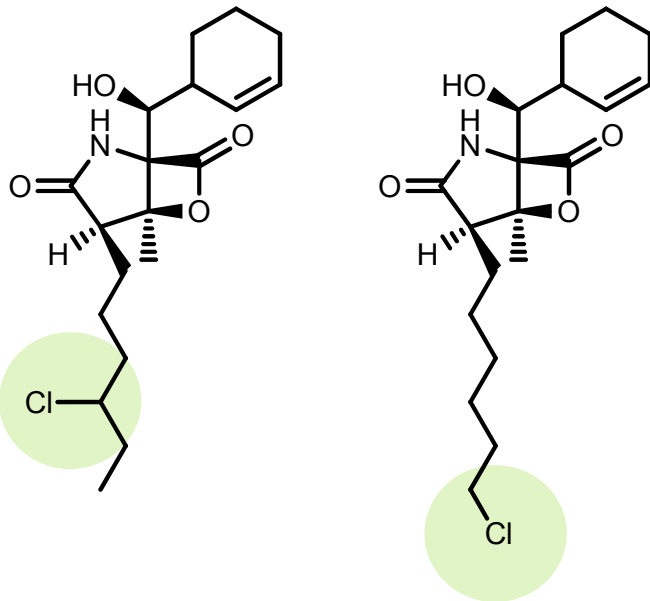
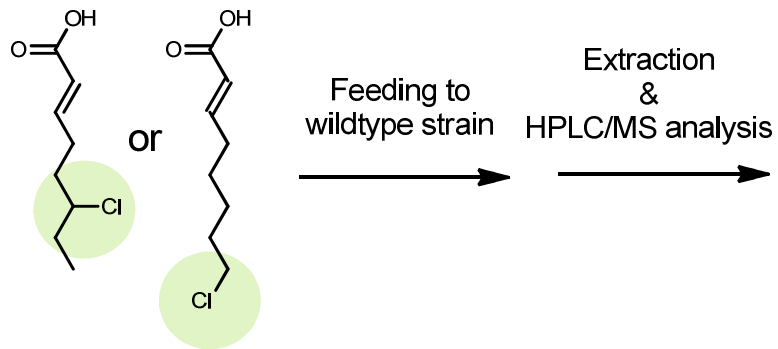
CinF: 2-octenoyl-CoA Carboxylase/Reductase

- Active site
- Substrate binding
- CO₂ binding
- Potential for engineering

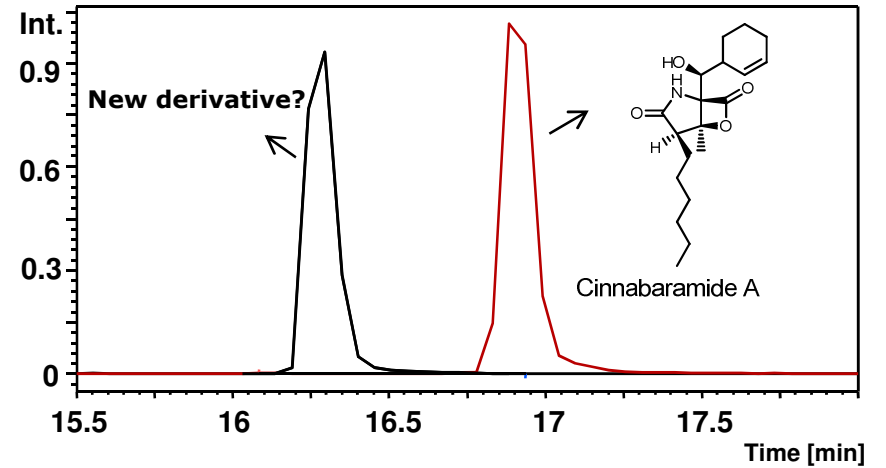


Quade N and Huo L *et al.*, *Nature Chem.Biol.* 2012

Precursor-directed biosynthesis



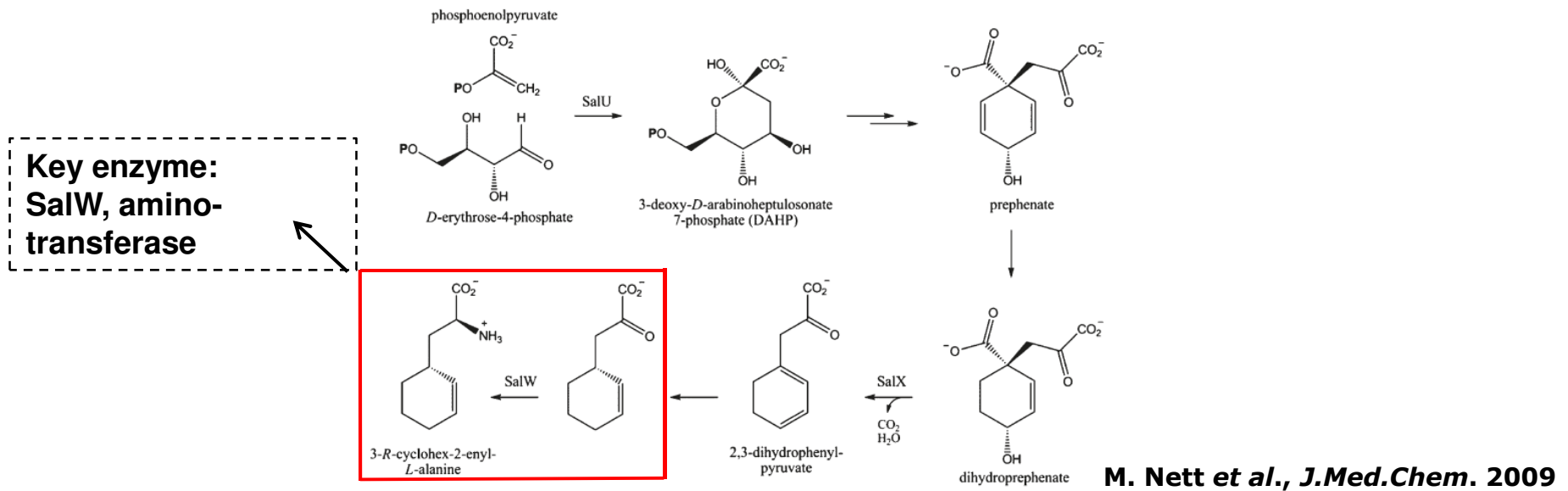
HPLC-MS (EIC) chromatograms of extracts obtained after feeding experiments with *S. sp* JS360:



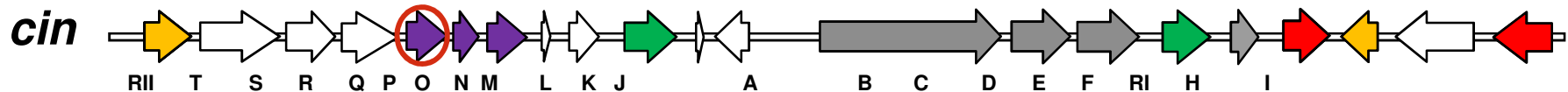
New chlorinated cinnabaramides

Precursor-directed biosynthesis

Proposed Biosynthesis of the cyclo-hexenylanaline of salinosporamides:



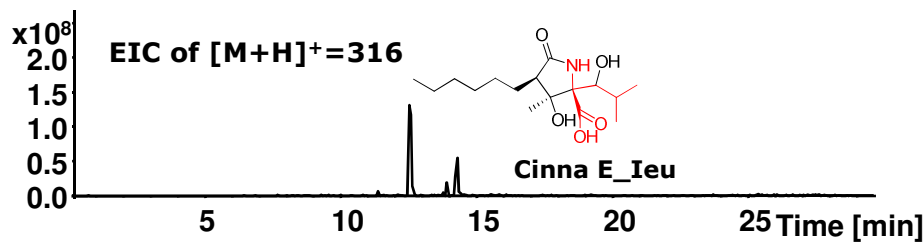
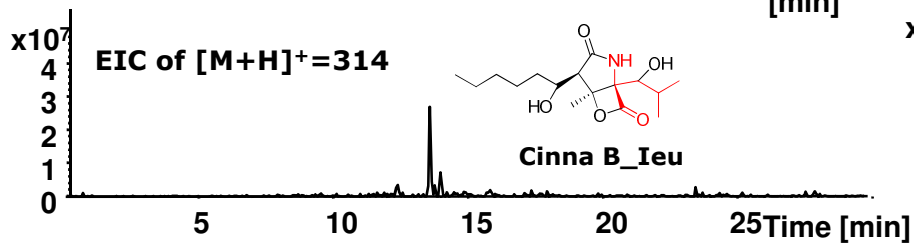
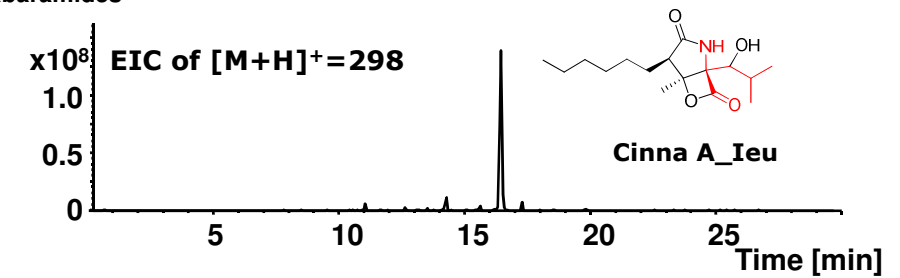
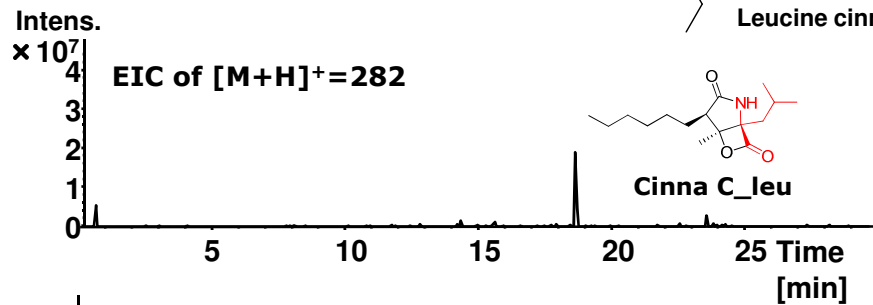
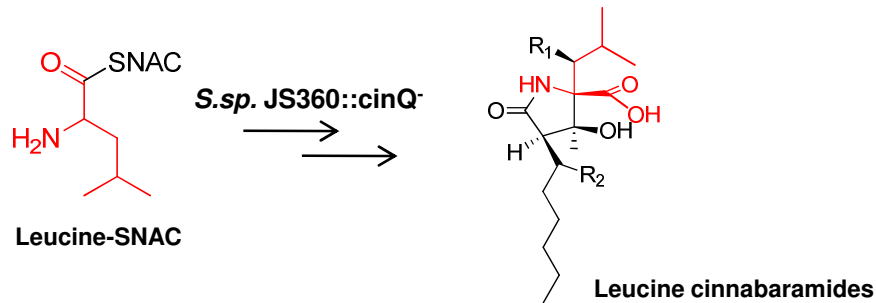
Streptomyces sp. JS360 – cosmid sequence



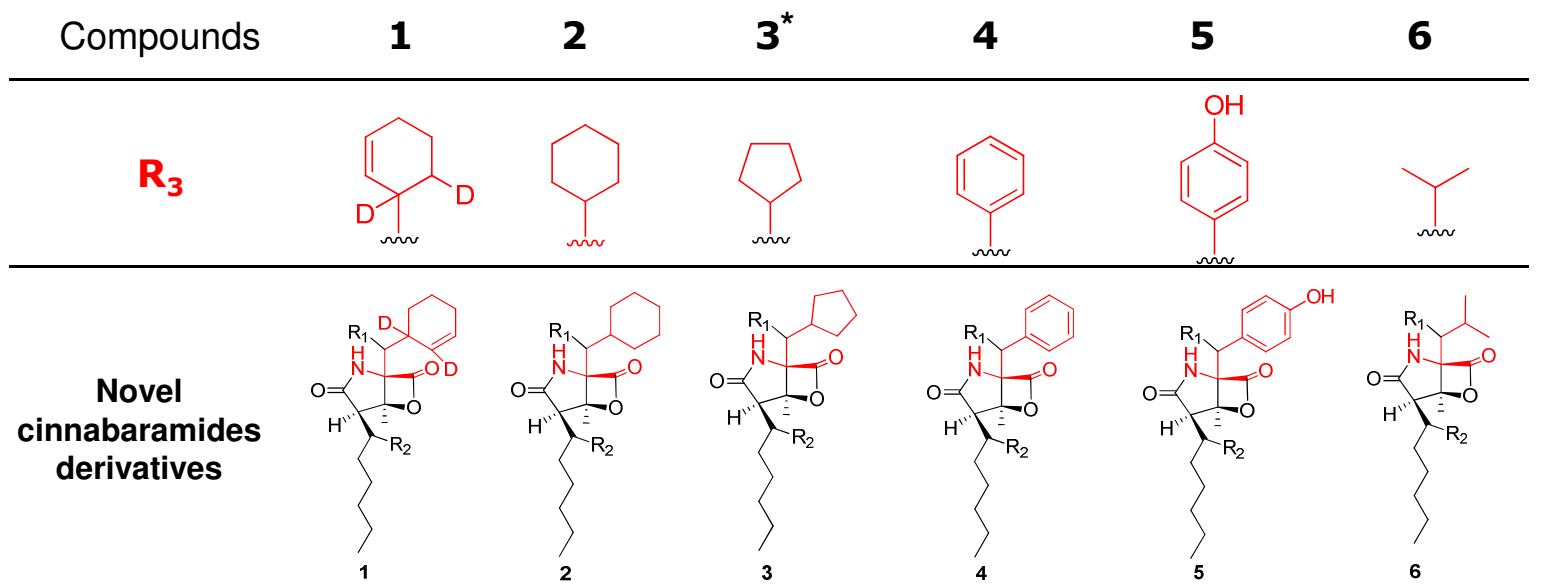
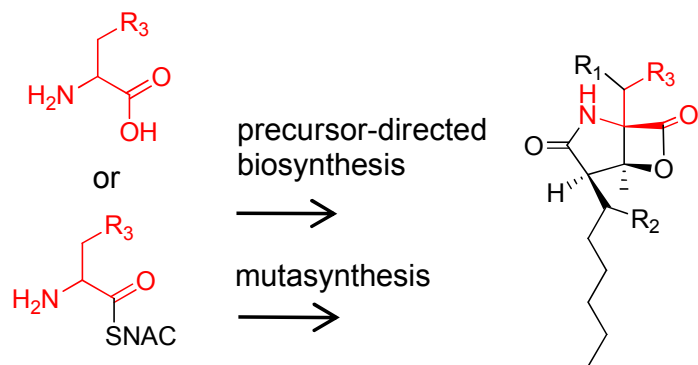
↳ CinQ as a putative L-amino acid aminotransferase shows **80%** similarity to SalW

Abolishment of cinnabaramide production in *S. sp* JS360::*cinQ* mutant

Mutasyntesis in *Streptomyces sp.* JS360::cinQ⁻ mutant



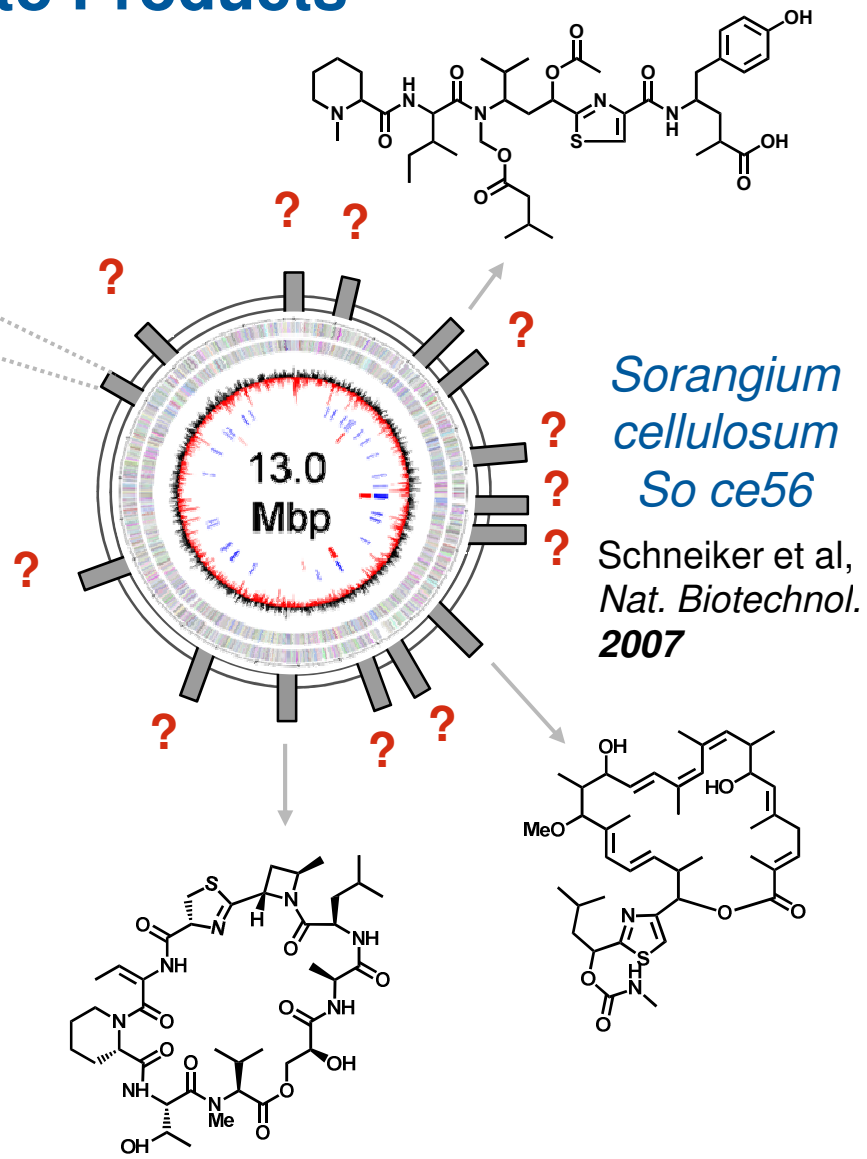
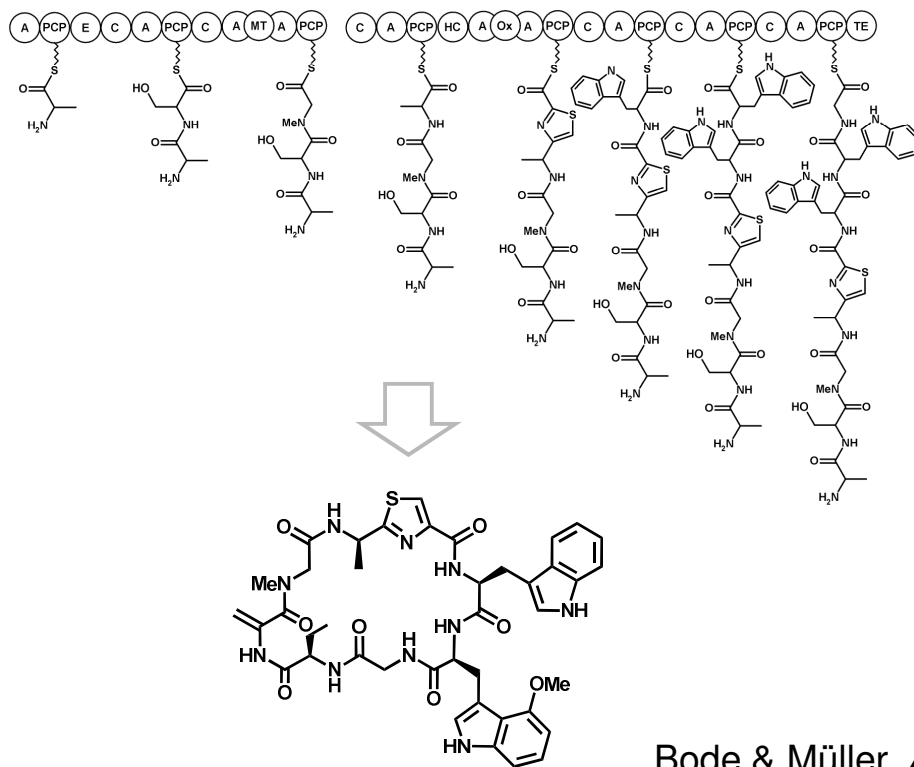
Summary for all obtained derivatives



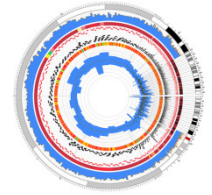
*: S. Rachid *et al.*, *ChembioChem*. 2011

Genome Mining: From Genes to Products

Biosynthetic gene cluster
(usually between 30-100 kb)

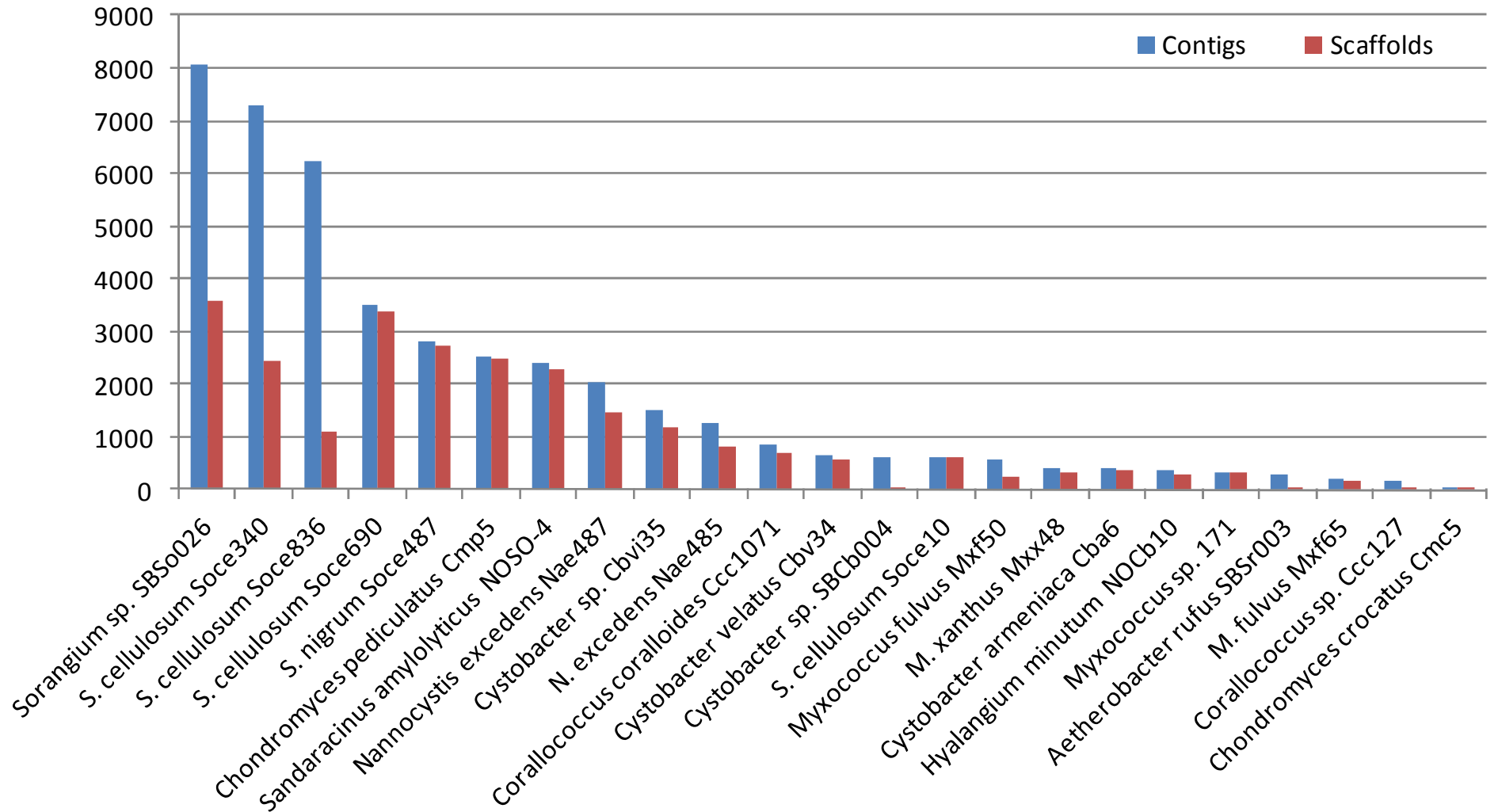


Bode & Müller, *Angew. Chem. Int. Ed.* 2005



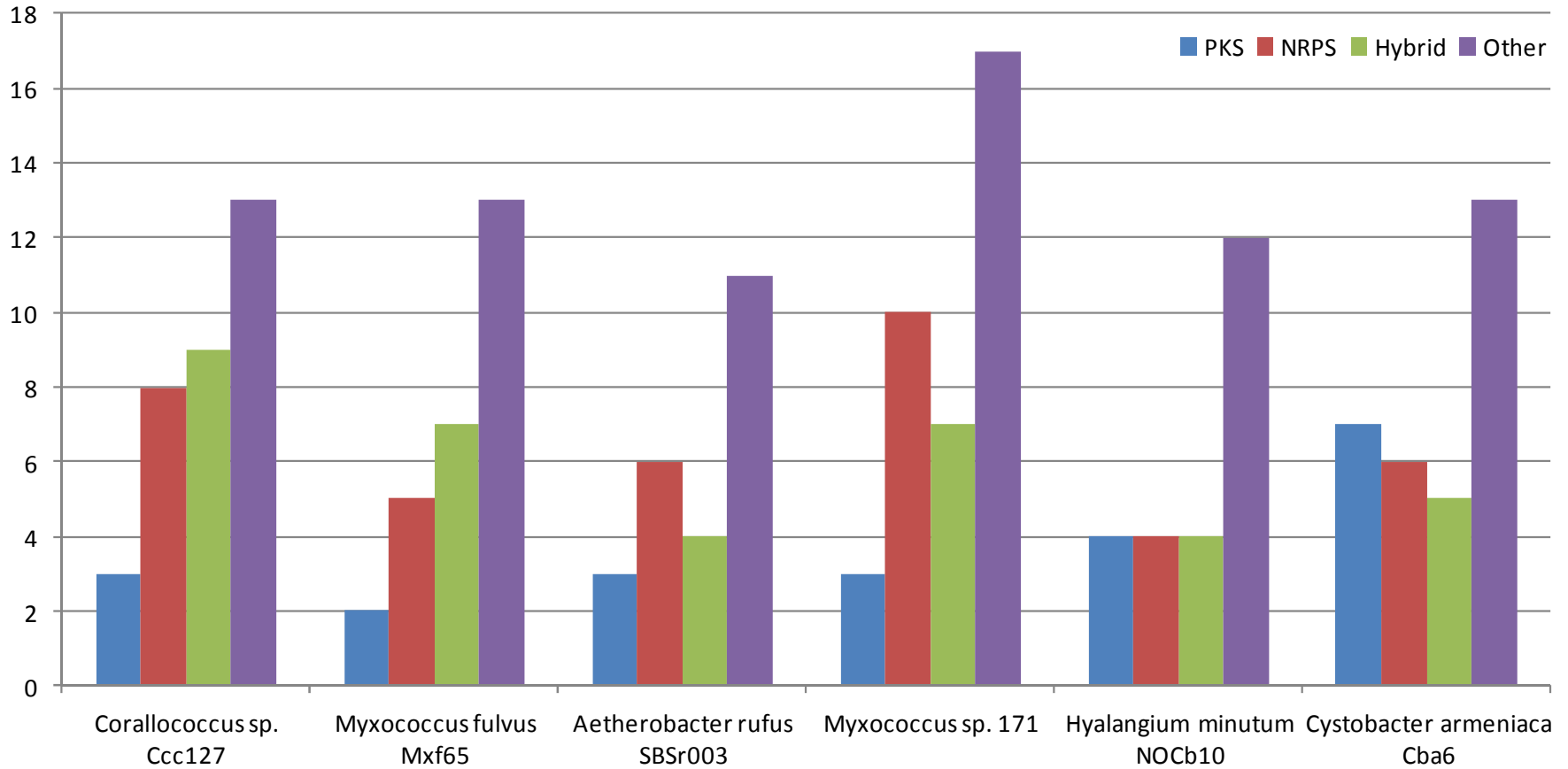
Myxobacterial Genome Projects

Contigs/Scaffolds

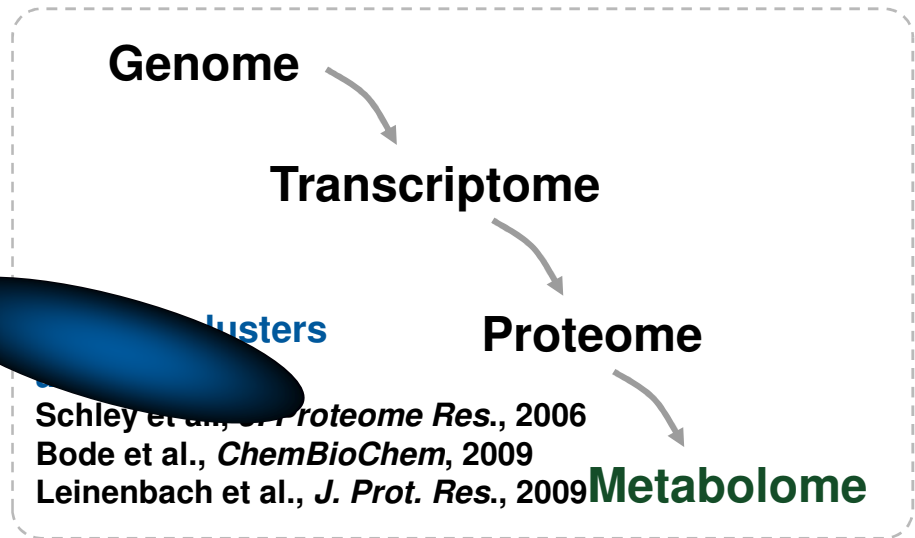
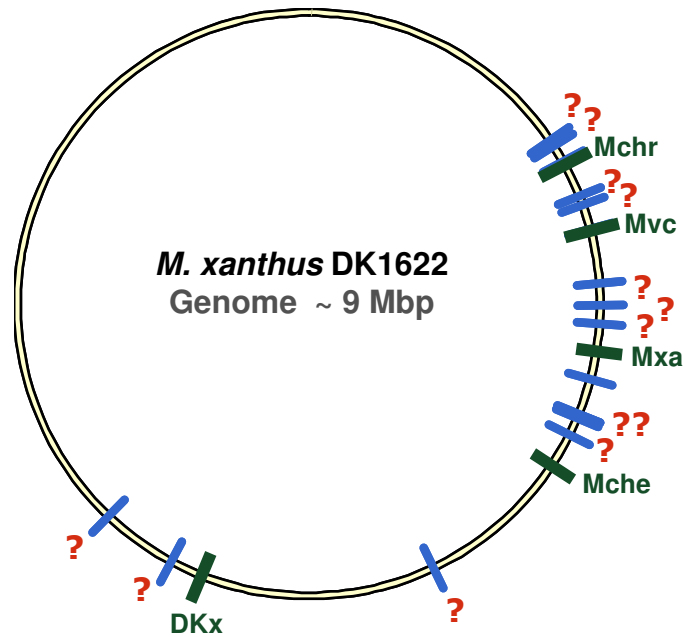


Natural Products Biosynthesis Potential

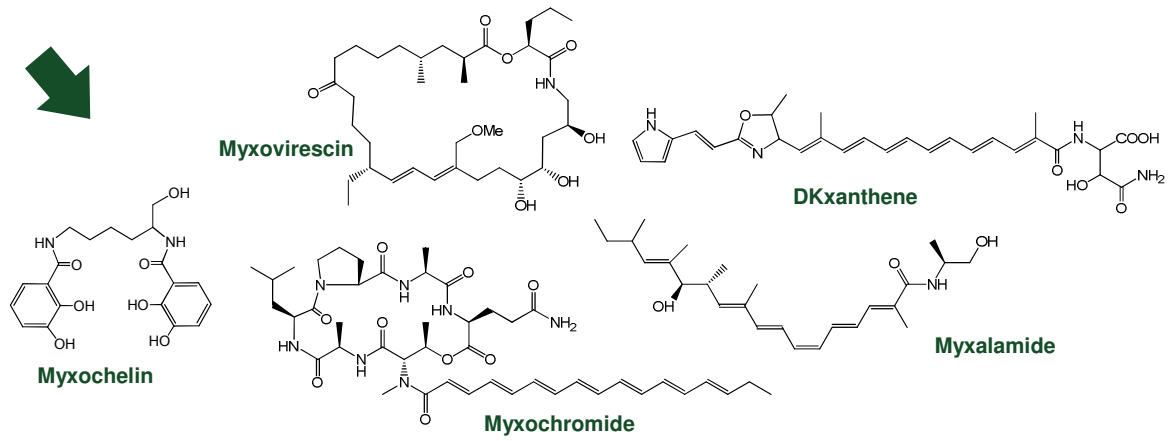
Number of clusters



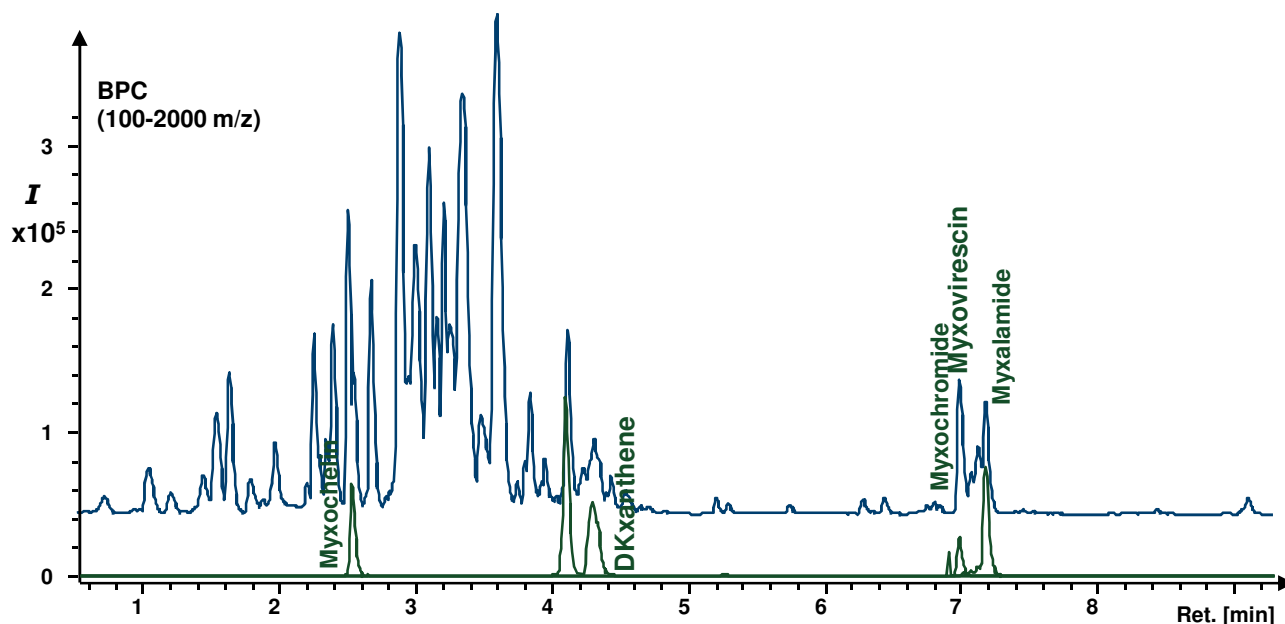
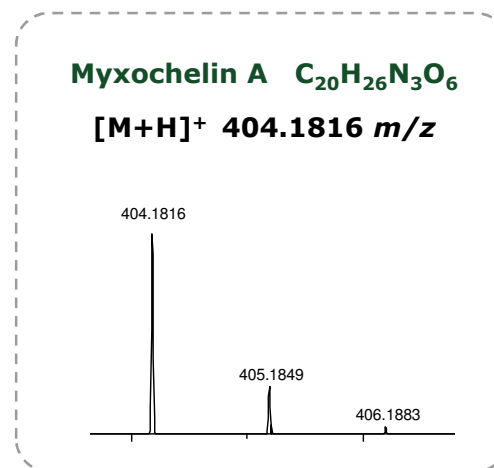
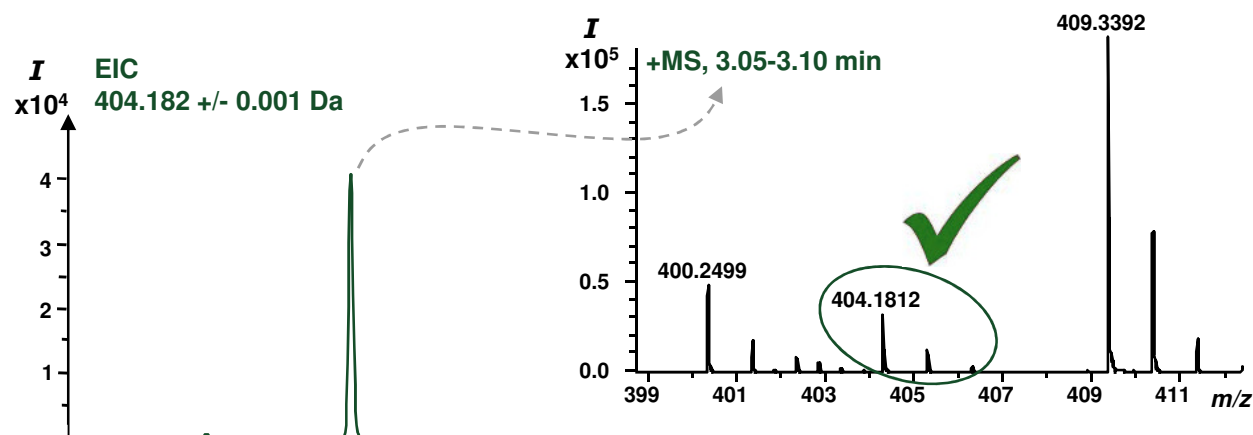
More Biosynthetic Gene Clusters than Compounds



**18 gene clusters,
5 known compounds**



Introducing an improved analytical platform: LC-coupled high-resolution electrospray mass spectrometry



Target screening



Krug et al., *AEM* 2008; Krug et al., *Anal. Chim. Acta* 2008

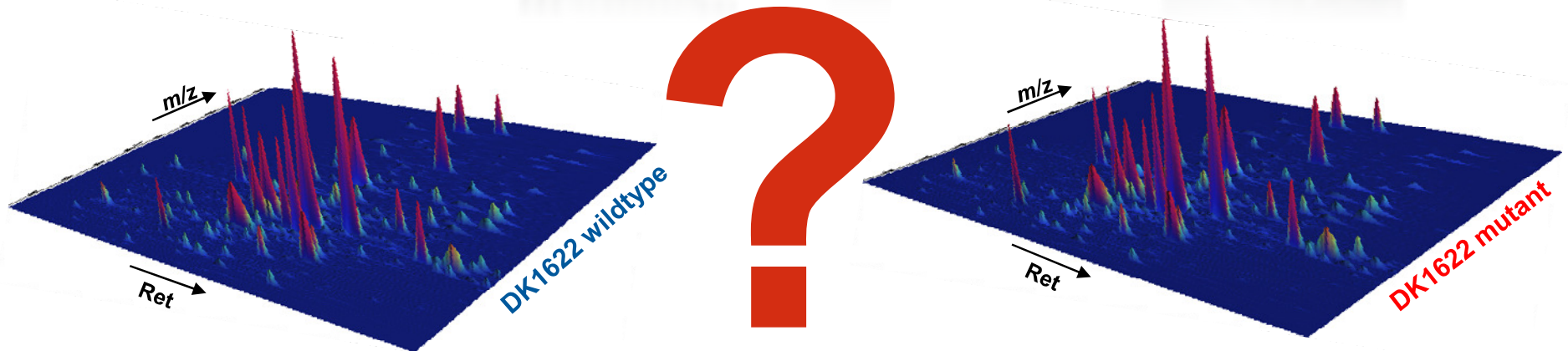
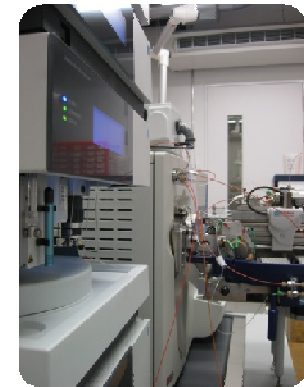
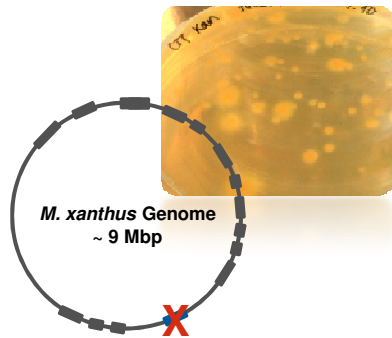
The Strategy

Targeted Inactivation

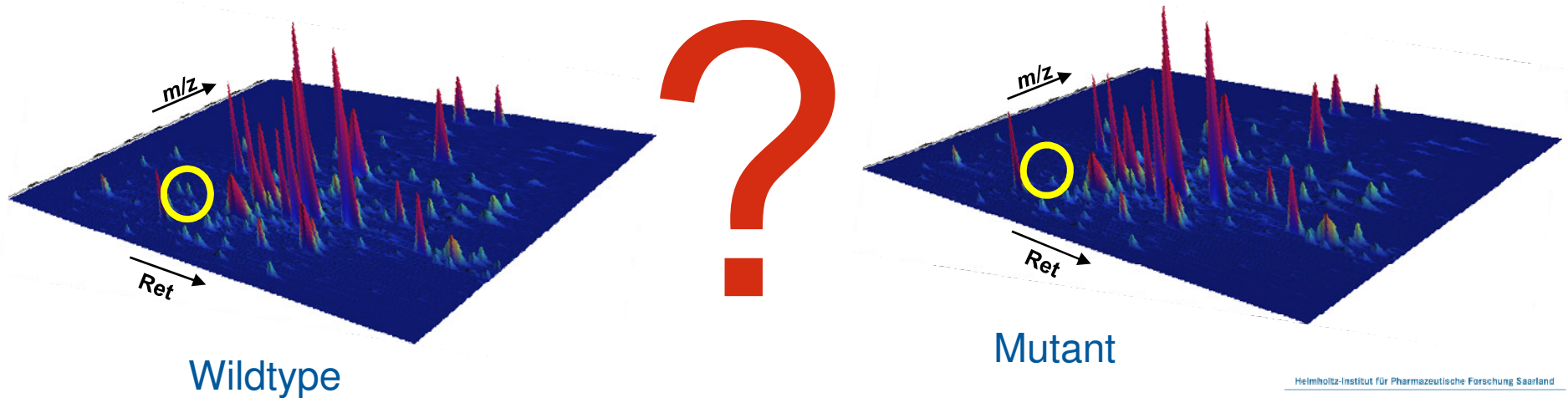
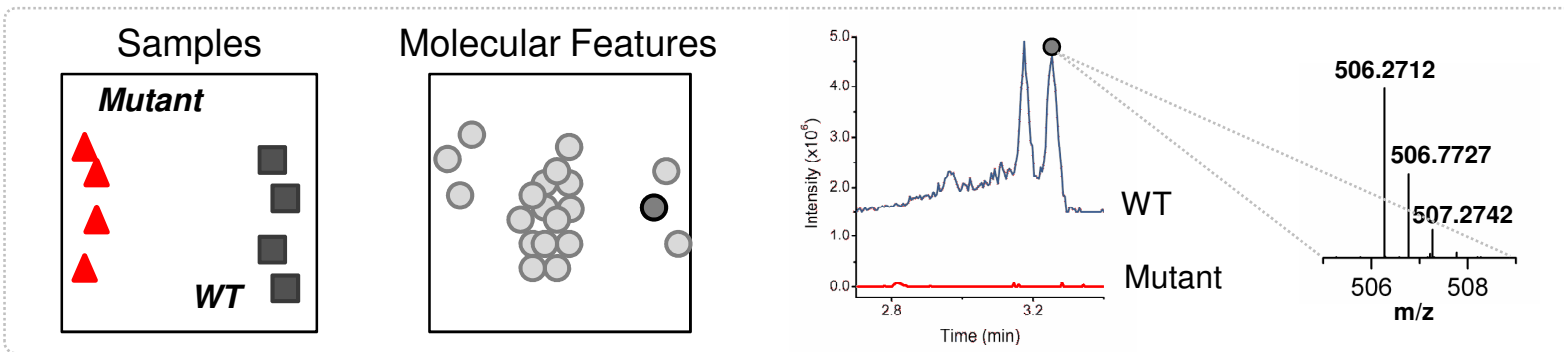
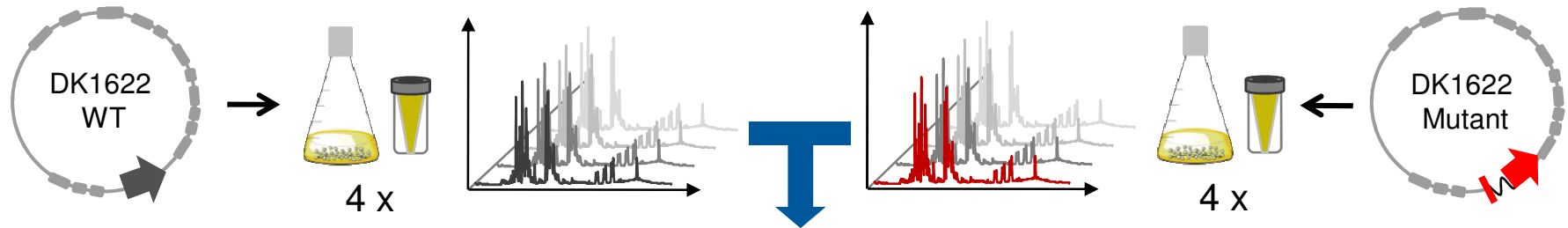
Cultivation
(50-ml scale + XAD-16 resin)

MeOH extraction
& concentration

LC- MS Analysis

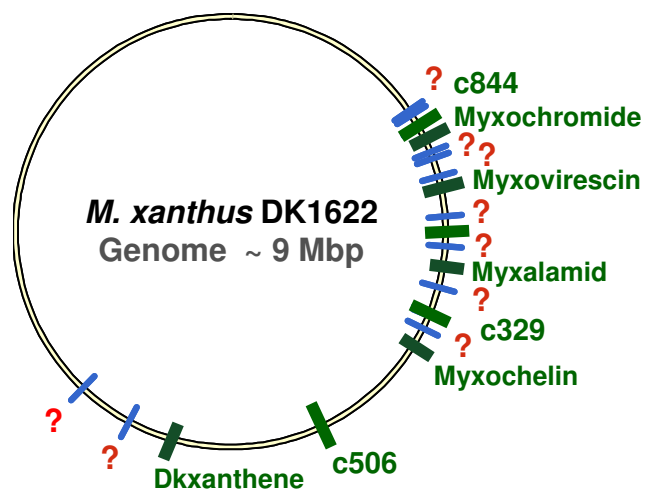


Genome based Strategy to find novel Compounds

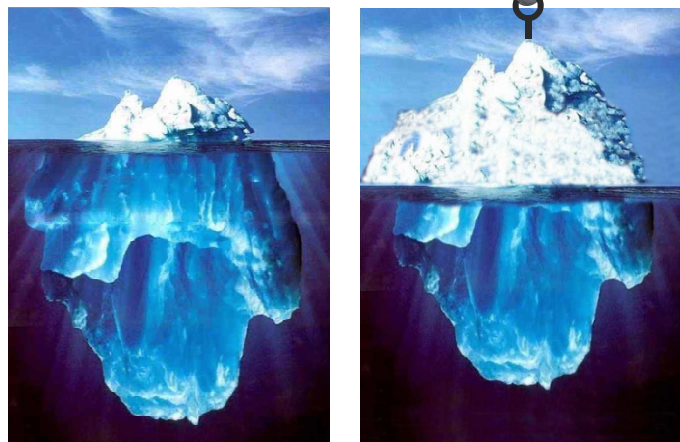


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Correlating genes to metabolites: Genomics and metabolomics for the discovery process

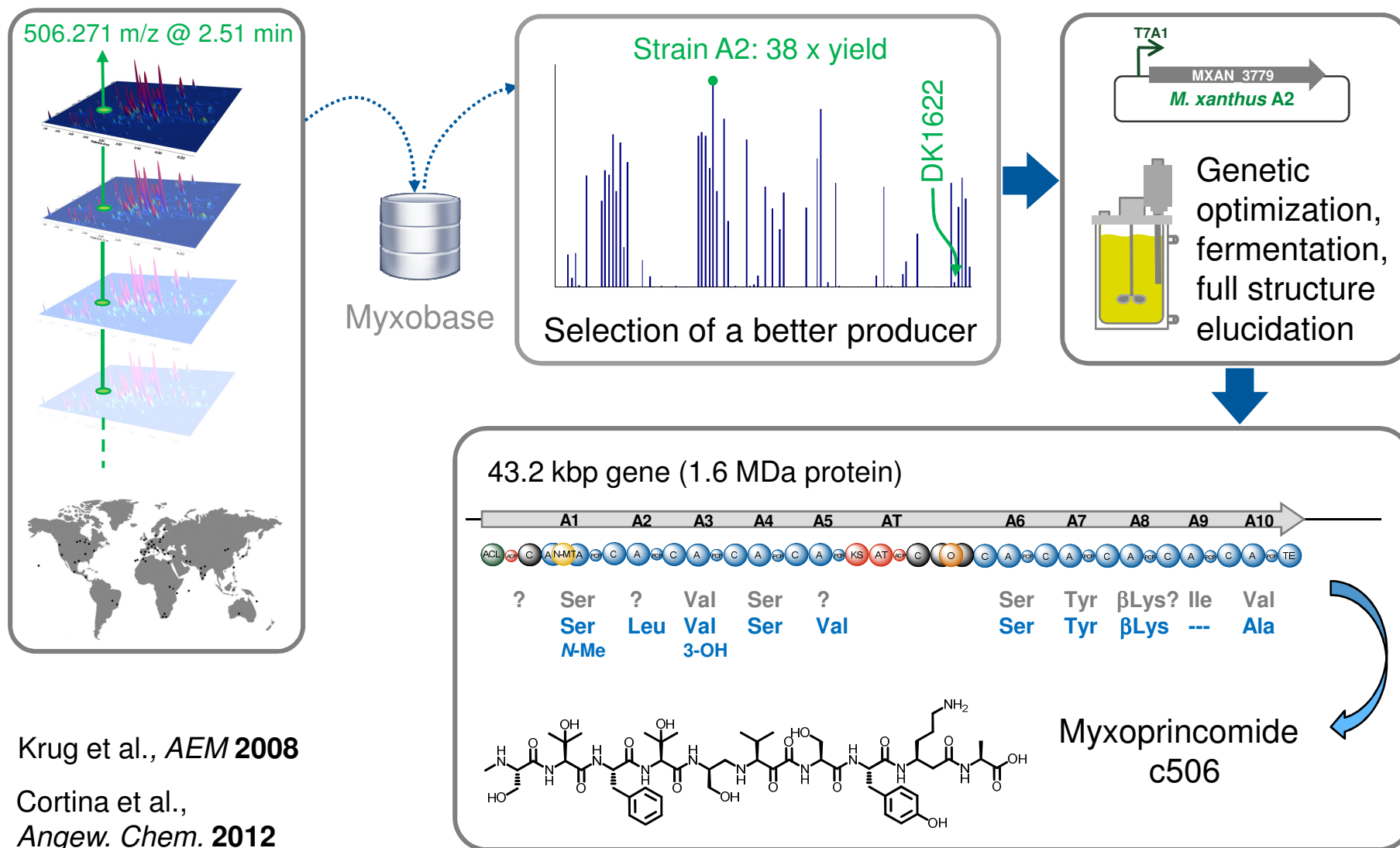


18 PKS/NRPS pathways



Product family (# compounds)	Discovery method	Analytical properties	abundance
Myxalamide (4)	genome-based	Intense yellow	very high
Myxochromide (3)	genome-based	Intense yellow	high
Myxochelin (2)	genome-based	UV 254 nm	high
Myxovioreascin (3)	genome-based	MS 624 m/z	fair
DKxanthene (11)	transposon mutagenesis	Intense yellow	fair
c506 (8) NRPS/PKS	metabolome mining	506.2713 ²⁺ m/z	low
c844 (1) NRPS	metabolome mining	844.3742 m/z	ultra low
c329 (1) PKS	metabolome mining	329.1861 m/z	ultra low

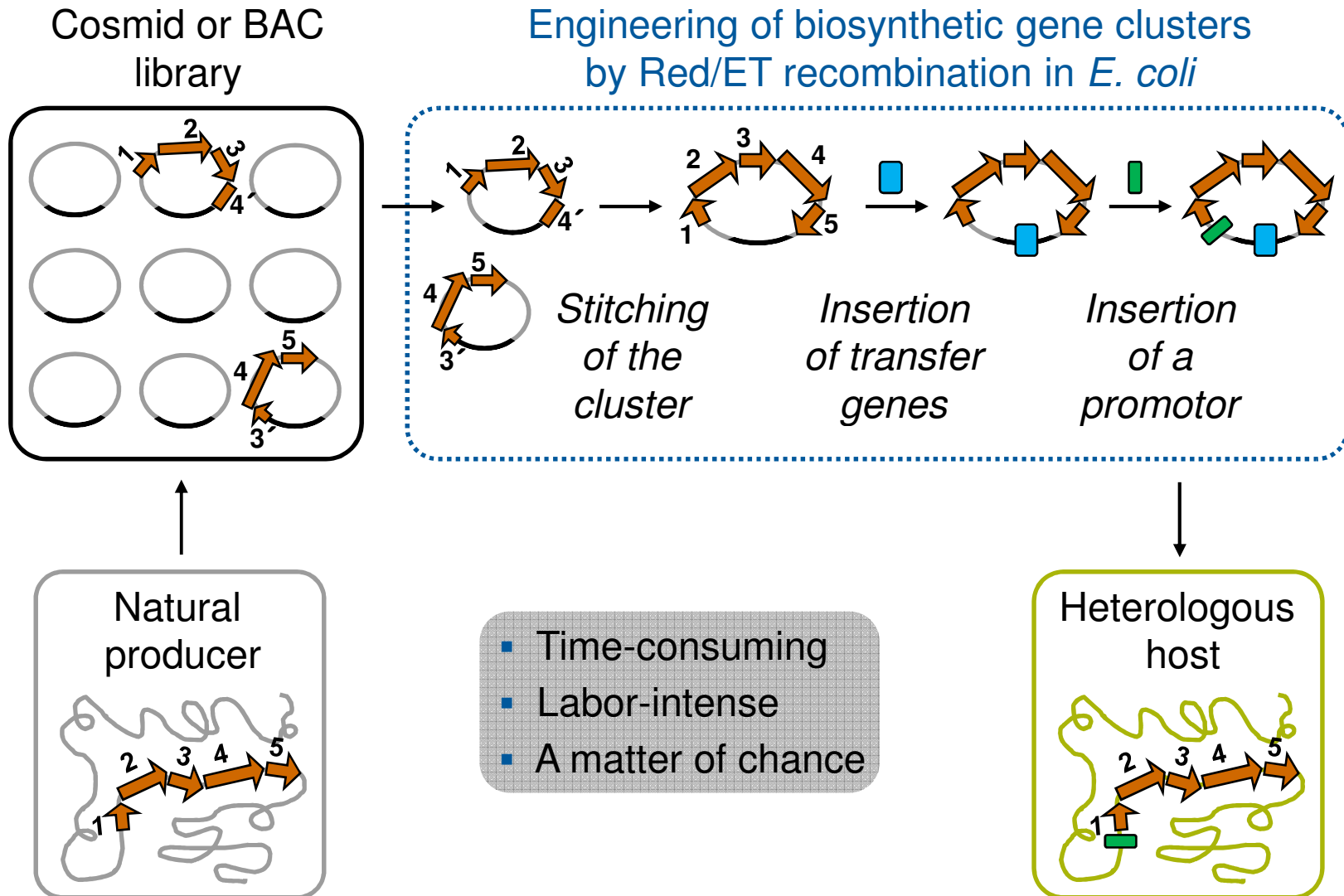
First Novel Secondary Metabolite found by Metabolome Mining: Myxoprincomides



Krug et al., *AEM* 2008

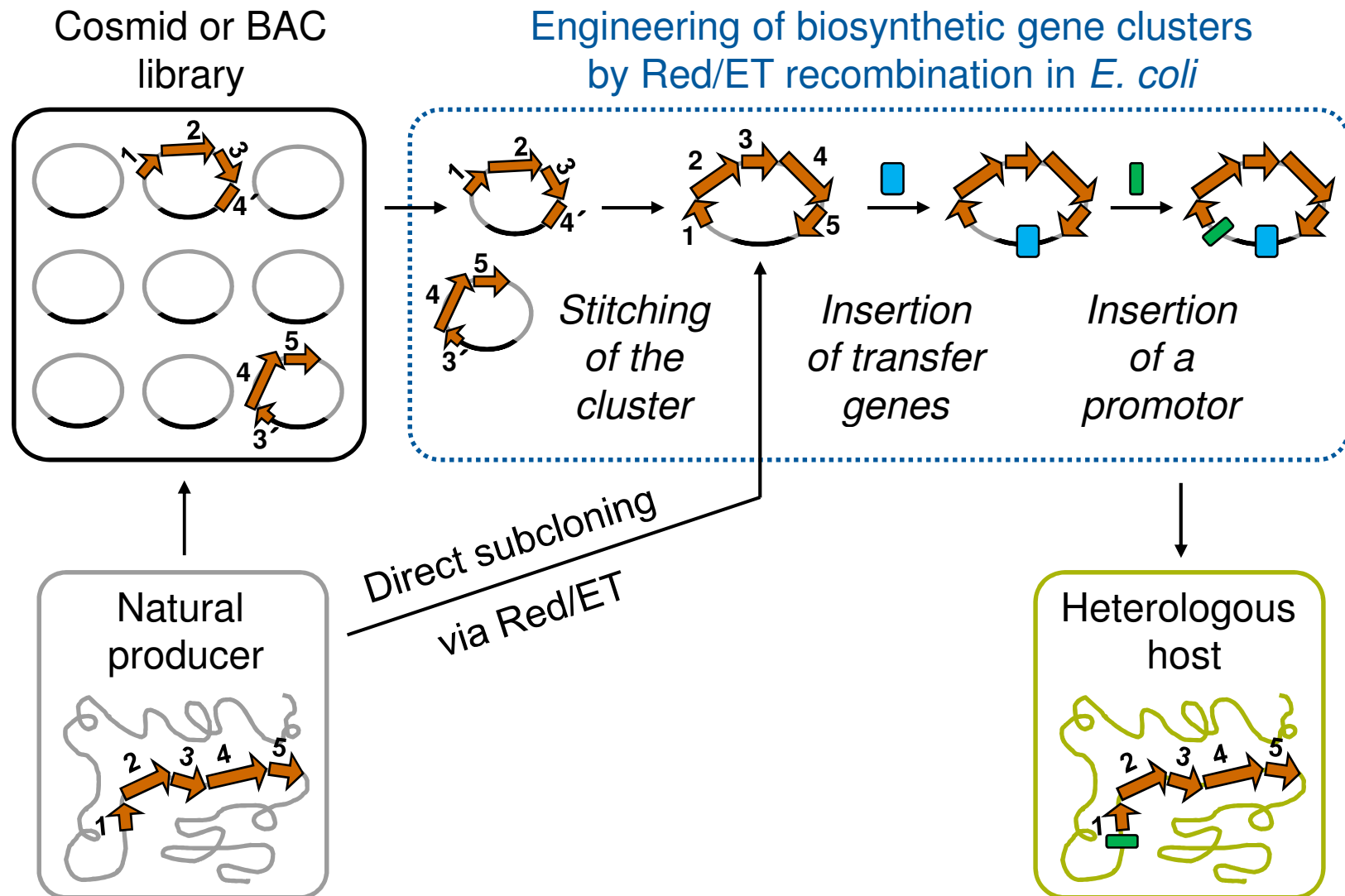
Cortina et al.,
Angew. Chem. 2012

Genome Mining by Heterologous Expression



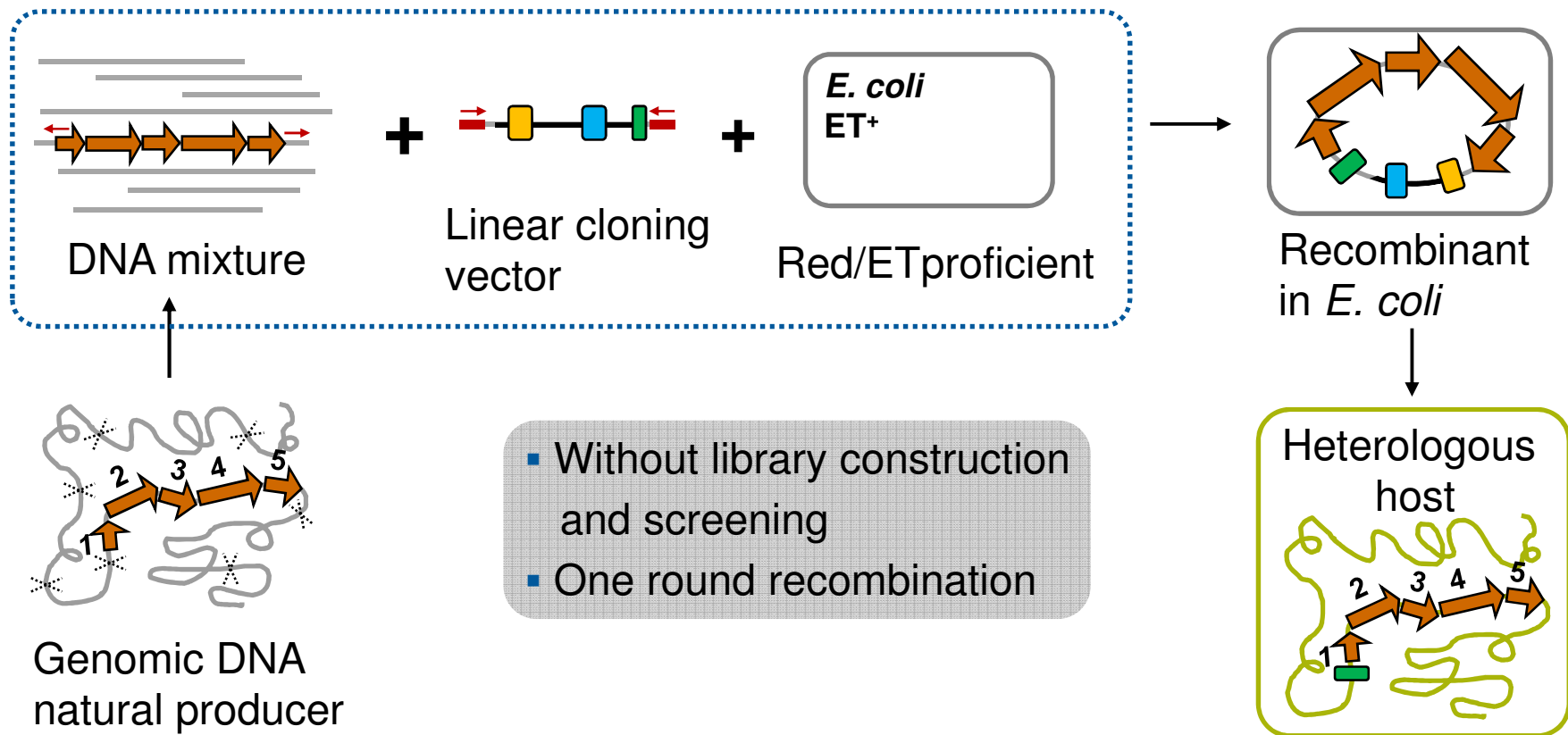
Wenzel et al., *Chem. Biol.* 2005

Genome Mining by Heterologous Expression



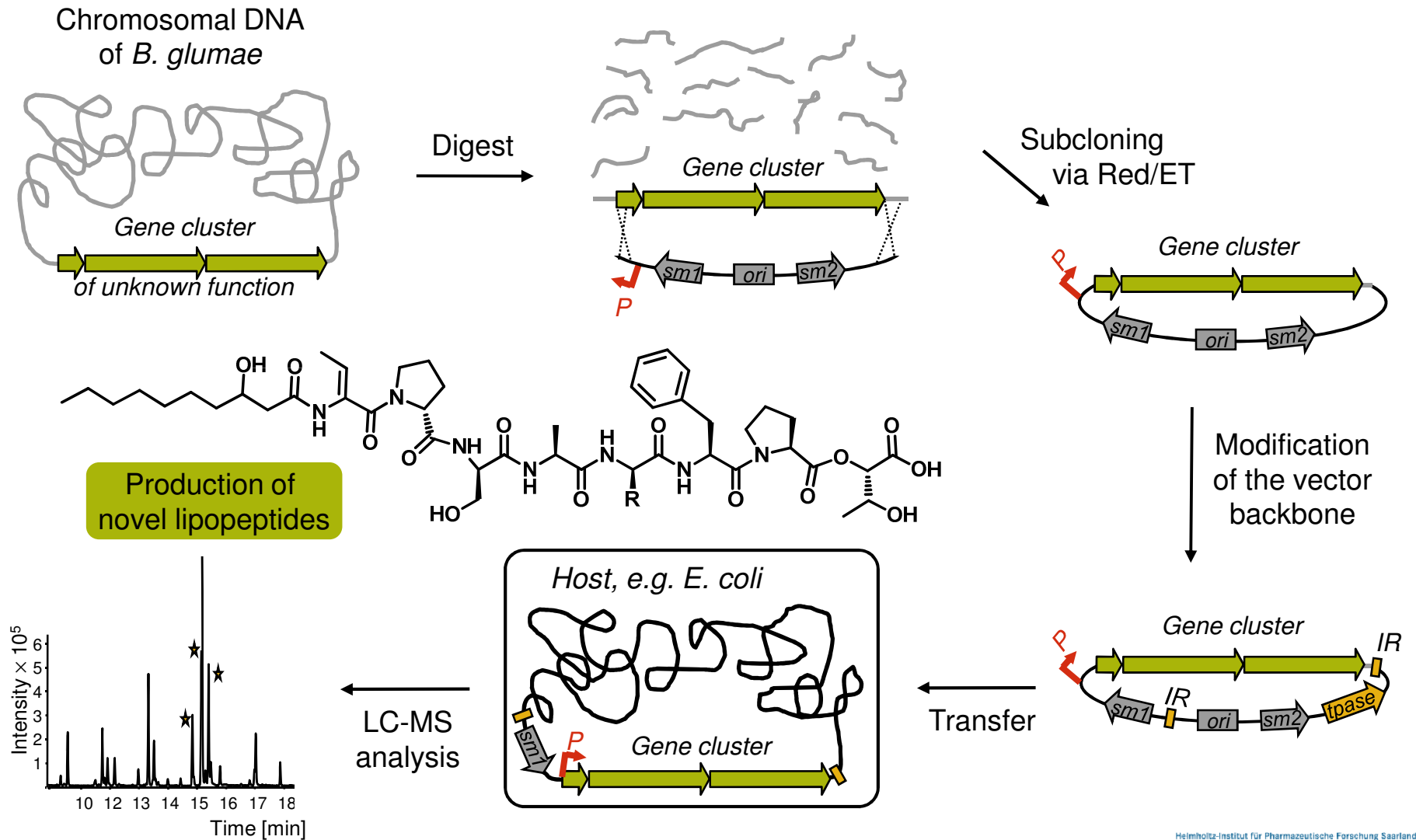
Wenzel et al., *Chem. Biol.* **2005** ; Fu et al., *Nat. Biotech.* **2012**

Direct Cloning by linear DNA Homologous Recombination



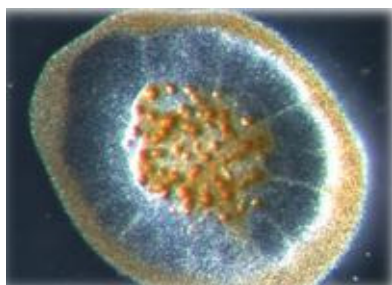
Fu et al., *Nat. Biotech.* 2012 highlighted in *Nat. Biotech* and *Nat. Methods*

Unknown Gene Cluster Generates Novel Lipopeptides

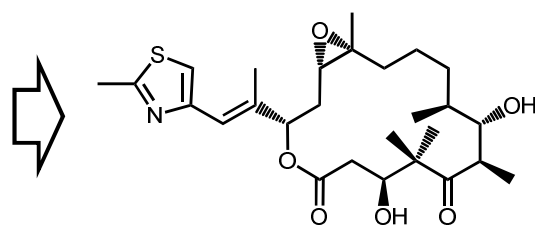


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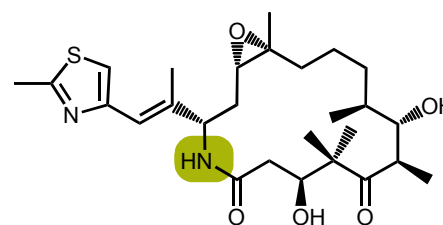
De novo Gene Cluster Synthesis for the Production of bioactive Polyketides/nonribosomal Peptides



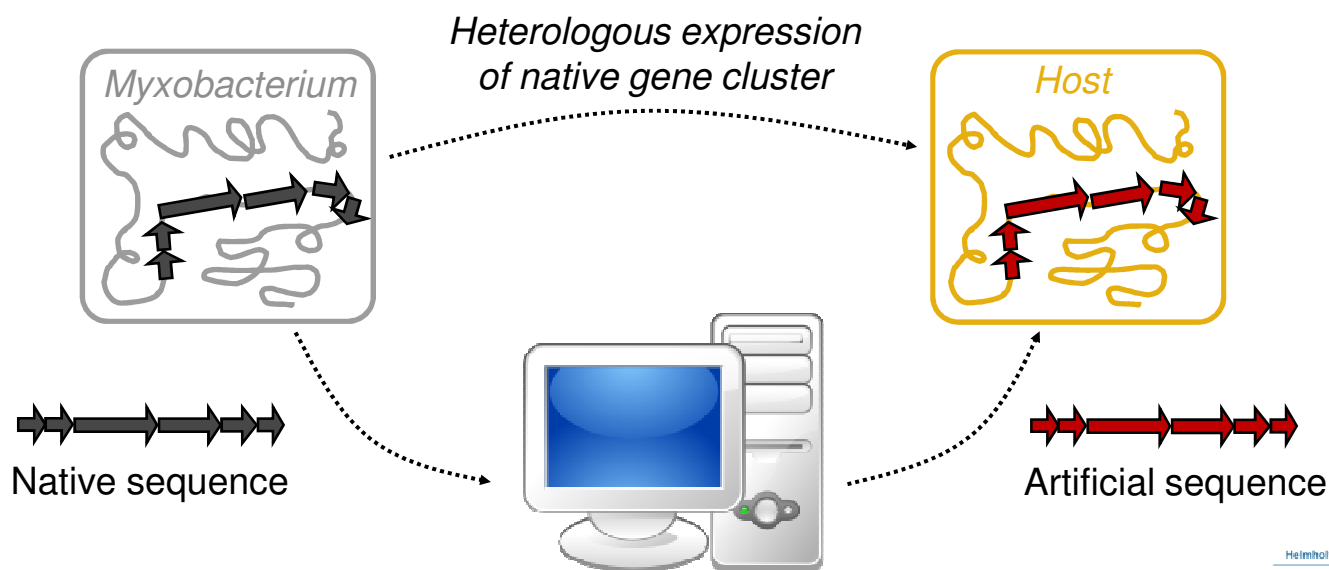
Sorangium cellulosum



Epothilone B



Ixabepilone

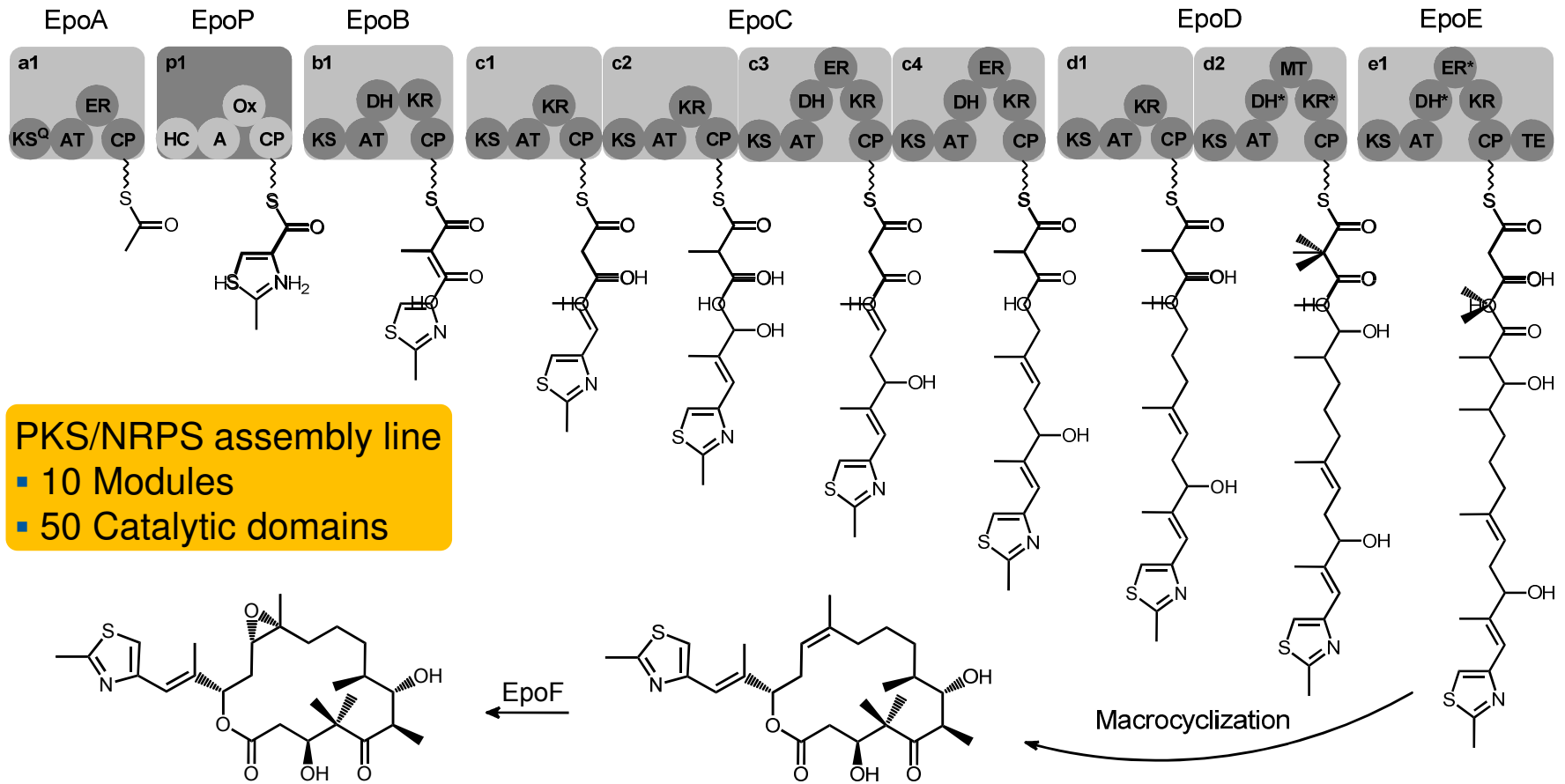


Epothilone Biosynthesis

Epothilone biosynthetic gene cluster (~ 60 kb)

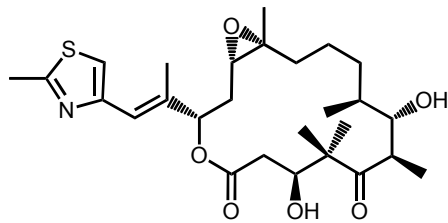


Epothilone assembly line



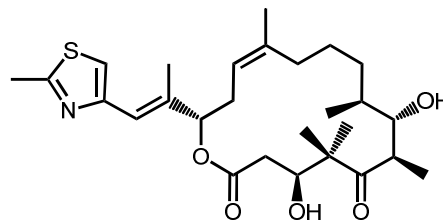
PKS/NRPS assembly line

- 10 Modules
- 50 Catalytic domains



Epothilone B

EpoF



Epothilone D

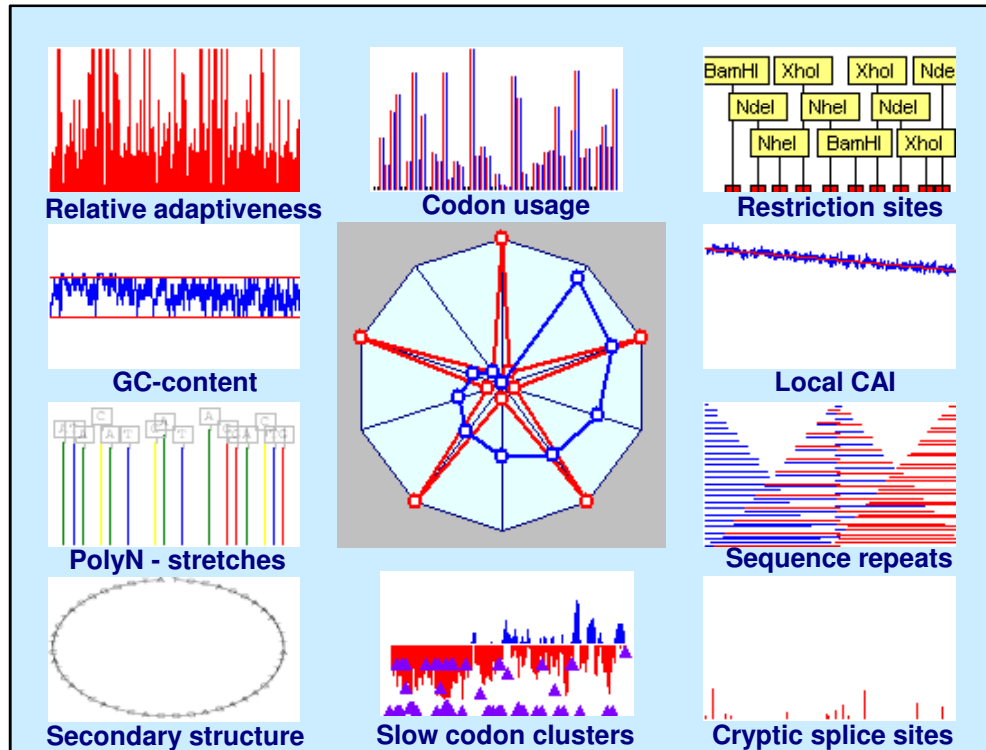
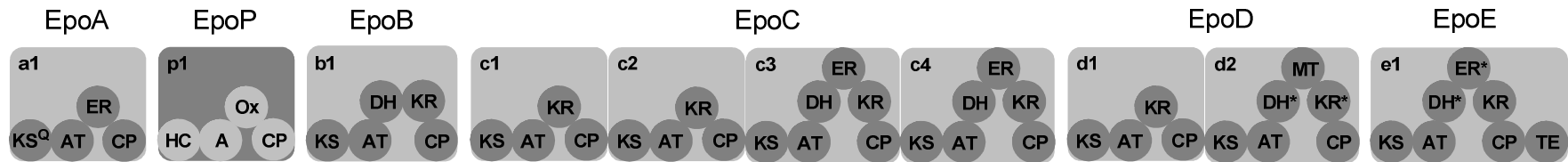
Macrocyclization

Design of an artificial Epothilone Pathway

Epothilone biosynthetic gene cluster (~ 60 kb)



Epothilone assembly line



Native gene cluster sequence
(from *Sorangium cellulosum*)



Design of an artificial
gene cluster sequence



Artificial gene cluster sequence
(optimized for *Myxococcus xanthus*)

Helmholtz-Institut für Pharmazeutische Forschung Saarland

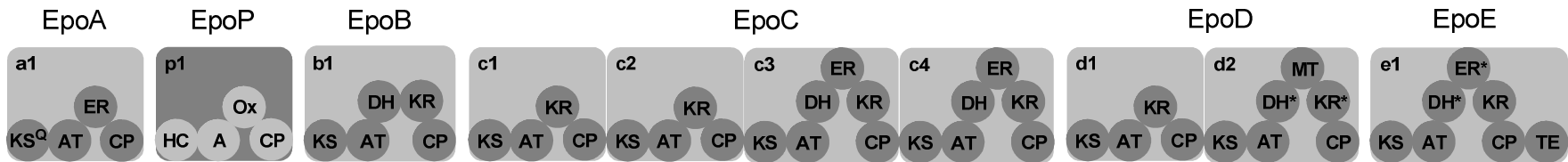


Assembly of an artificial Epothilone Pathway

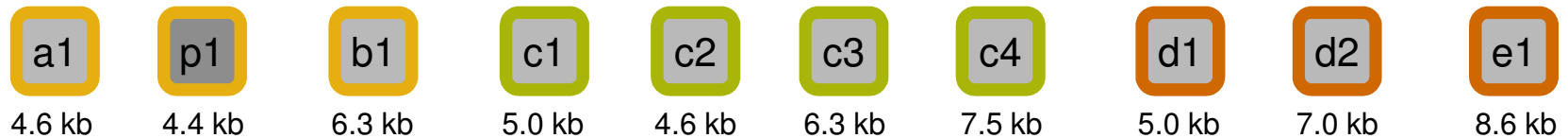
Epothilone biosynthetic gene cluster (~ 60 kb)



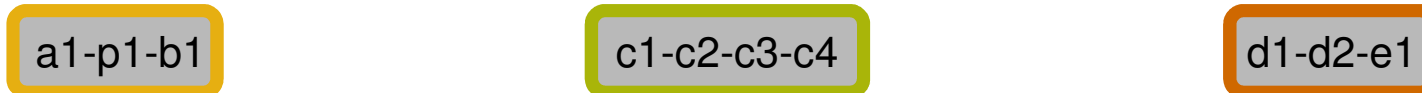
Epothilone assembly line



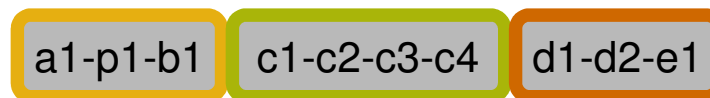
Total synthesis of modules



Assembly of operons



Assembly of the gene cluster

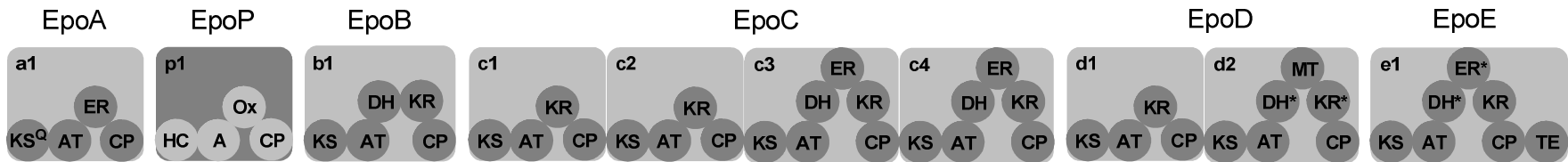


Assembly of an artificial Epothilone Pathway

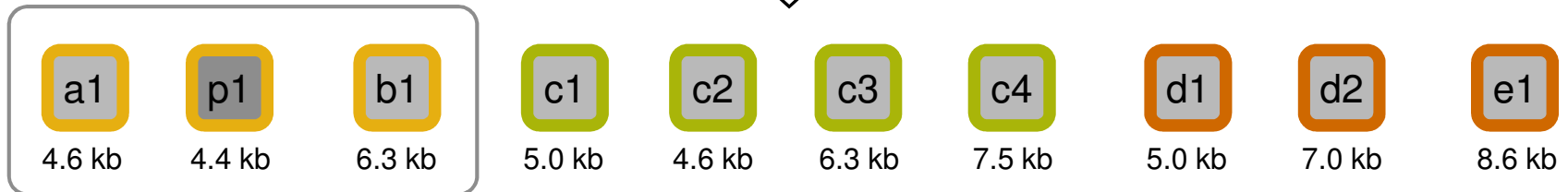
Epothilone biosynthetic gene cluster (~ 60 kb)



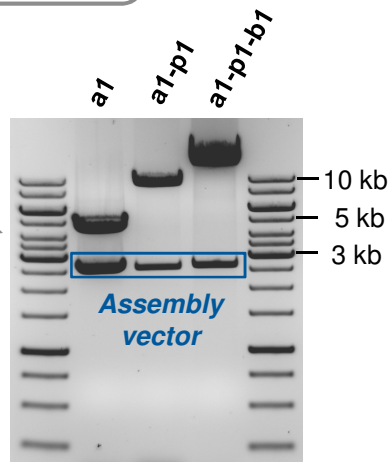
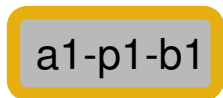
Epothilone assembly line



Total synthesis of modules



Assembly of the first transcription unit



Sequence design allows for various assembly strategies, including

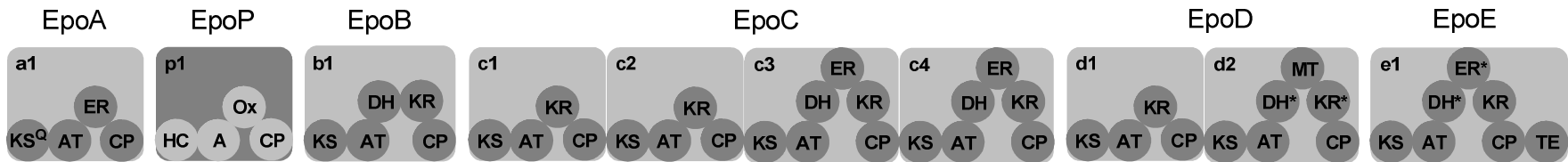
- Conventional cloning
- Recombination technologies (*in vivo* & *in vitro*)

Assembly of an artificial Epothilone Pathway

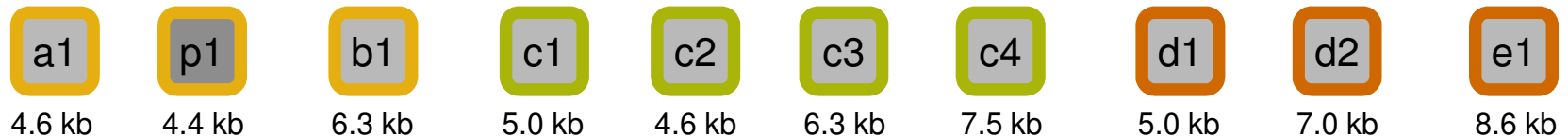
Epothilone biosynthetic gene cluster (~ 60 kb)



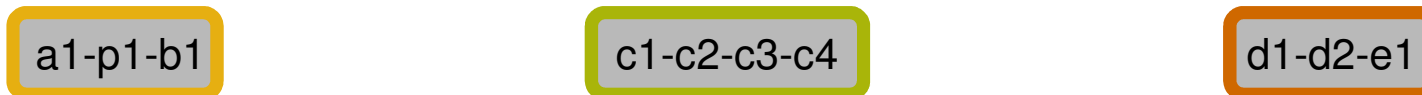
Epothilone assembly line



Total synthesis of modules

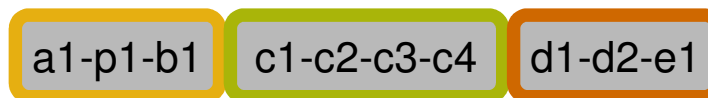


Assembly of operons



Assembly of the gene cluster

Unstable!

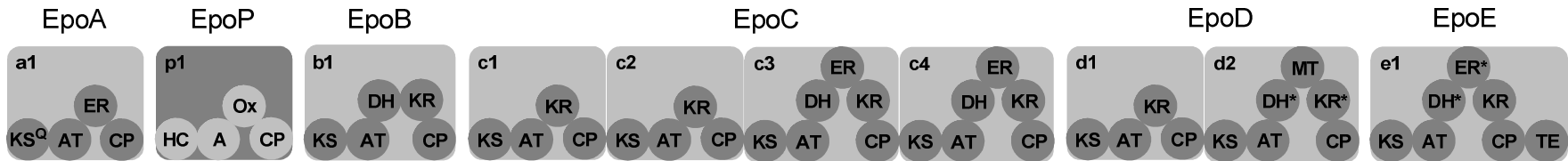


Assembly of an artificial Epothilone Pathway

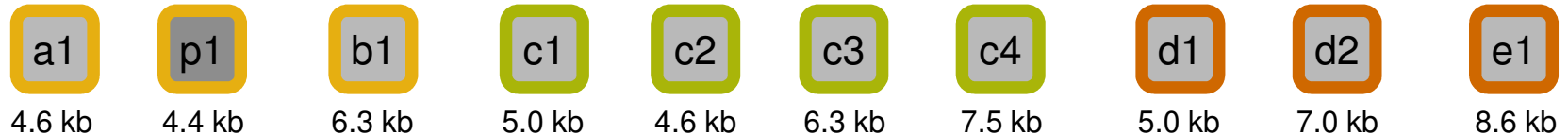
Epothilone biosynthetic gene cluster (~ 60 kb)



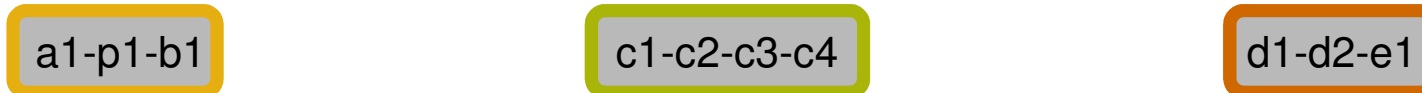
Epothilone assembly line



Total synthesis of modules



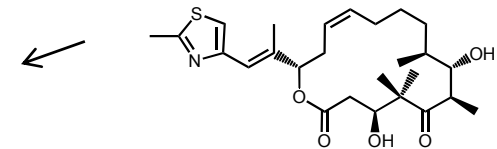
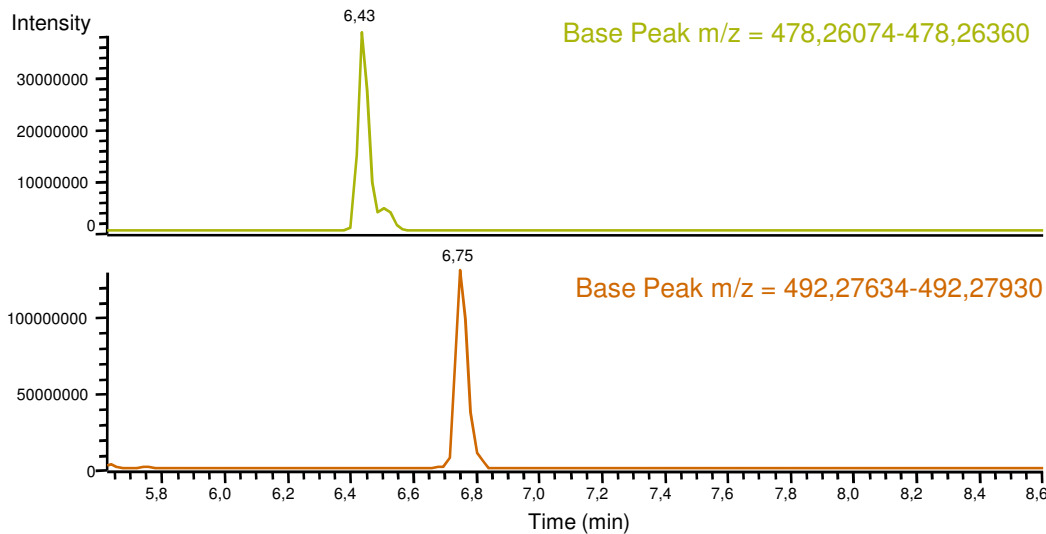
Assembly of operons



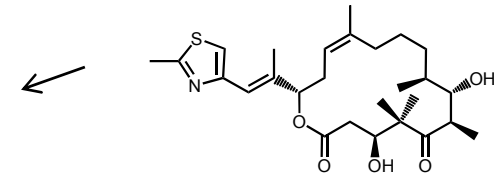
Assembly of the gene cluster



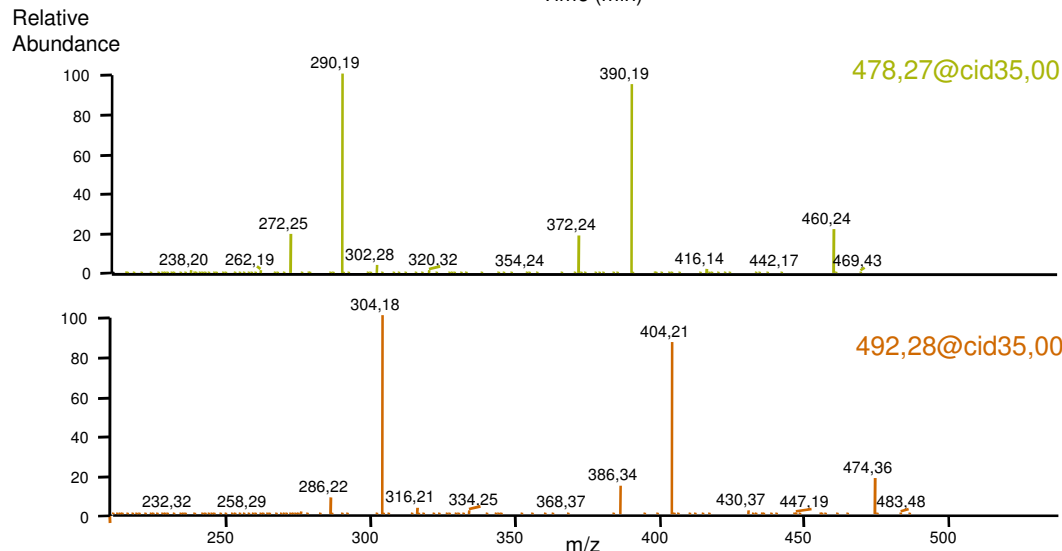
Heterologous Epothilon Production mediated by an artificial Biosynthetic Gene Cluster



Epothilon C ($m/z [M+H]^+ 478,26$)



Epothilon D ($m/z [M+H]^+ 492,27$)

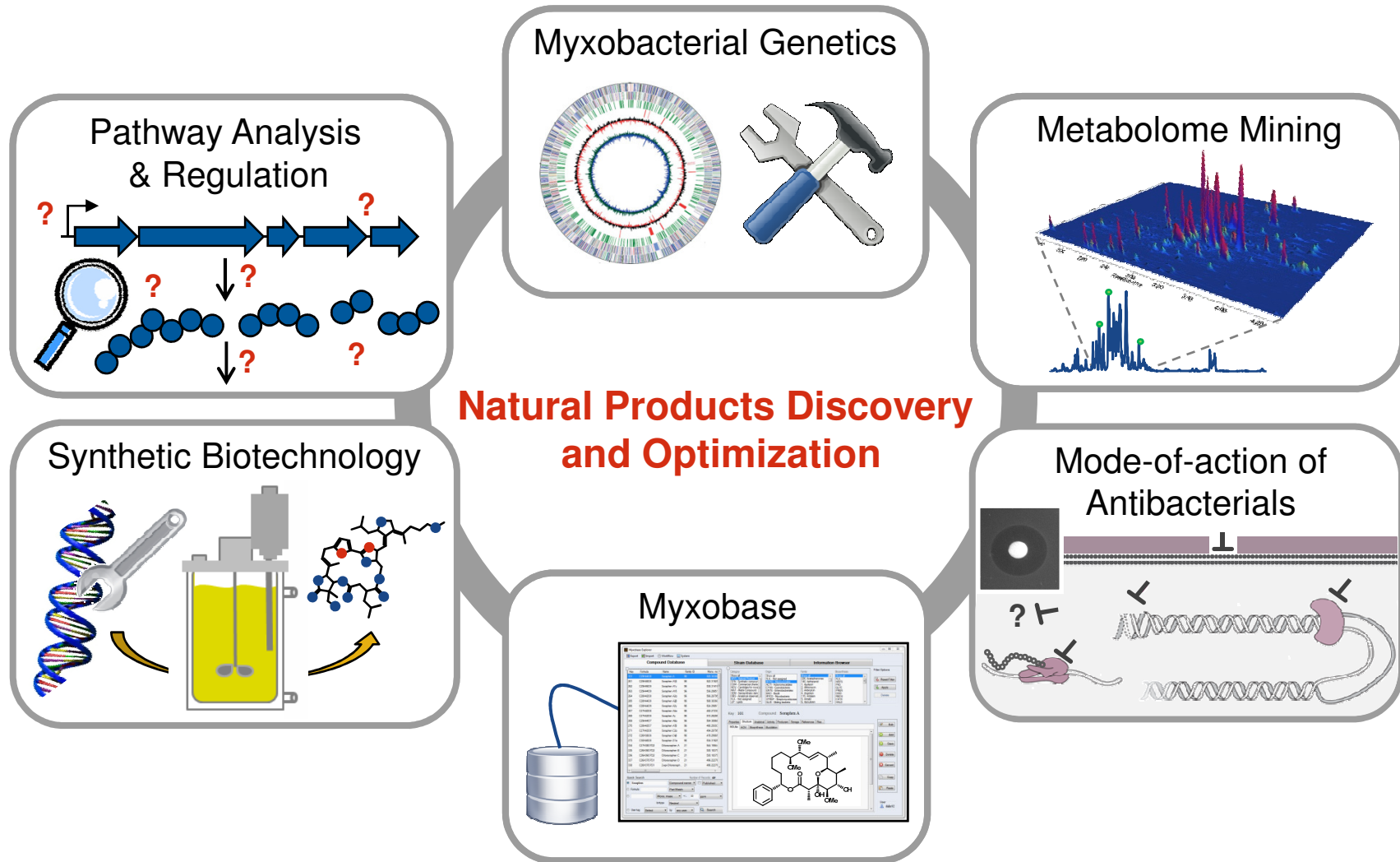


Production yield
~ 100 $\mu\text{g/L}$

→ bottlenecks?

→ improvement of
production yields

Functional Genomics for novel Anti-Infectives



Helmholtz Institute for Pharmaceutical Research

Department of Microbial Natural Products



- **HZI Braunschweig:** Susanne Häussler, Heinz, Nick Quade, Klaus Gerth, Helmut Blöcker, Rolf Jansen, Heinrich Steinmetz, Wolfgang Kessler, Florenz Sasse, Marc Stadler
- **Saarland University:** Uli Kazmaier, Johann Jauch, Elmar Heinzle
- **TU Braunschweig:** Stefan Schulz, Christoph Wittmann
- **GeneBridges:** Youming Zhang, Francis Stewart
- **Bielefeld University:** Alf Pühler, Alex Goesmann, Susanne Schneiker
- **Hannover University:** Andreas Kirschning, Markus Kalesse
- **Intermed Discovery:** Marc Stadler, Jens Bitzer
- **Bruker Daltonik:** Gabriela Zurek
- **ATG Biosynthetics:** Hubert Bernauer

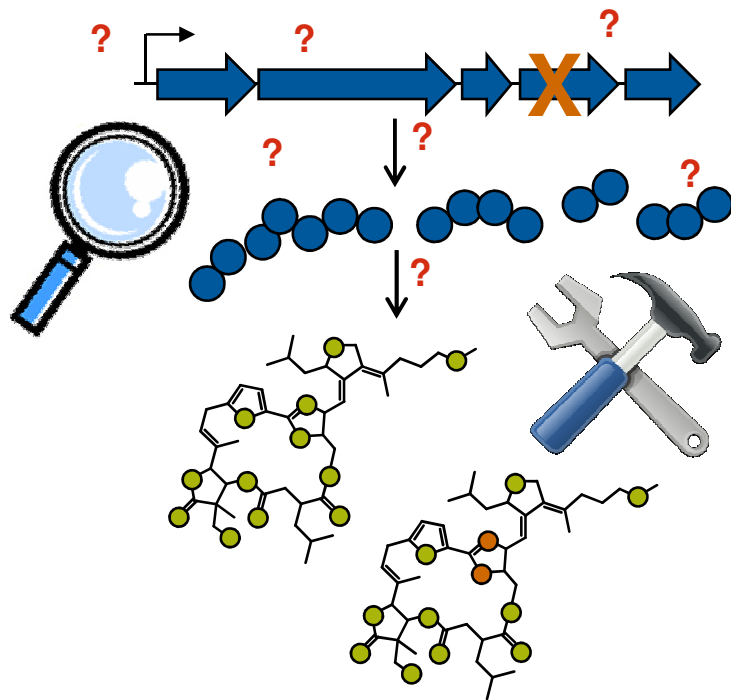
Financial support: BMBF, DFG, Gebiotec



Helmholtz-Institut für Pharmazeutische Forschung Saarland

Challenges in Developing Natural Products

- *Structural complexity*
- *Fermentative yield*
- *Backbone modifications*
- ...



Structure & Yield optimization

How are these compounds made?

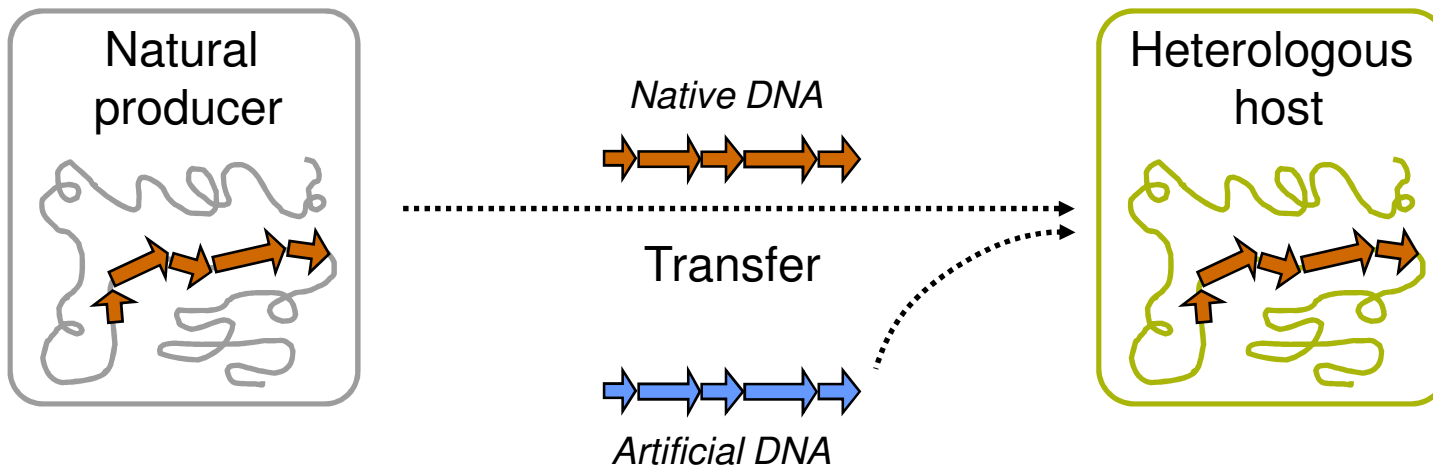
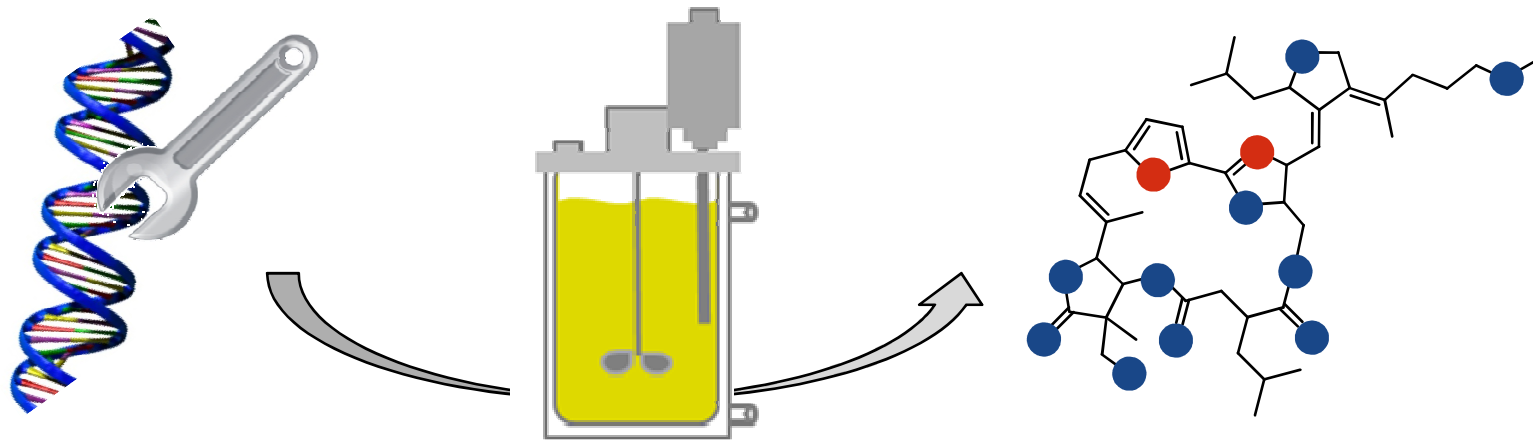
- Identify biosynthetic pathways (*Bacterial genomics*)
- Study biosynthesis & gene regulation

How can we engineer production?

- Manipulate the producer strains
- Express pathways in suitable hosts

... **Synthetic Biotechnology**

'Synthetic Biotechnology'



Mutasyntesis in *Streptomyces sp.* JS360::cinQ⁻ mutant

