GenScript
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A Guide to DNA Assembly for Drug Discovery

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Assistant Professor
University of Minnesota

May 15, 2018

Leveraging DNA Assembly for Natural Product Biosynthesis

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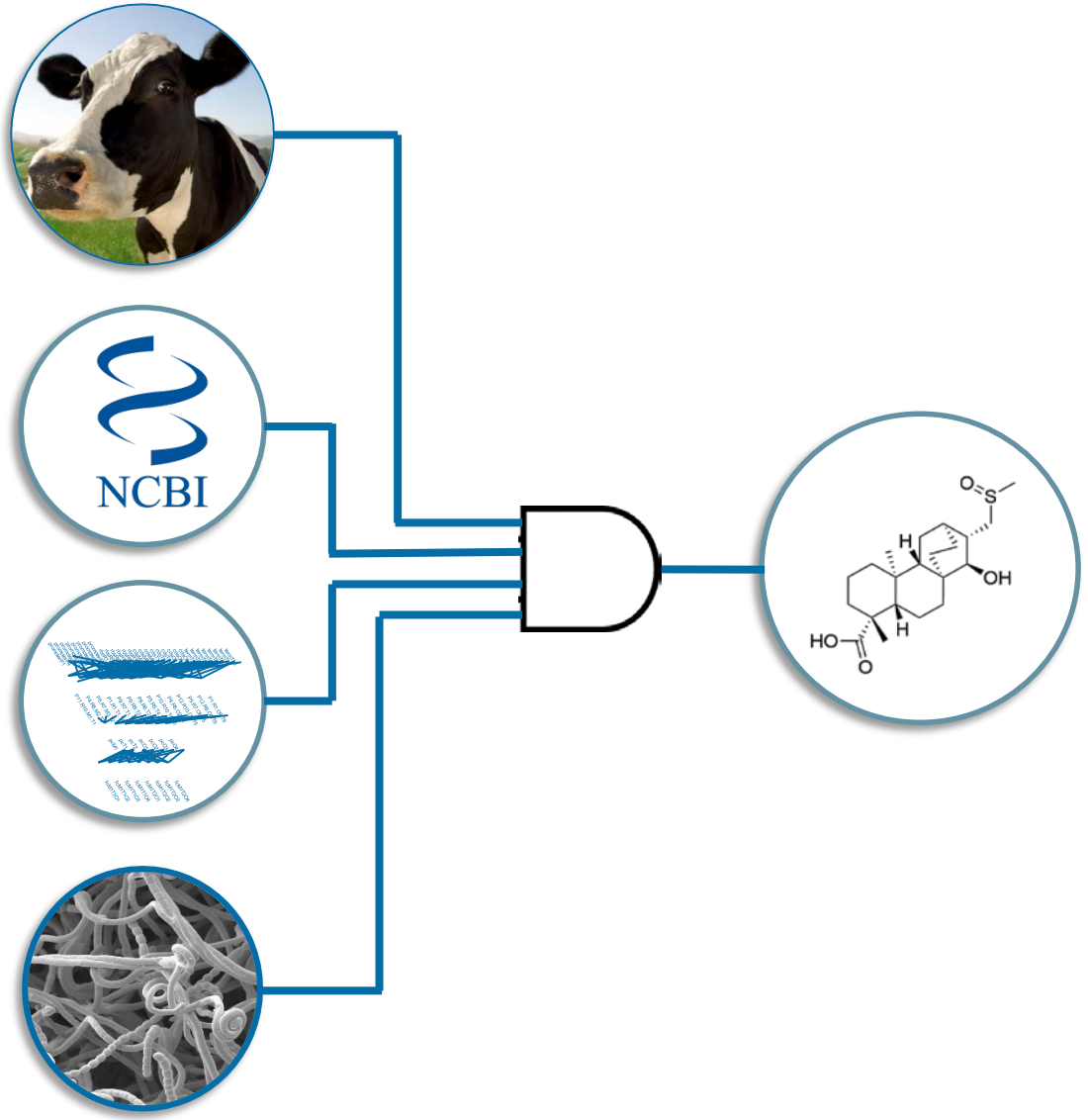


BitesizeBio

Goals for the Webinar

- Introduction to **key concepts** in DNA assembly as it relates to engineering metabolic pathways
- Argument for the **importance of combinatorial DNA assembly** projects in natural product biosynthesis
- **Example application** to illustrate how DNA assembly methods were used to produce a valuable natural product scaffold
- **New directions/questions** that are important for pushing genetic engineering into more complex multi-part systems

“DNA Synthesis” versus “DNA Assembly”

IDT INTEGRATED DNA TECHNOLOGIES

Agilent Technologies

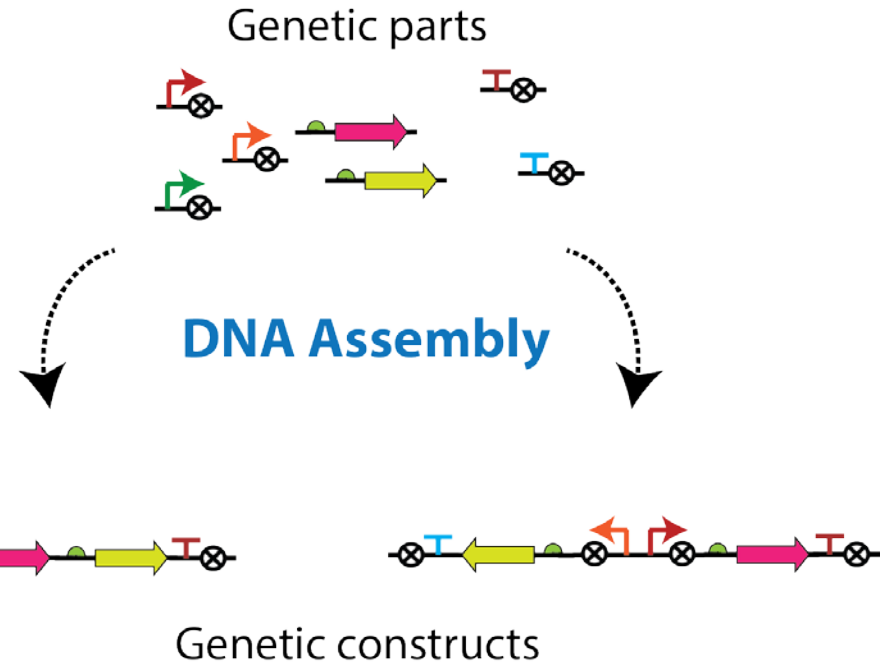
GenScript Make Reseach Easy

eurofins

TWIST BIOSCIENCE

JGI JOINT GENOME INSTITUTE DEPARTMENT OF ENERGY

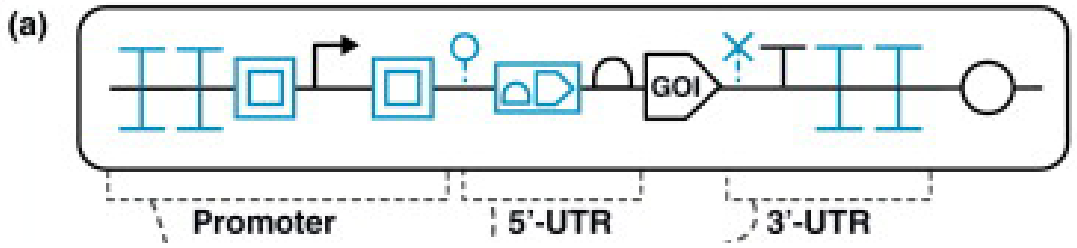
GENEART THE GENE OF YOUR CHOICE



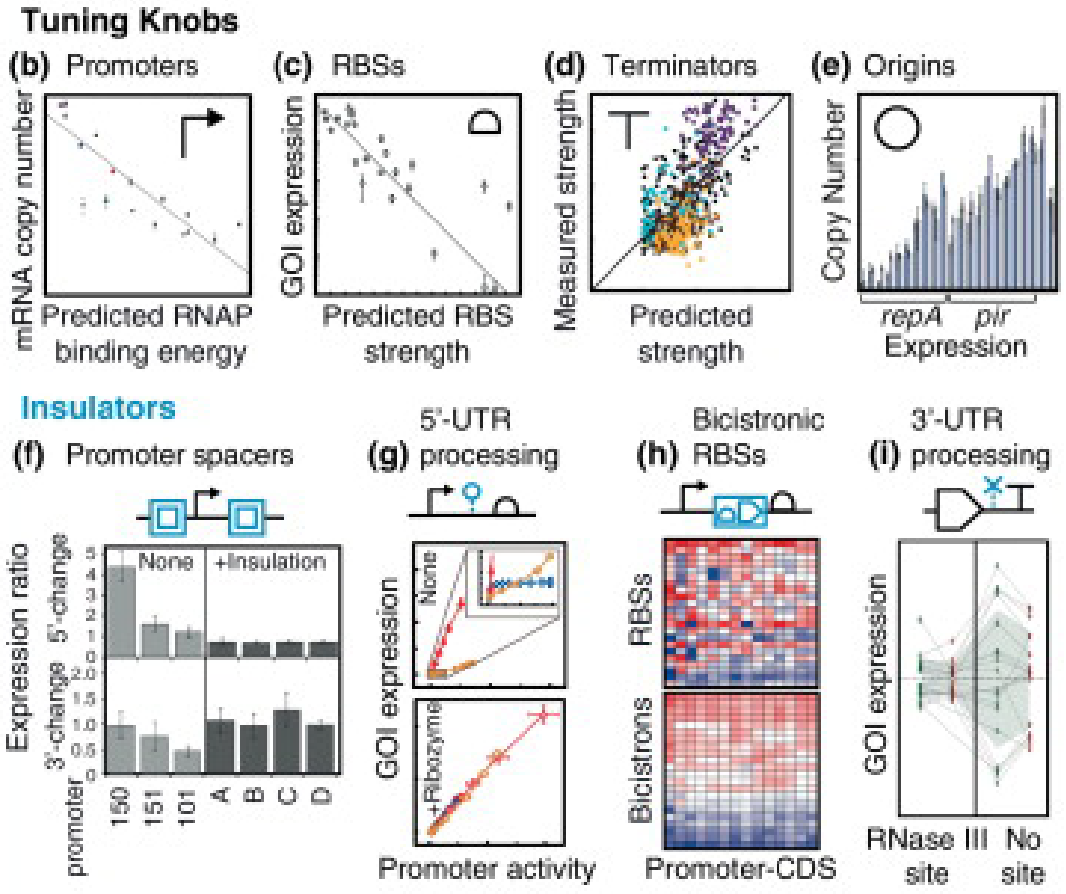
DNA Synthesis: de novo construction of oligonucleotides and larger molecules from nucleotide monomers

DNA Assembly: combinatorial concatenation of pre-synthesized parts to produce functional genetic constructs

What do We Mean by “Genetic Parts”?

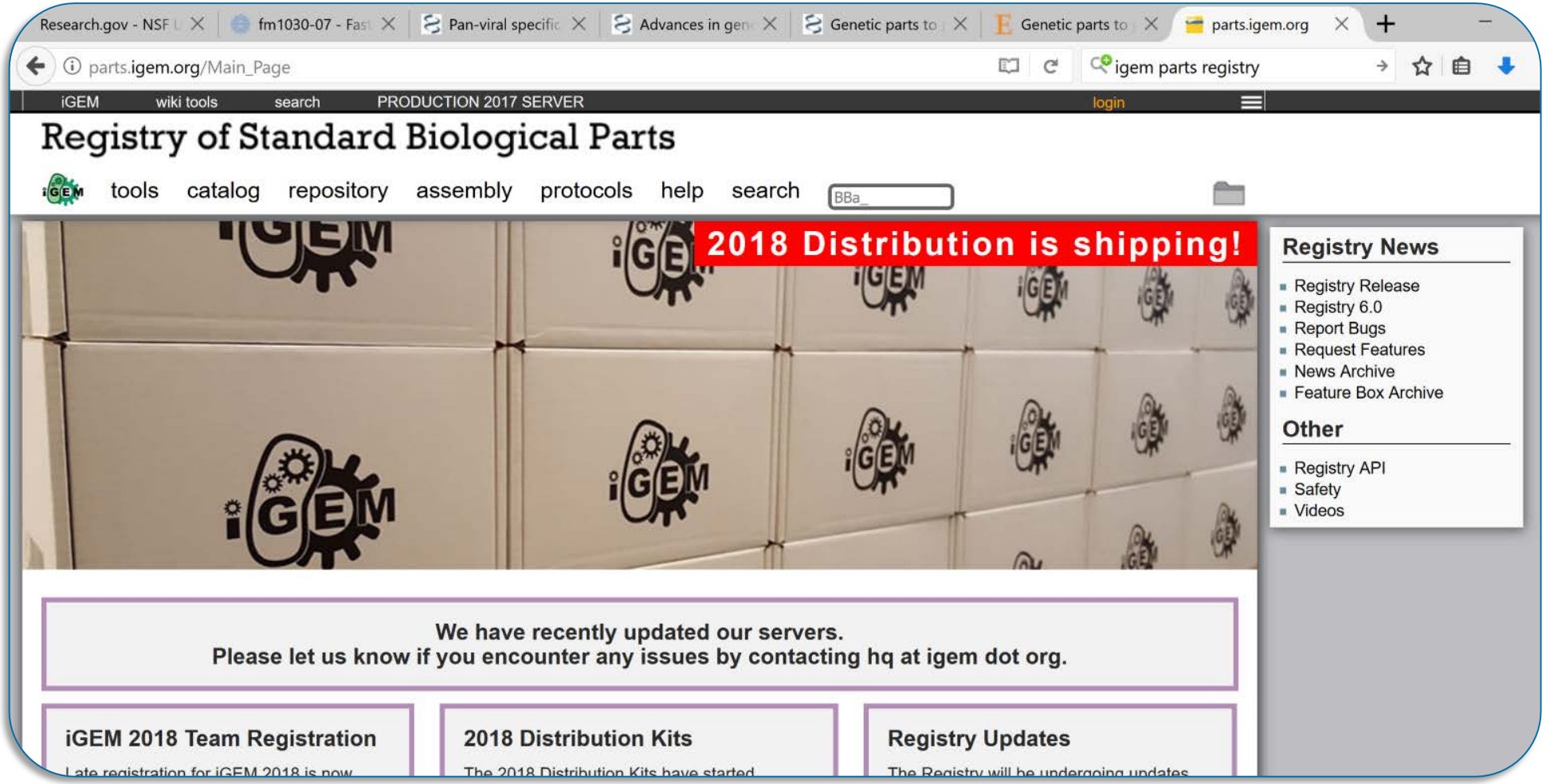


Genetic parts: a sequence of DNA that encodes a biological function or behavior. This can include information storage (*e.g.* CDS), transcription or translation control (*e.g.* promoter), etc. Genetic parts can be combined to make more complex functional units.

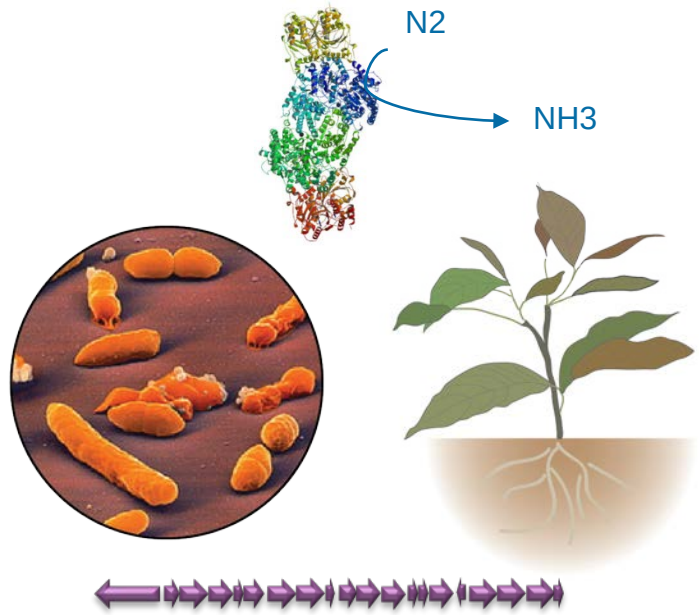


Nielsen AAK, Segall-Shapiro TH, Voigt CA ***Curr. Opin. Chem. Biol.*** (2013)

The iGEM Registry of Standard Biological Parts

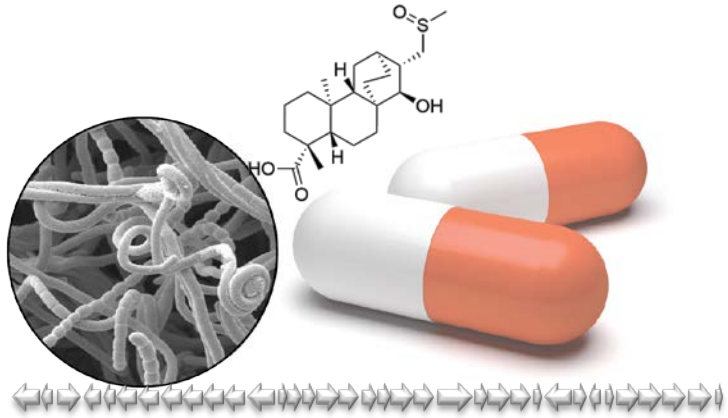


Important Biological Capabilities Require Massively Multi-Part Genetic Systems

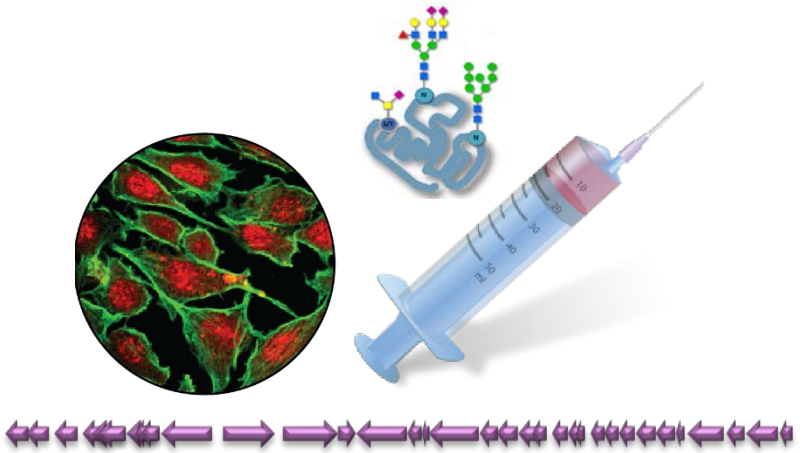


Nitrogen Fixation

Electron transfer

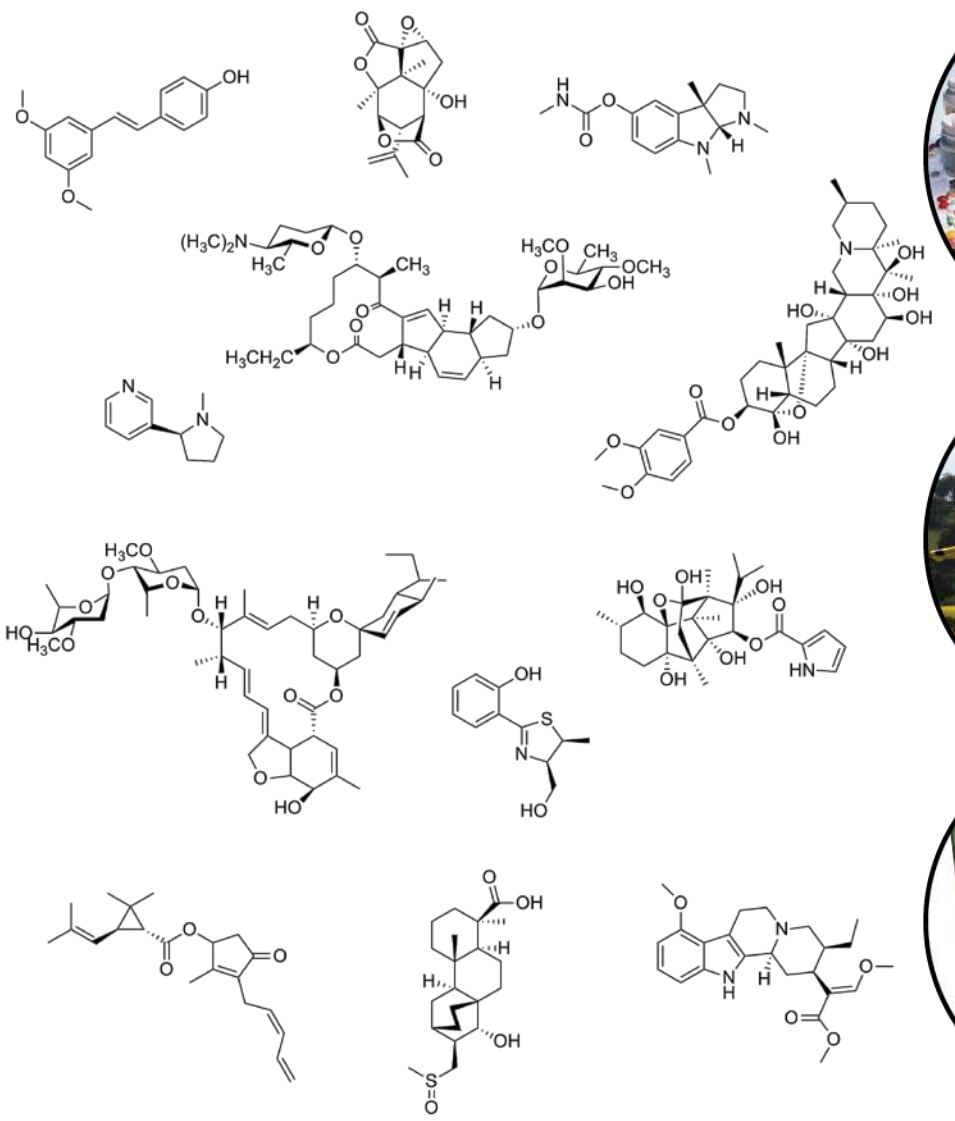


Natural product biosynthesis

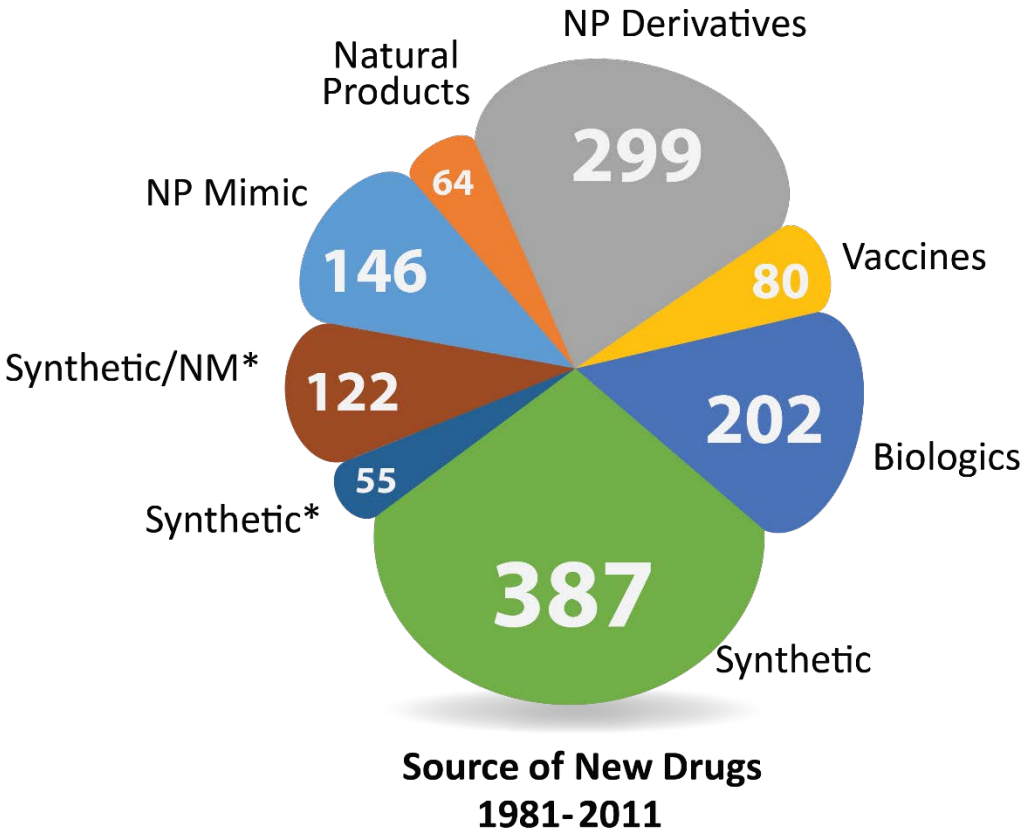


Protein glycosylation

What are “Natural Products”

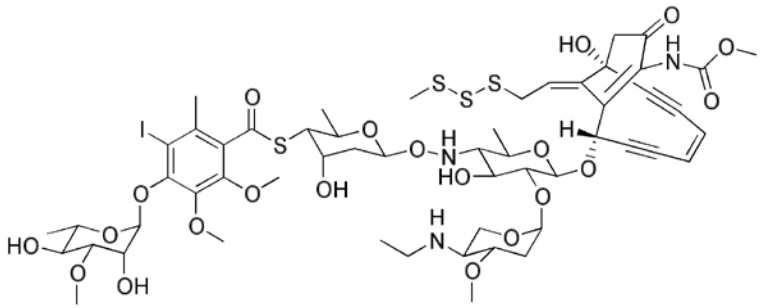


Natural Products are Privileged Structures for Drug Discovery

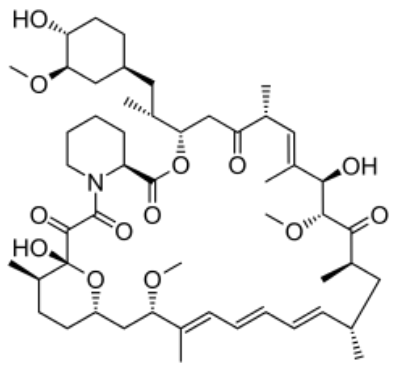


Source of New Drugs 1981-2011

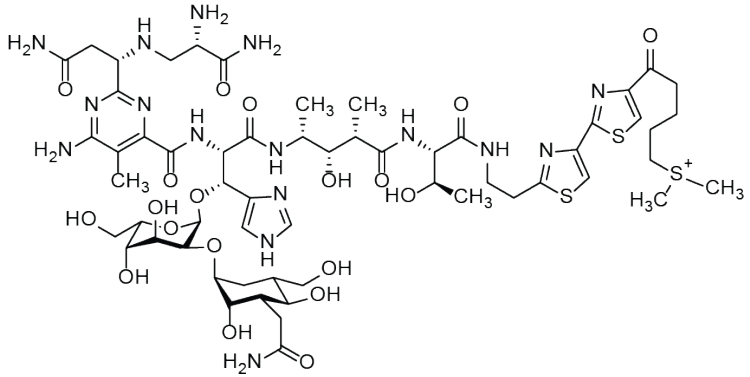
Newman & Cragg (2012) *J. Nat. Prod.* **75**:311-335



Calicheamicin (Mylotarg®)

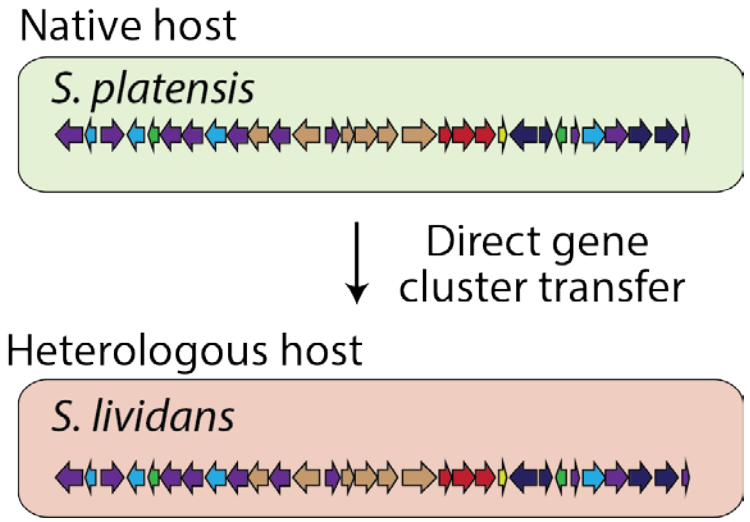


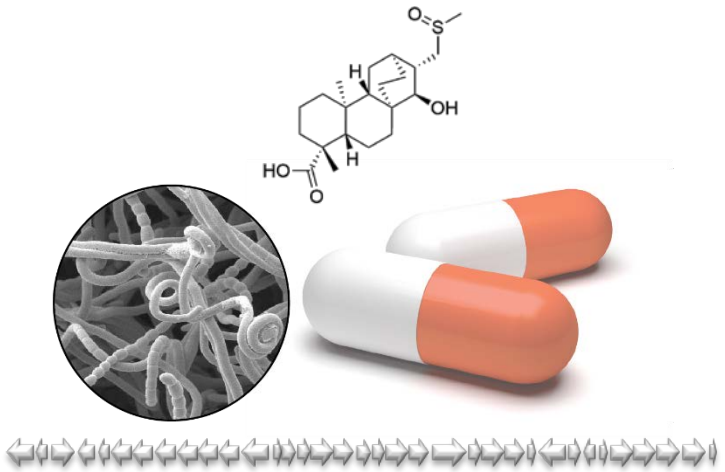
Rapamycin (Rapamune®)



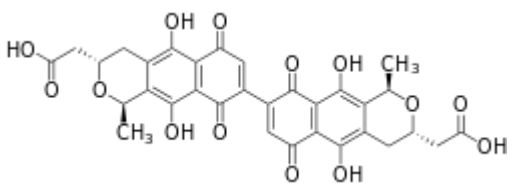
Bleomycin (Blenoxane®)

Heterologous Production of Natural Products

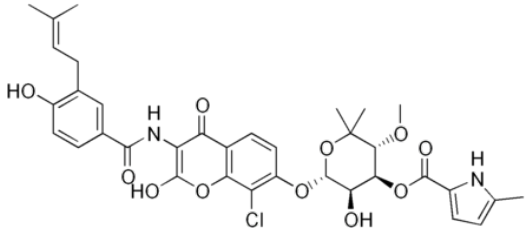




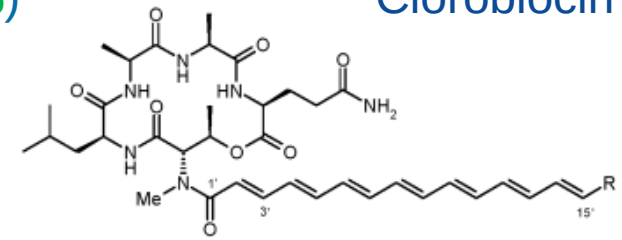
Natural product biosynthesis



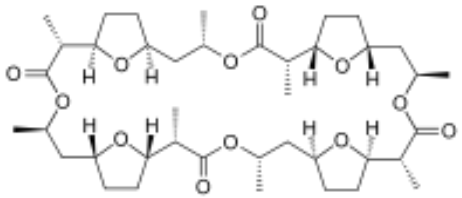
Actinorhodin (**100%**)



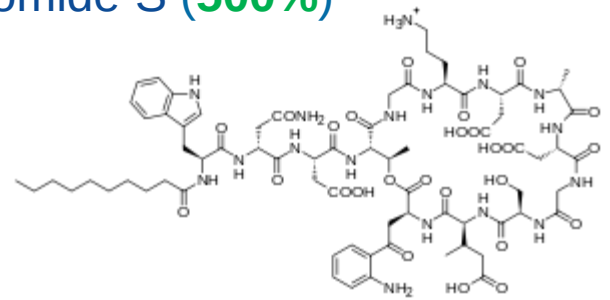
Clorobiocin (**104%**)



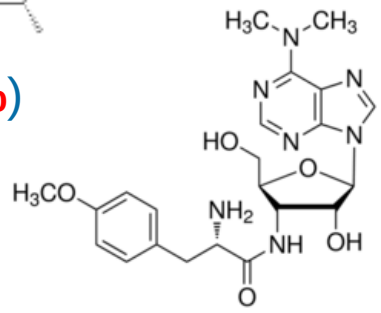
Myxochromide S (**500%**)



Nonactin (**25%**)



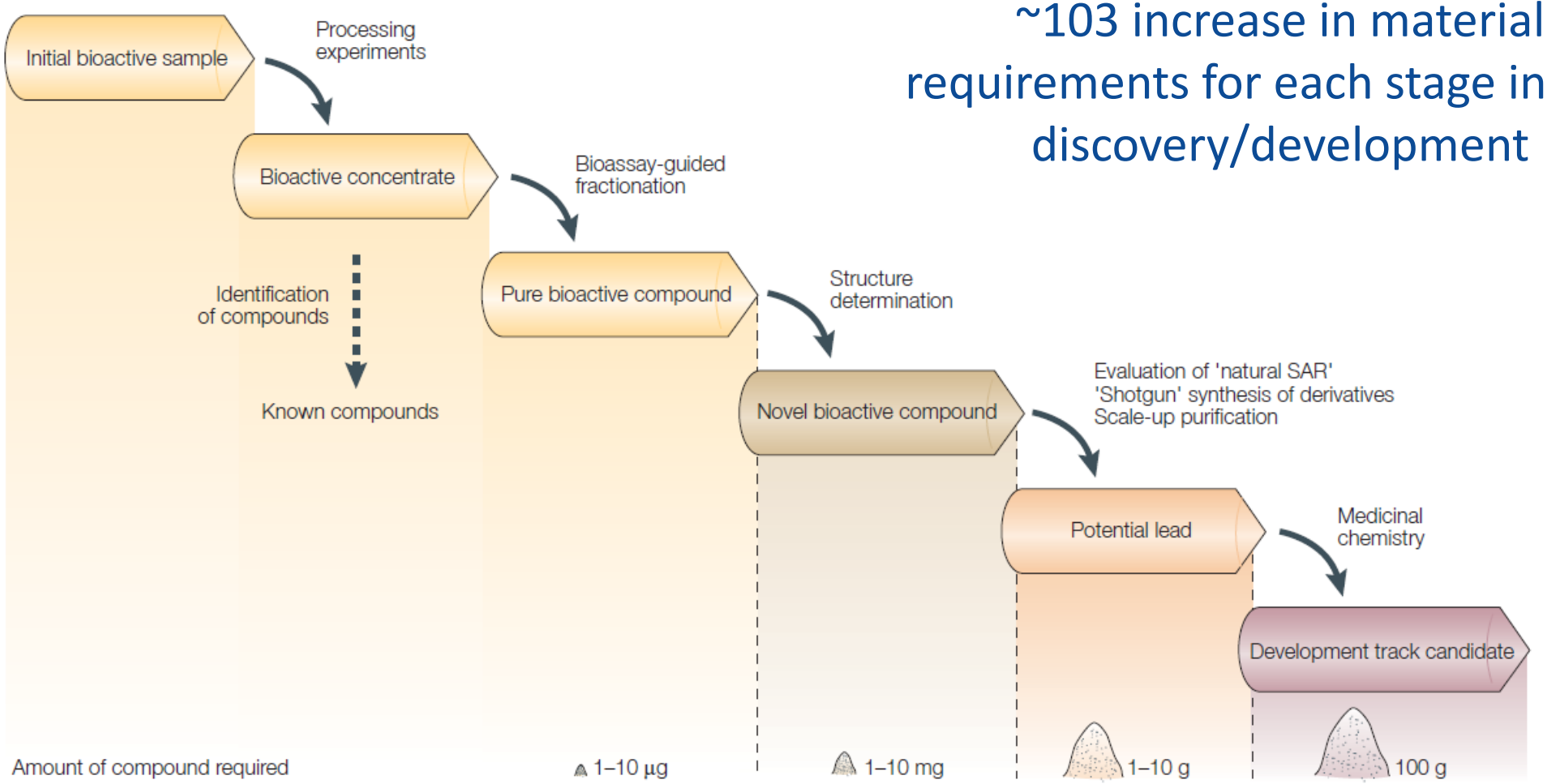
Daptomycin (**2%**)



Puromycin (**10%**)

Expert Opin. Drug Discov. (2006) **1**(5)

Drug Discovery Pipelines Require Access to Hundreds of Grams of Material

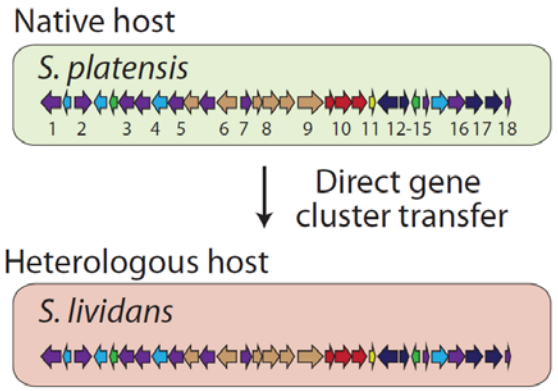


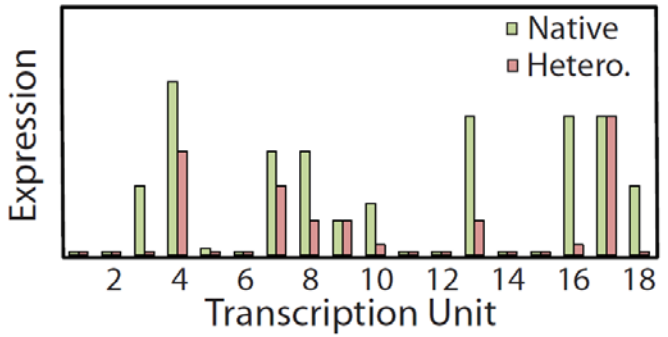
~103 increase in material requirements for each stage in drug discovery/development

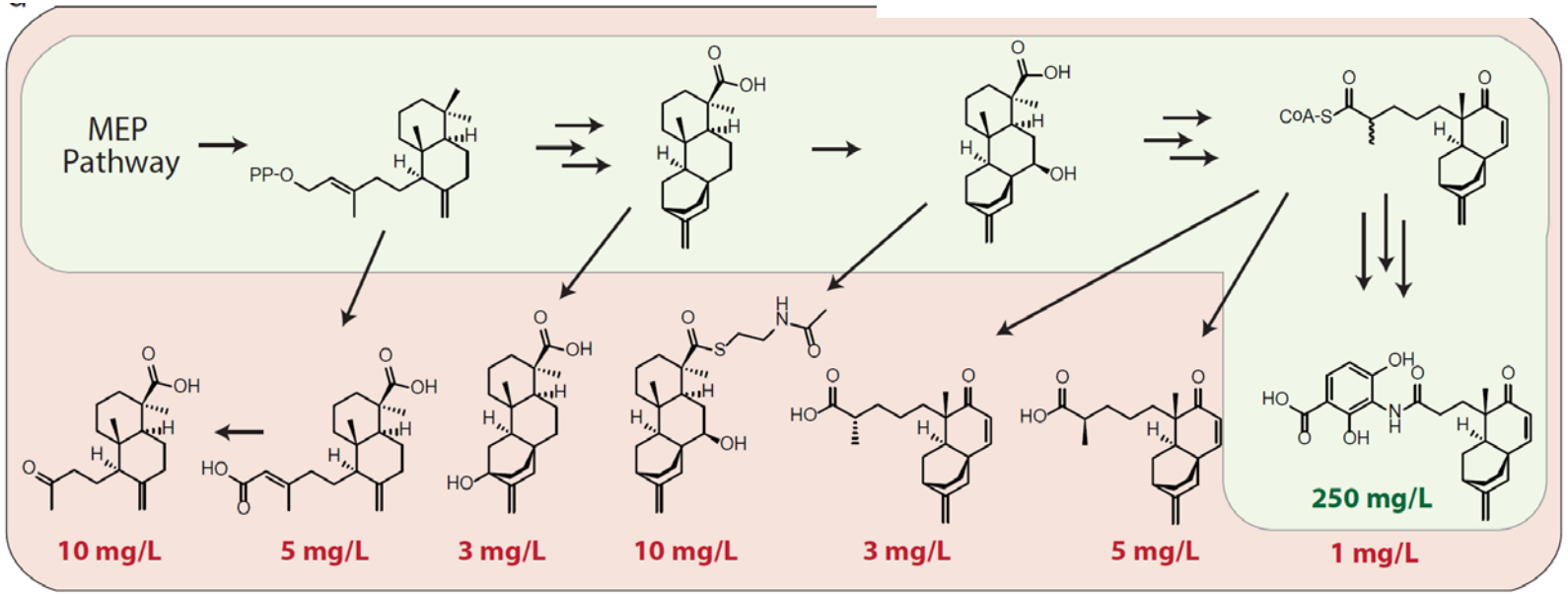
Koehn and Carter (2005) *Nat. Rev. Drug Disc*. 4:206-220

Gene Expression is Finicky but Important for Heterologous Production

- Gene expression changes upon host transfer



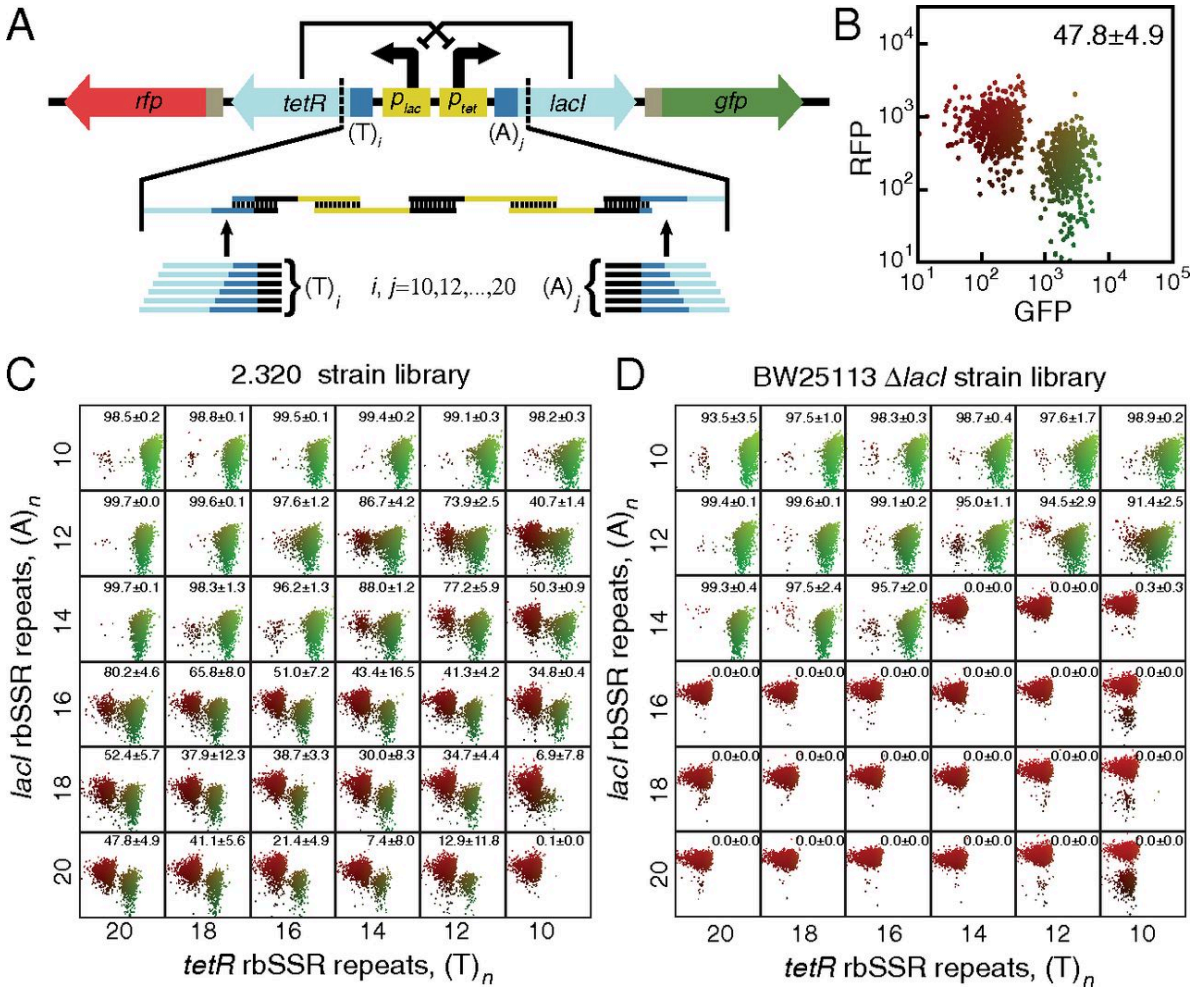




Smanski et al. Shen (2012) *J. Nat. Prod.* **75**:2158-2167

Gene Expression is Finicky but Important for Heterologous Production

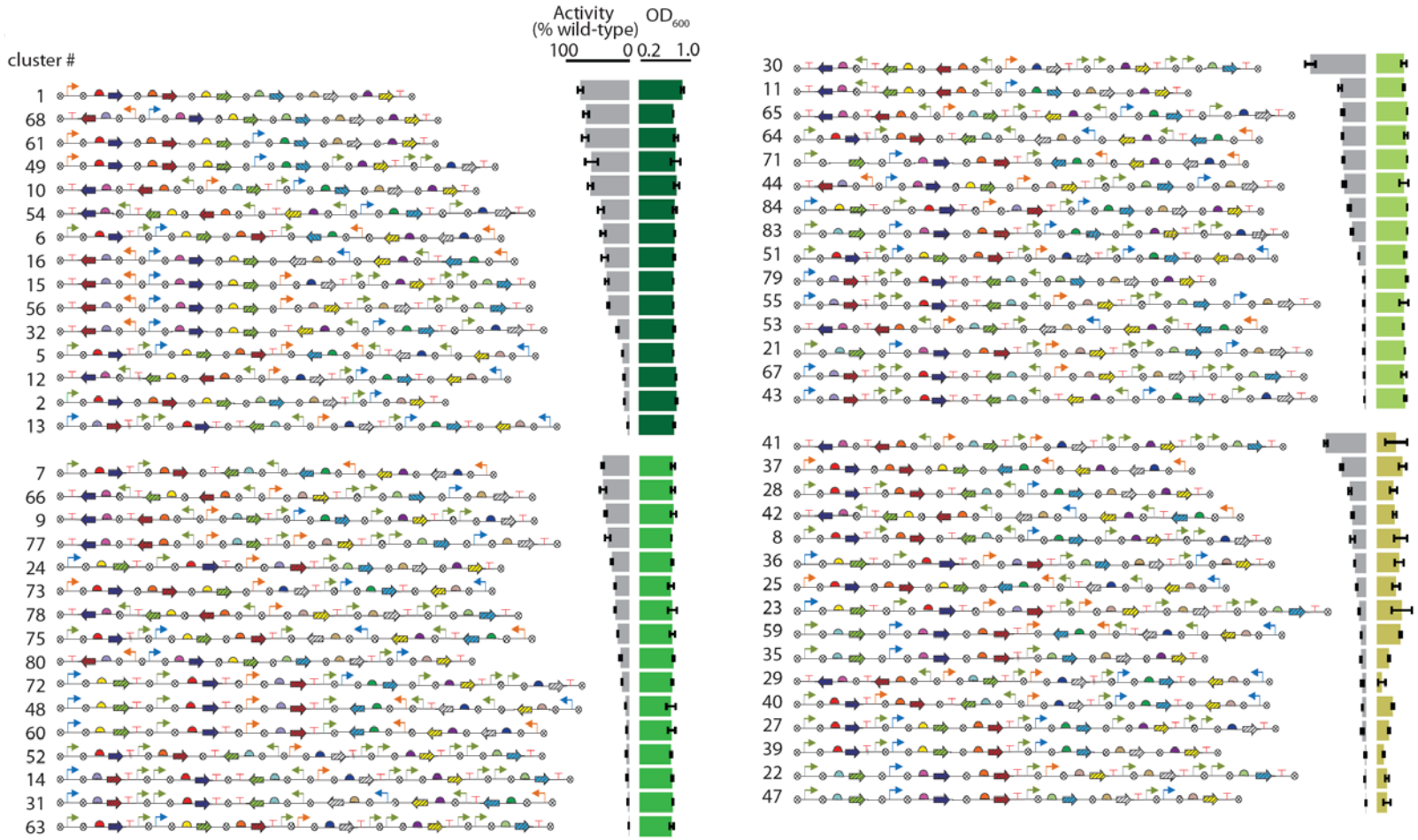
- Gene expression changes upon host transfer
- Even conservative host changes can break a system



Robert G. Egbert, and Eric Klavins PNAS 2012;109:16817-16822

Gene Expression is Finicky but Important for Heterologous Production

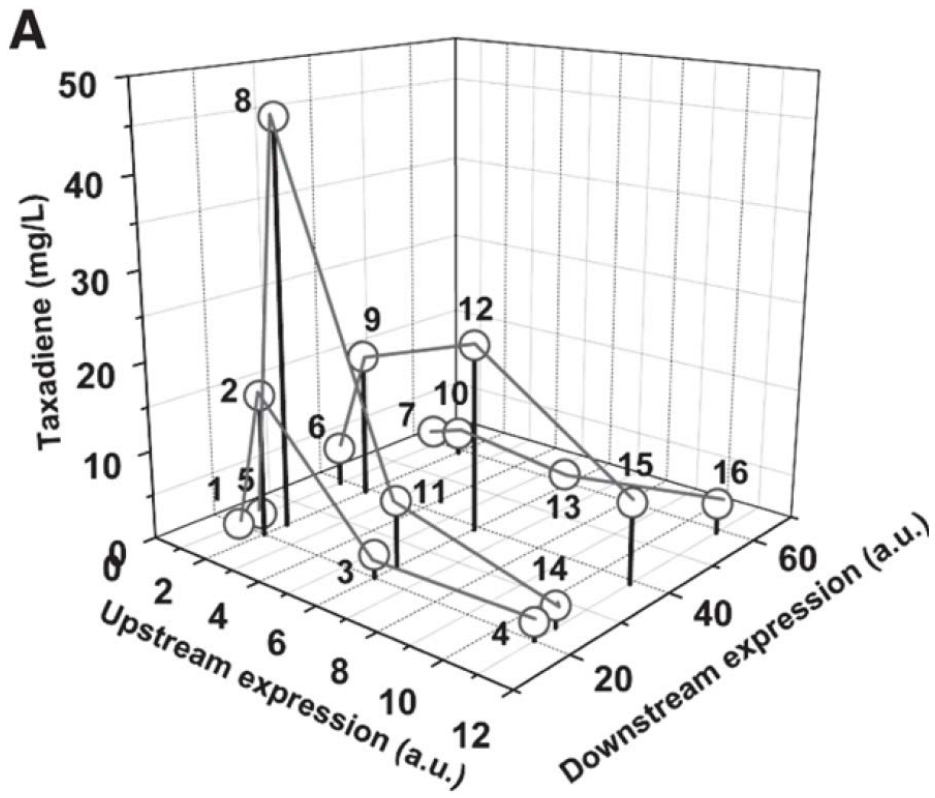
- Gene expression changes upon host transfer
- Even conservative host changes can break a system
- Permuting expression over multiple genes dramatically impacts system performance



Smanski et al. Voigt (2014) Nat. Biotechnol. 32:1241-1249

Gene Expression is Finicky but Important for Heterologous Production

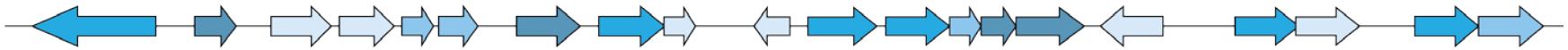
- Gene expression changes upon host transfer
- Even conservative host changes can break a system
- Permuting expression over multiple genes dramatically impacts system performance
- Optimal gene expression is nuanced and non-obvious



Ajikumar PK and Stephanopoulos G (2010) Science 330:70-74

Approach: Reconstructing Biosynthetic Gene Clusters from Parts

Natural gene cluster



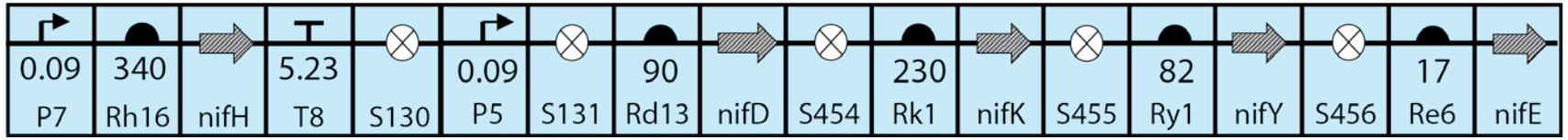
1. Remove non-coding DNA
2. Eliminate non-essential genes
3. Remove transcription factors
4. Re-design CDSs

5. Clone/Synthesize genes
6. Add synthetic regulation
7. Organize into operons
8. Control with synthetic circuits

Advantages:

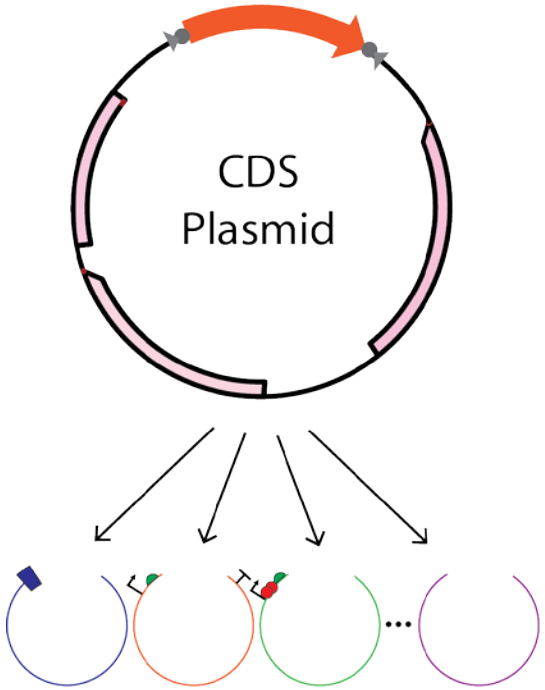
- Role of every element is known
- Independent control of gene expression levels
- Ability to build and test many variant designs

Refactored gene cluster

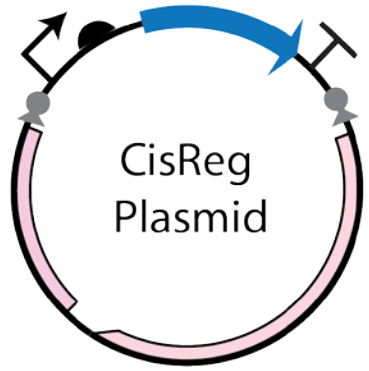


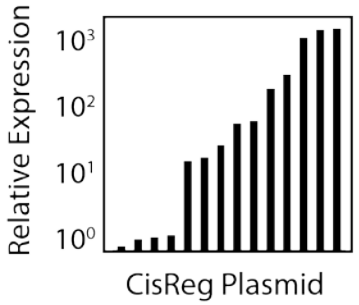
Algorithmic DNA Assembly Pipeline: Key Features

1. Standardized CDS Domestication

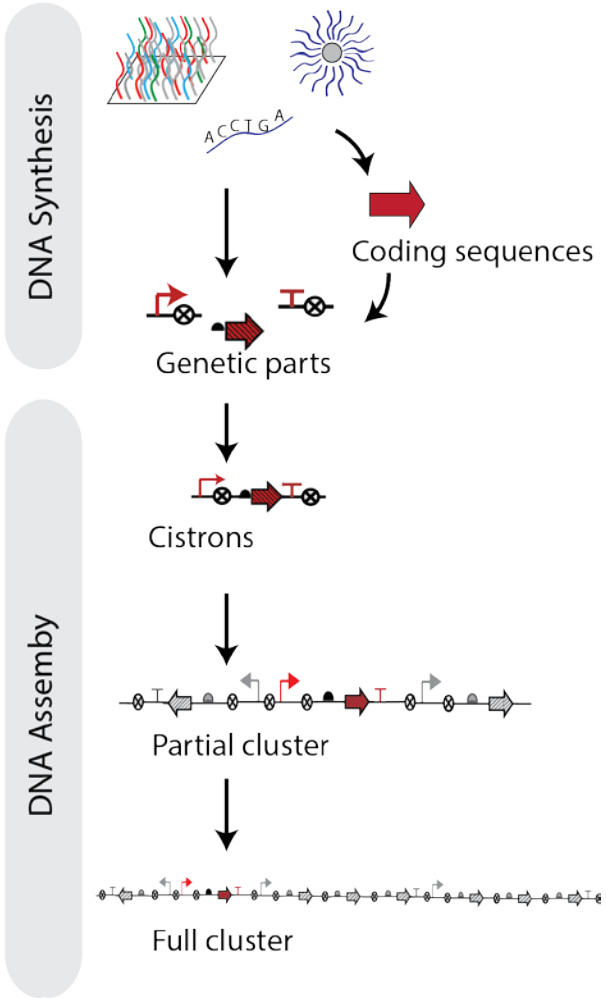


2. Combinatorial Quantification of Cis Regulatory Elements





3. Hierarchical Assembly



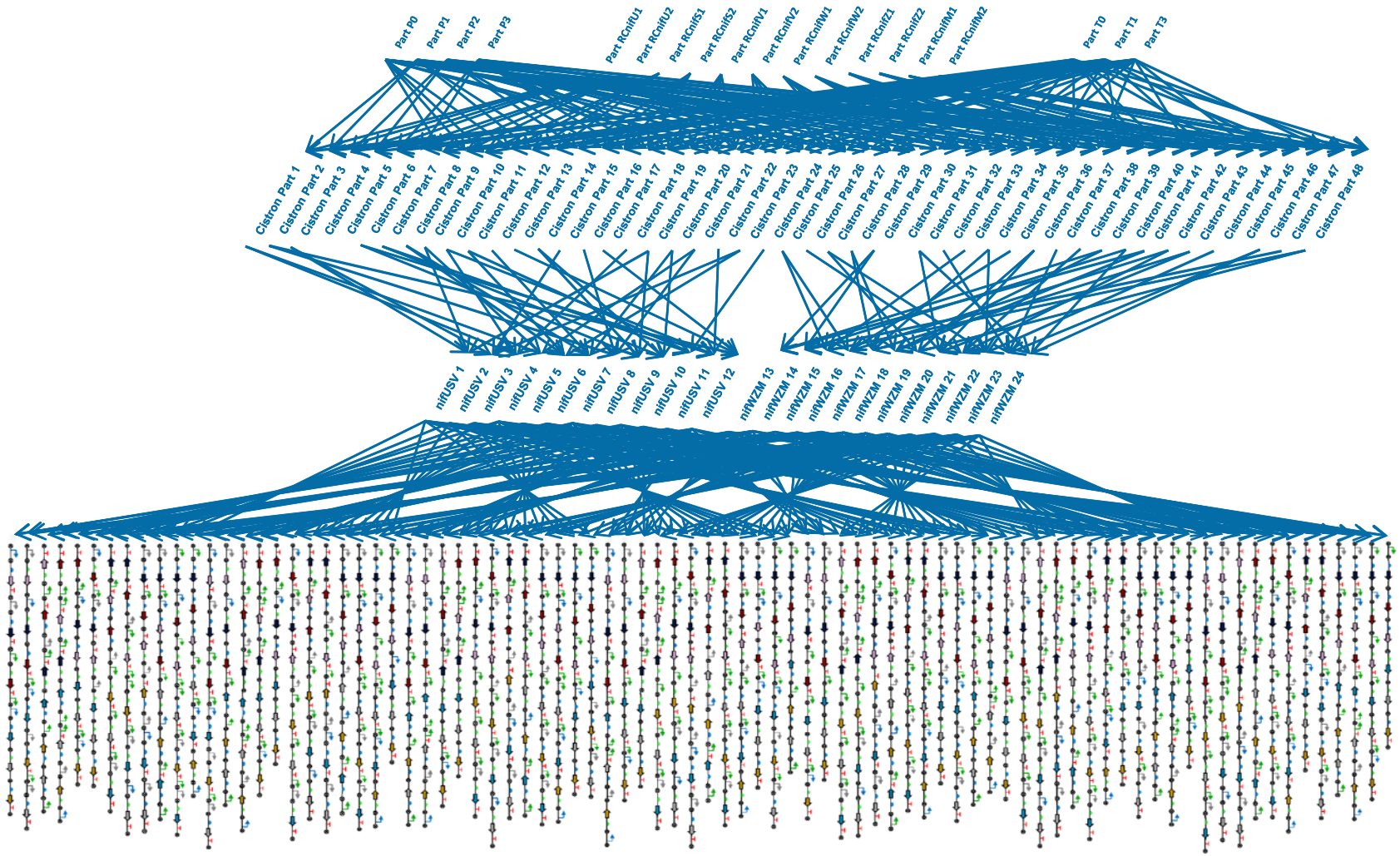
Algorithmic DNA Assembly Pipeline For High-Throughput Plasmid Construction

Genetic Parts:

Cistron-level Constructs:

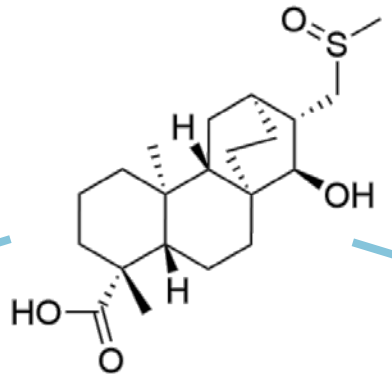
Partial Gene Clusters:

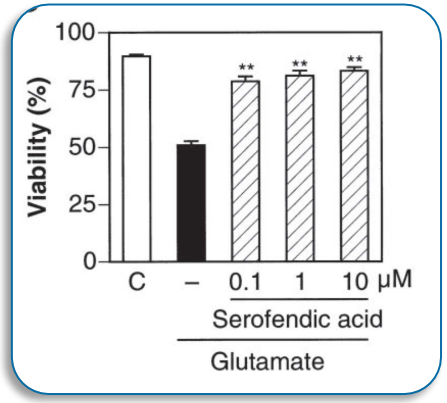
Full Gene Clusters:



Proof-of-concept: Biosynthesis of a Natural Product of Unknown Origin

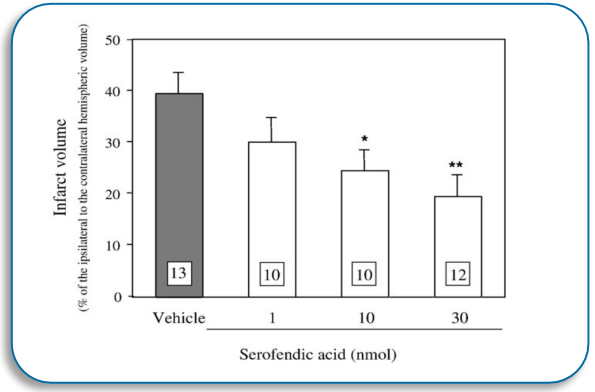
Serofendic Acid has Diverse Therapeutic Potential





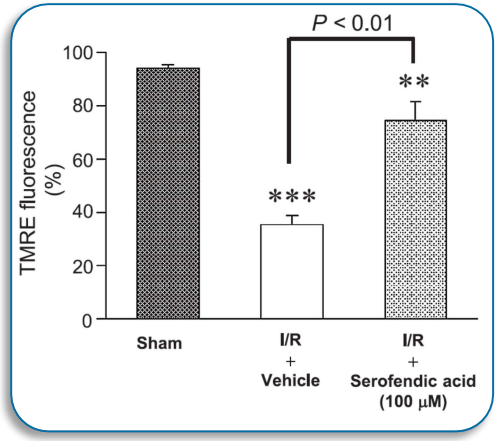
Prevents glutamate neurotoxicity

Kume, T et al. (2002) *PNAS* **99**:3288-3293.



Decreases neurological damage from stroke

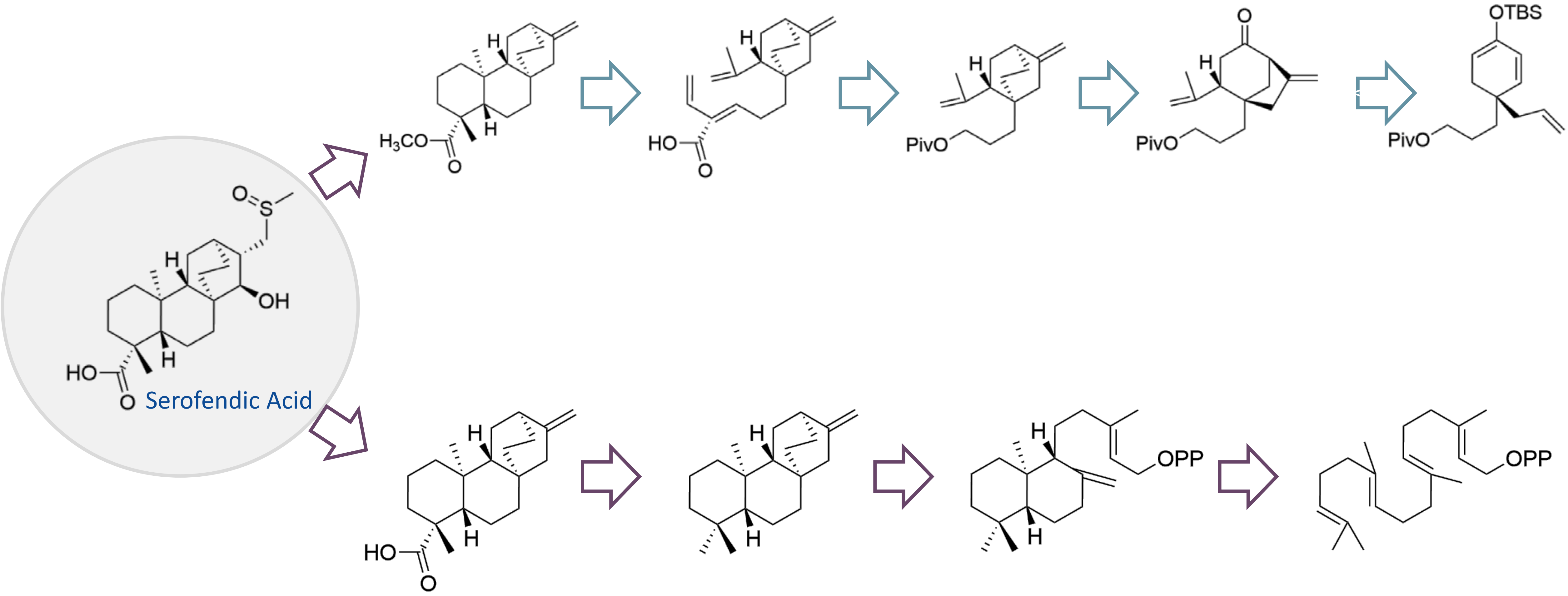
Nakamura, T et al. (2008) *Eur J Pharmacol* **586**:3288-3293.



Decreases damage from myocardial ischemia

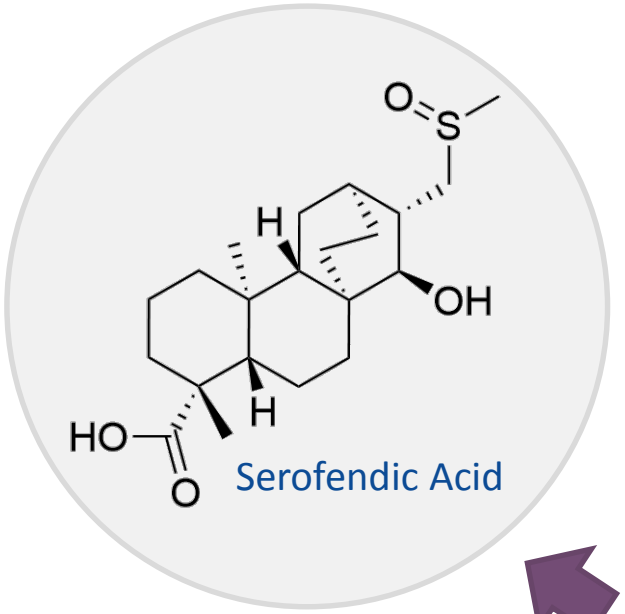
Ioroi, T et al. (2014) *J Pharmacol Sci* **126**:274-280.

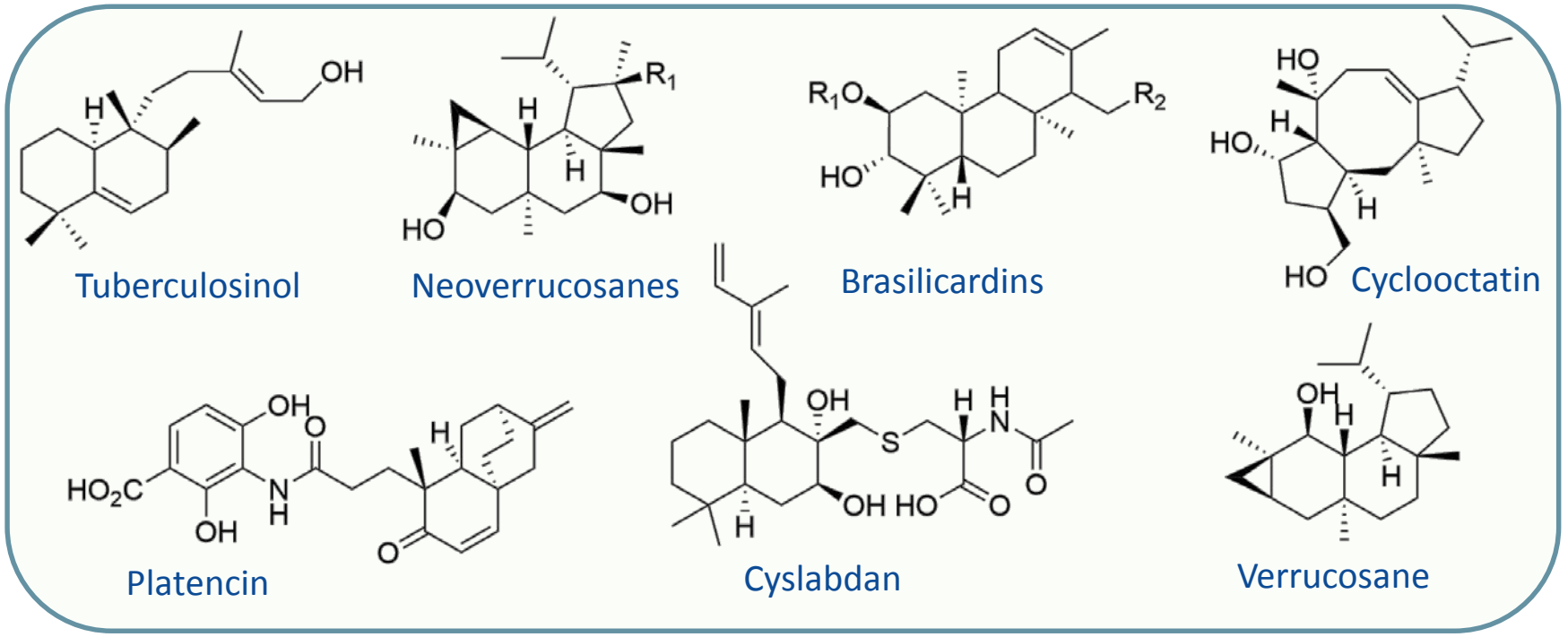
Retro- Biosynthetic Approach to Design a Pathway Towards Serofendic Acid

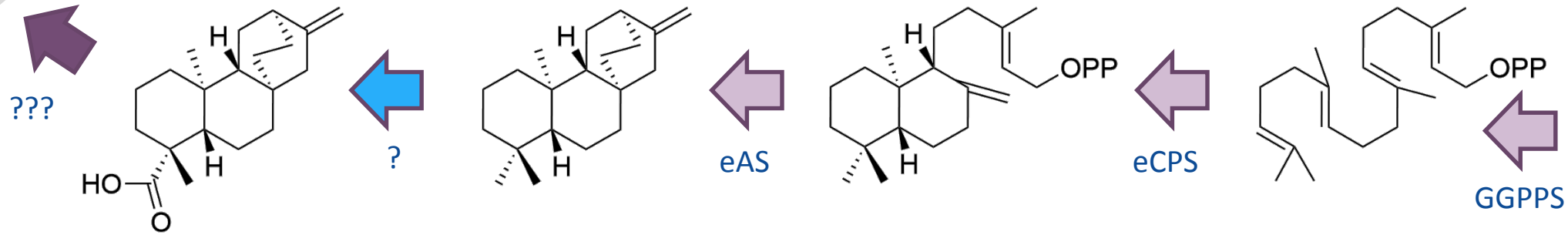


Toyota, M et al. (2005) *Org Lett* **7**:3929-3932.

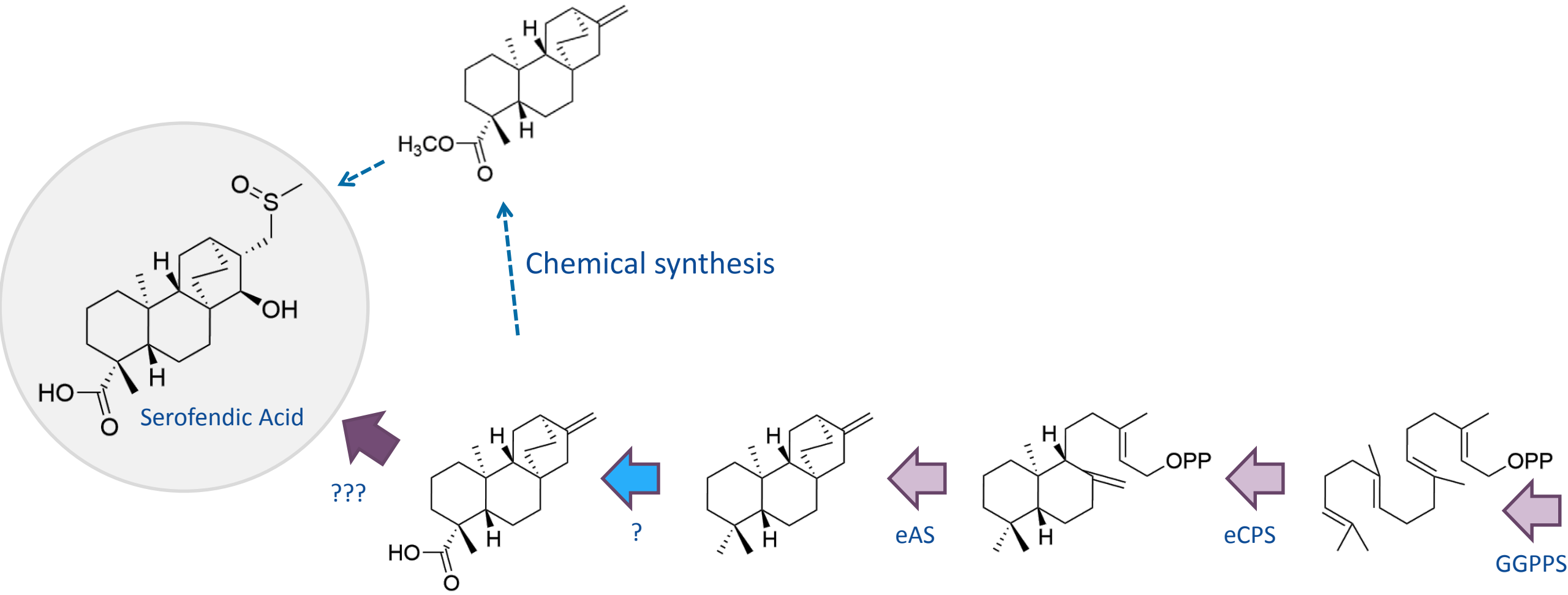
Retro- Biosynthetic Approach to Design a Pathway Towards Serofendic Acid



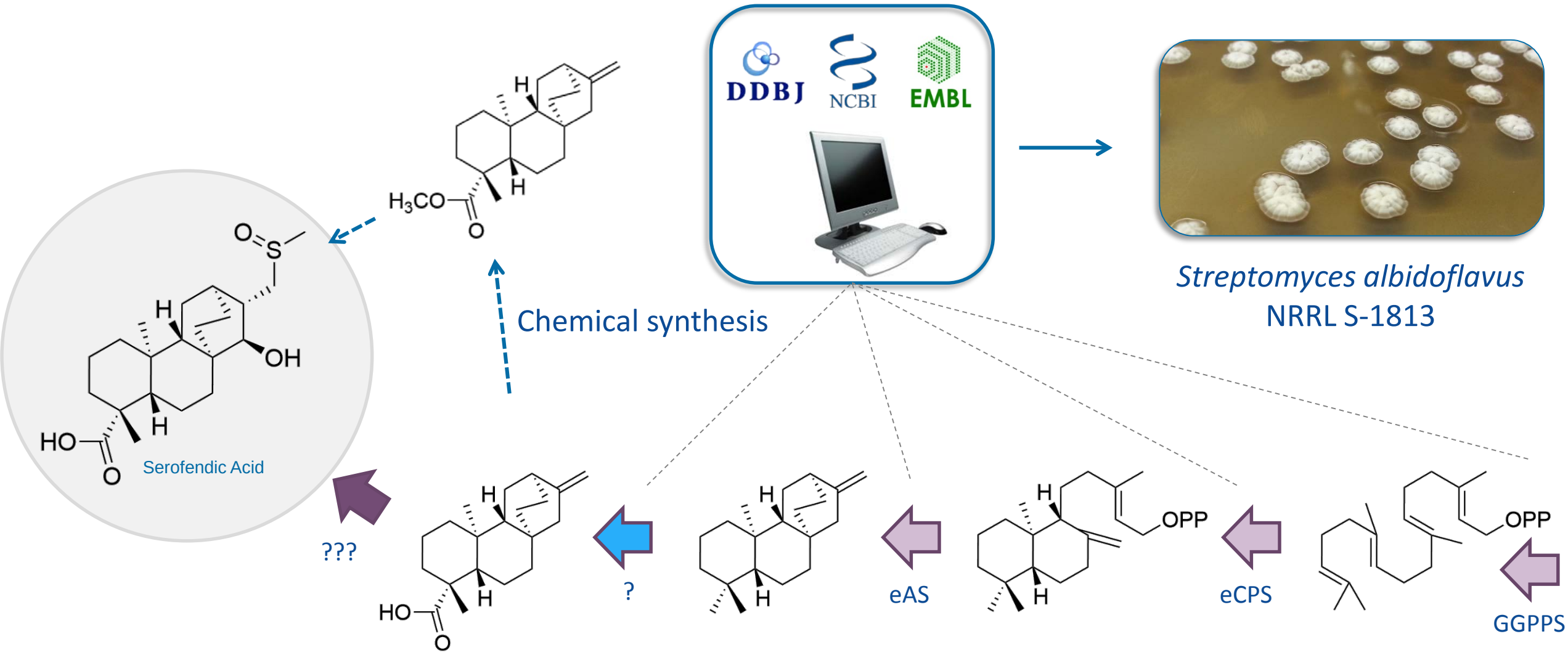




Retro- Biosynthetic Approach to Design a Pathway Towards Serofendic Acid



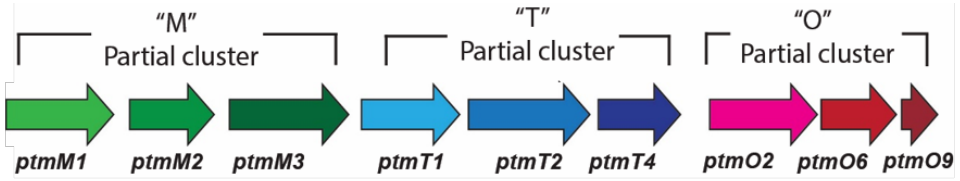
Retro- Biosynthetic Approach to Design a Pathway Towards Serofendic Acid

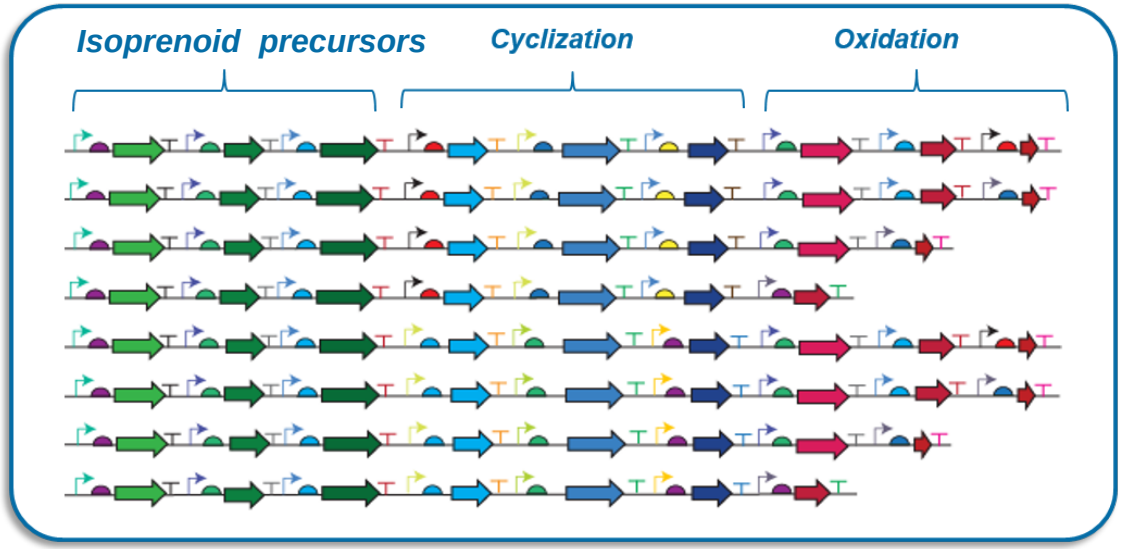


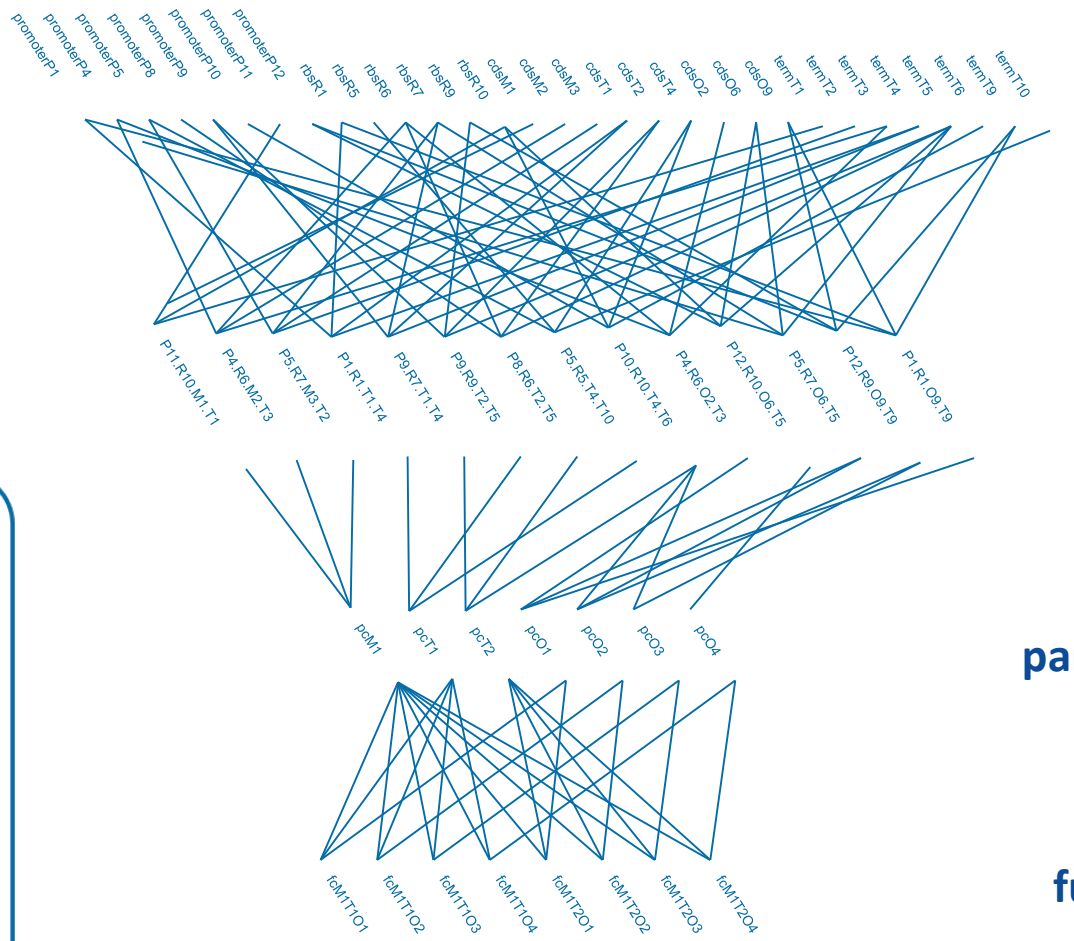
Mini-Library Designed and Built to Screen for *Ent*-atiserenoic Acid Production

8 designs vary according to:

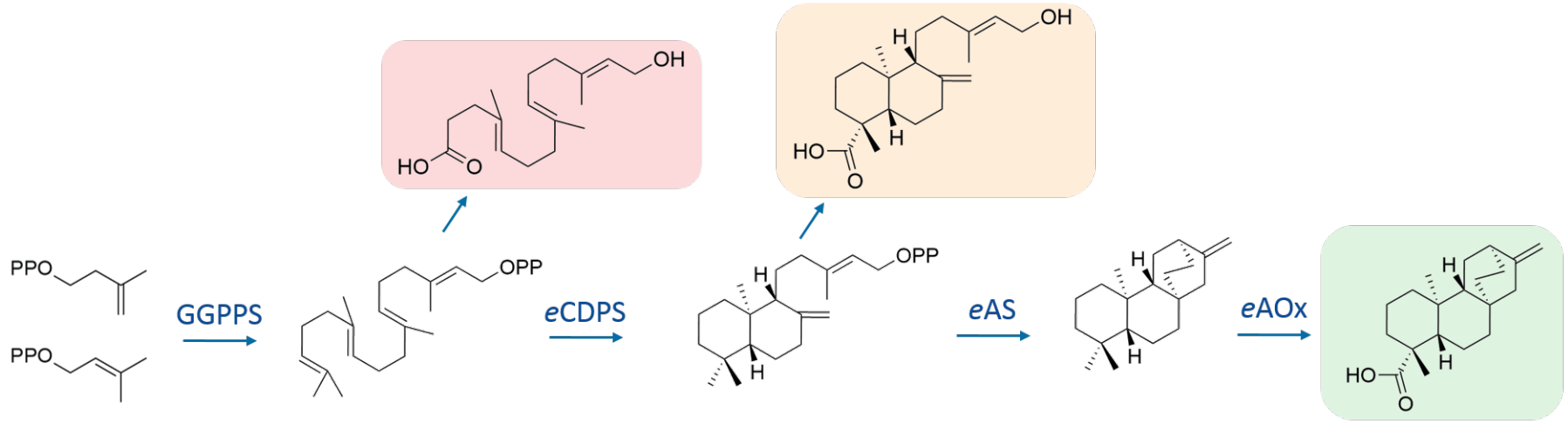
- Promoter strength
- Ribosome binding site strength
- Gene content



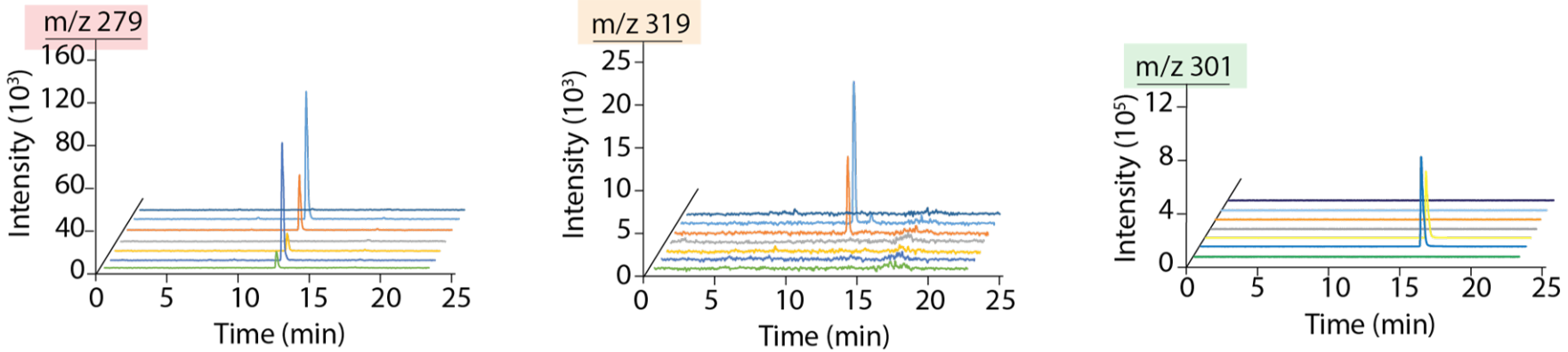




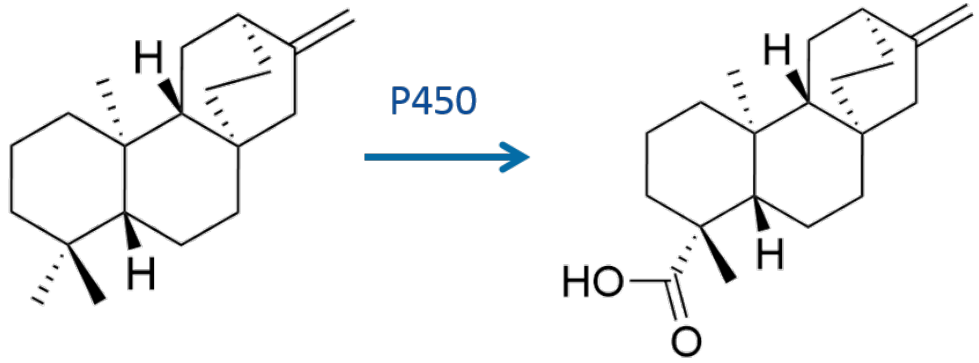
Initial Library of Synthetic Gene Clusters Produces *Ent*-atiserenoic Acid & Congeners



>40 mg/L isolated yield

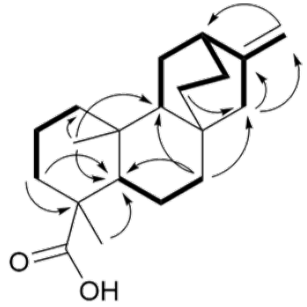


(A) 6-Electron Oxidation of Methyl Group by P450 Monooxygenase PtnO2

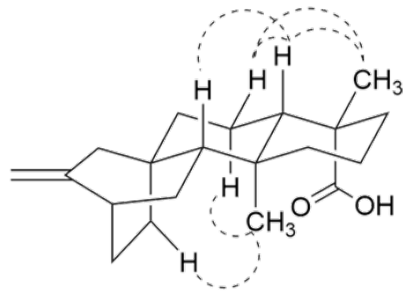


ent-Atiserene
[M-H] = 271

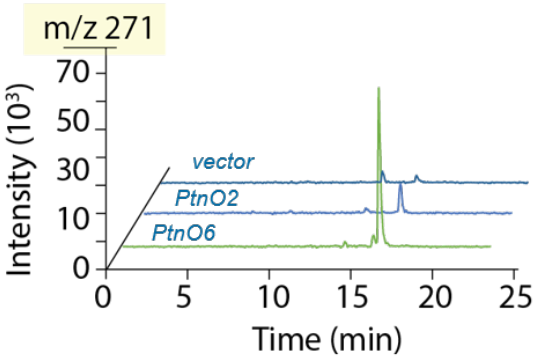
ent-Atiserenoic Acid
[M-H] = 301

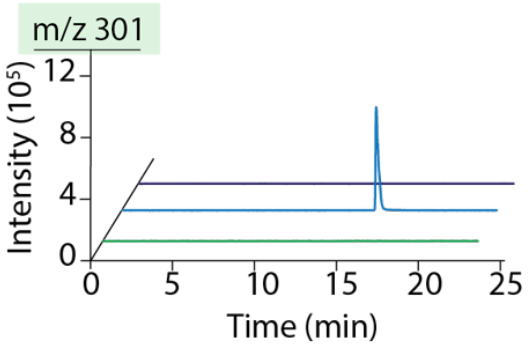


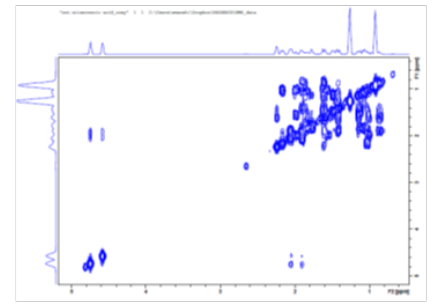
[COSY, HMBC]

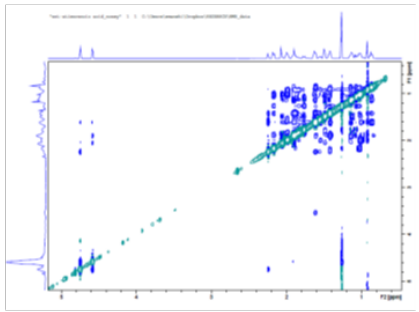


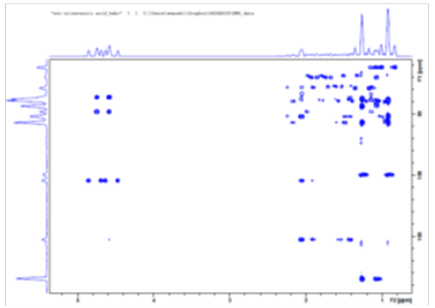
[NOESY]







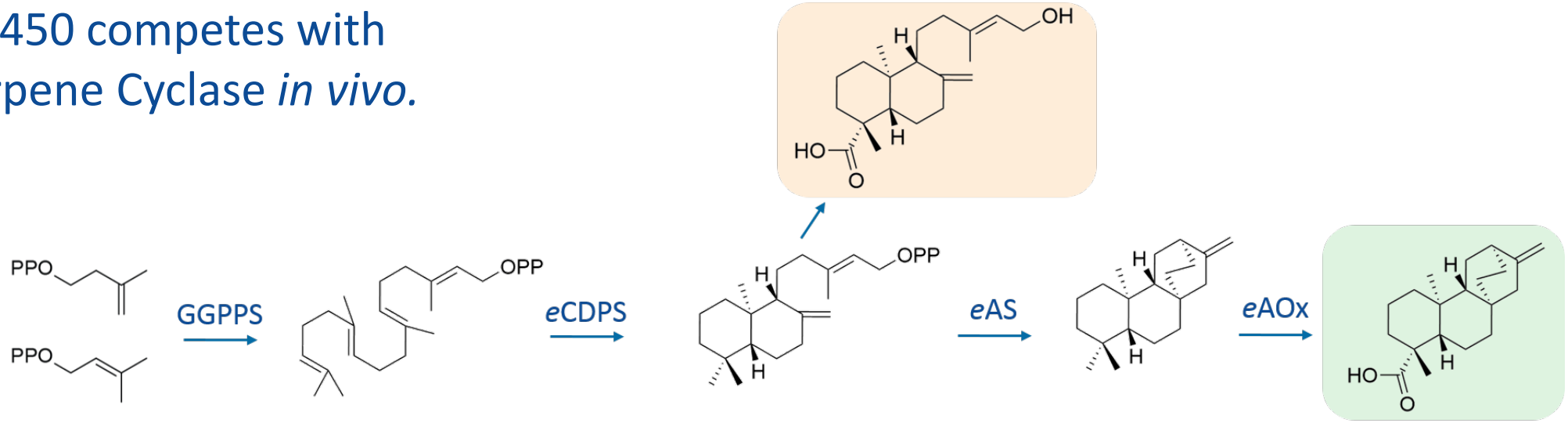


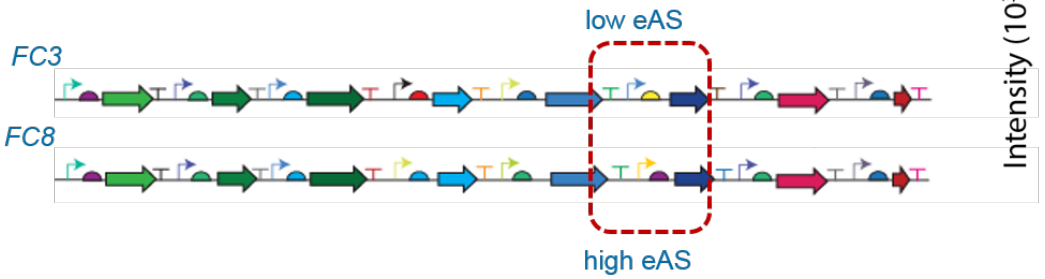


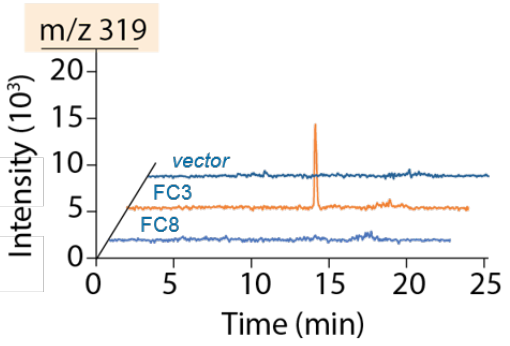
Oxidation by PtnO2, a new cytochrome P450, shows high regioselectivity.

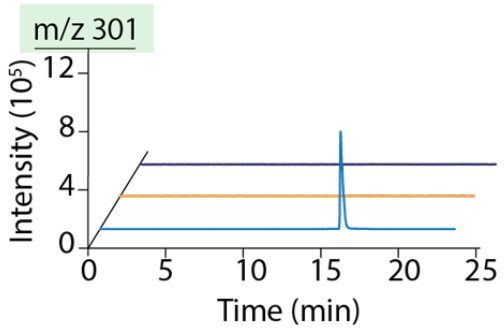
(B) Poorly-Tuned Gene Expression Leads to Shunt Metabolite Production

P450 competes with
Terpene Cyclase *in vivo.*



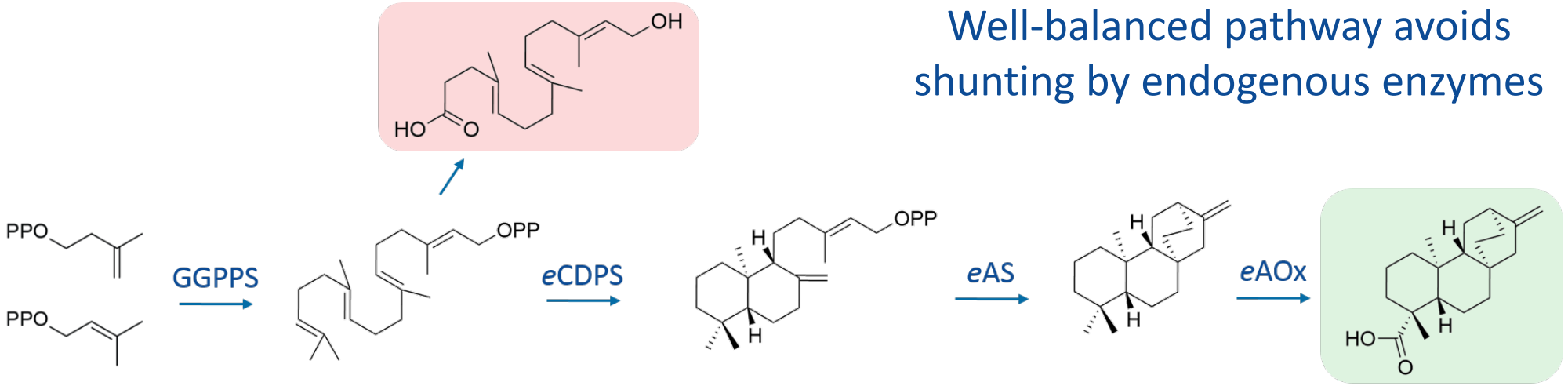


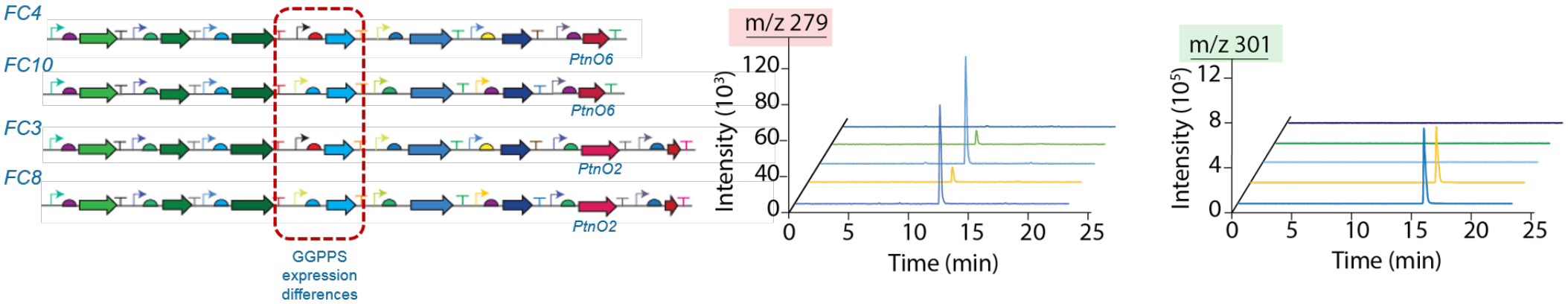




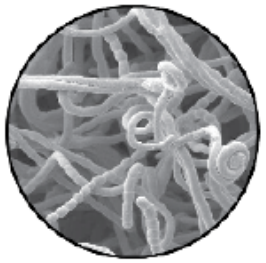
(C) Poorly-Tuned Gene Expression Allows Interference by Non-Pathway Enzymes

Well-balanced pathway avoids shunting by endogenous enzymes



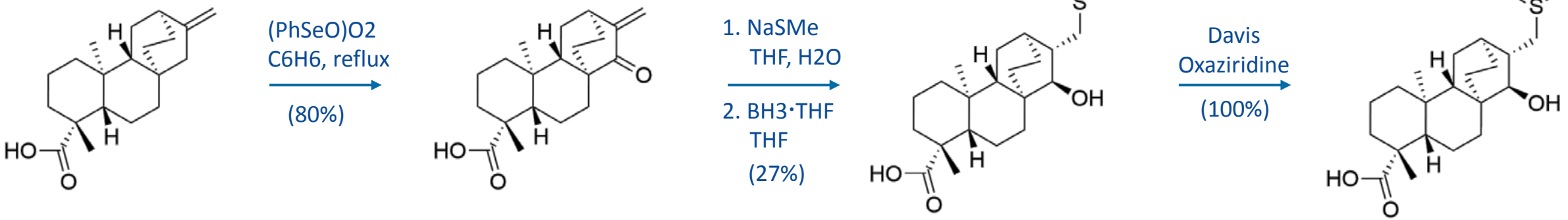


Semi-Synthesis of Serofendic Acid and Derivatives



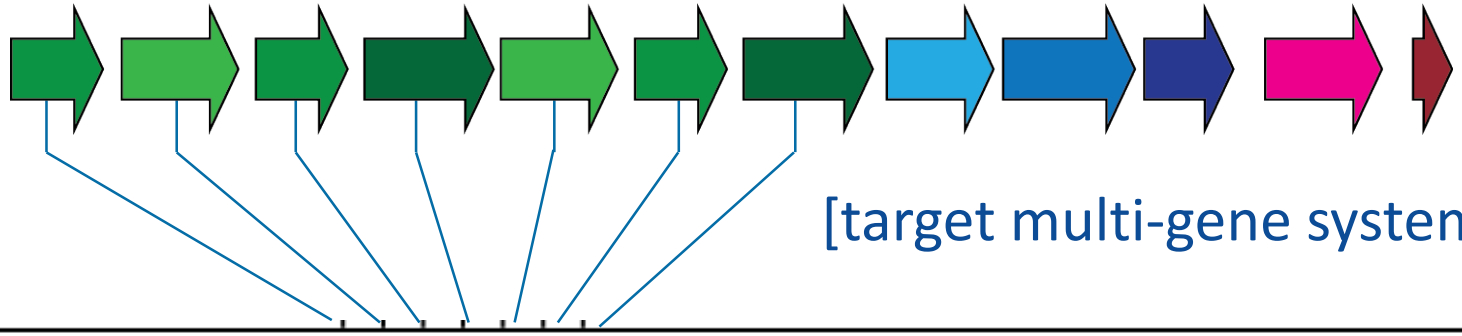
recombinant *Streptomyces*

- Initial extraction yielded 40 mg / L *ent*-Atiserenoic acid.
- Formal synthesis completed without methylester protecting group
- Semi-synthesis reduced complexity from 17 to 4 steps and increased yield ~10-fold
- Facile derivatization of *ent*-Atiserenoic acid demonstrated

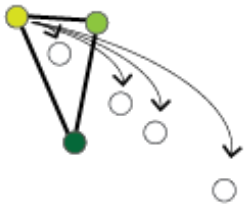


Toy, A. M. et al., *Org. Letters*(2005) 7:3929

Current Efforts are Focused on Improving Titer Through Multivariate Optimization

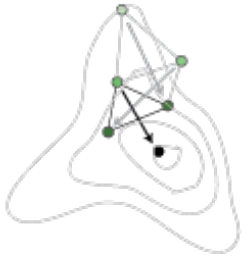


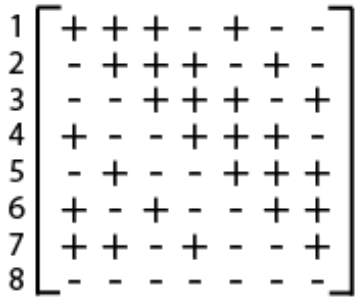
[target multi-gene system]



Nelder-Mead simplex algorithm

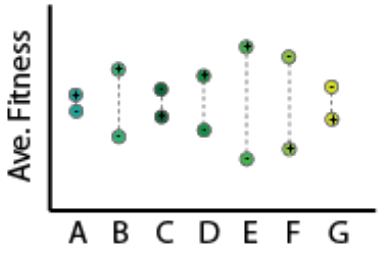
[hill climbing]

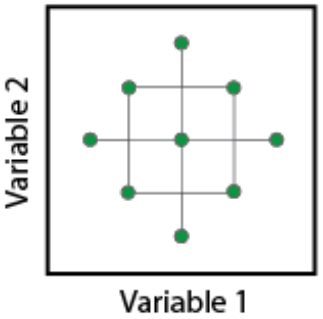




Plackett-Burman model

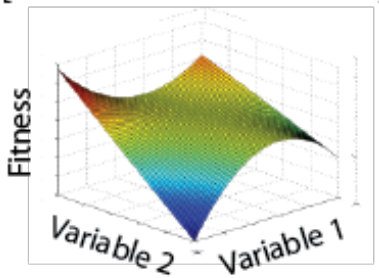
[variable prioritization]

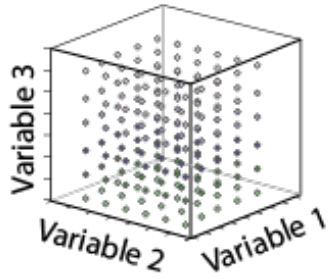




Central Composite design

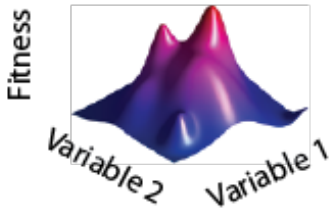
[interaction estimation]





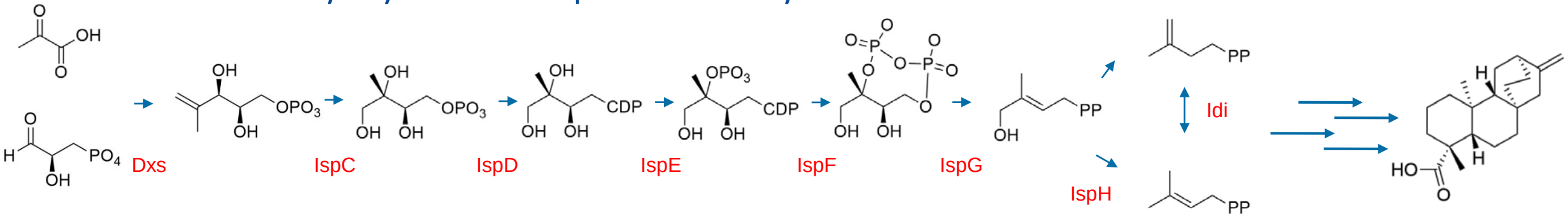
Full Factorial design

[full landscape visualization]

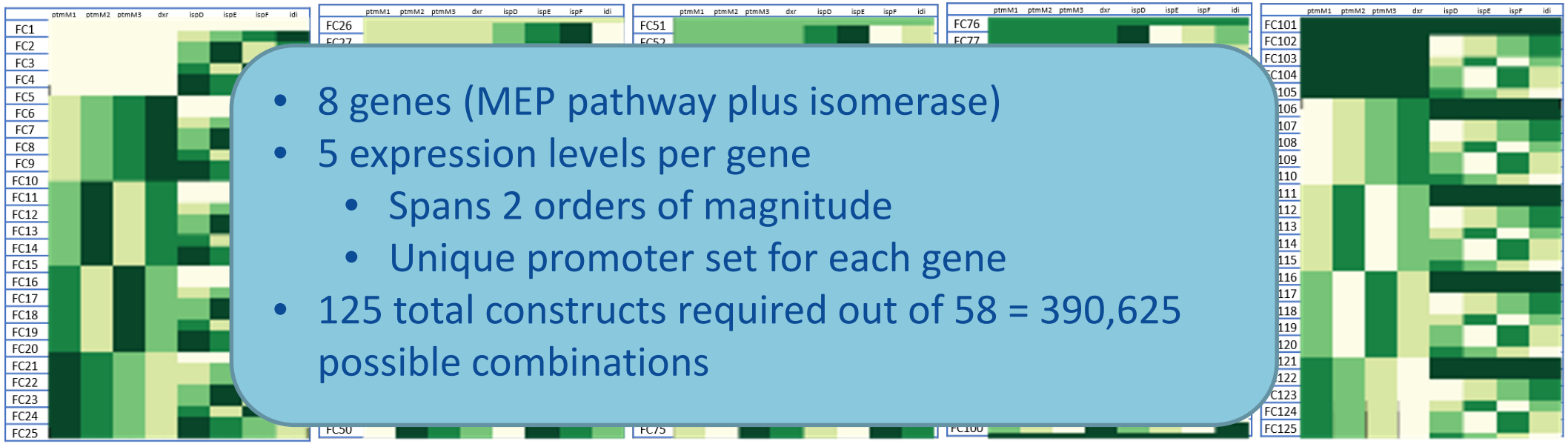


Current efforts are focused on improving titer through multivariate optimization

Methylerythritol Phosphate Pathway

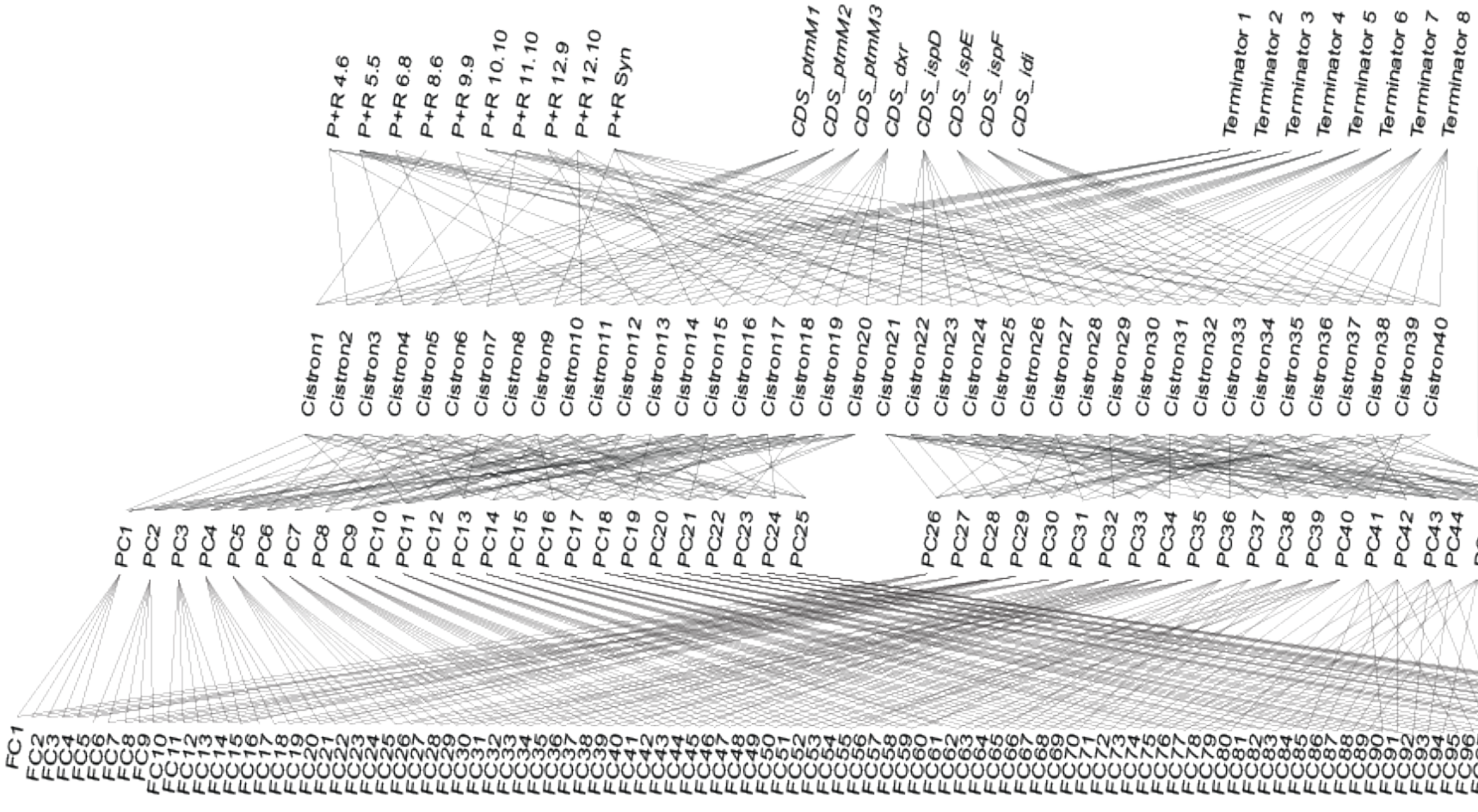


5-level fractional factorial design:



- 8 genes (MEP pathway plus isomerase)
- 5 expression levels per gene
  - Spans 2 orders of magnitude
  - Unique promoter set for each gene
- 125 total constructs required out of 58 = 390,625 possible combinations

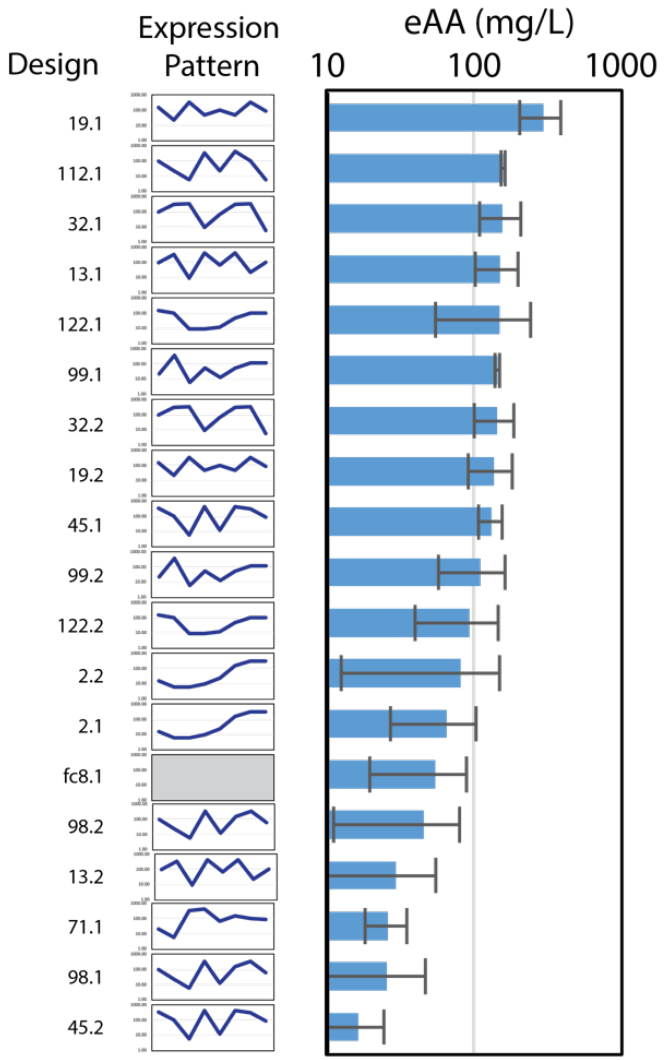
Current Efforts are Focused on Improving Titer Through Multivariate Optimization

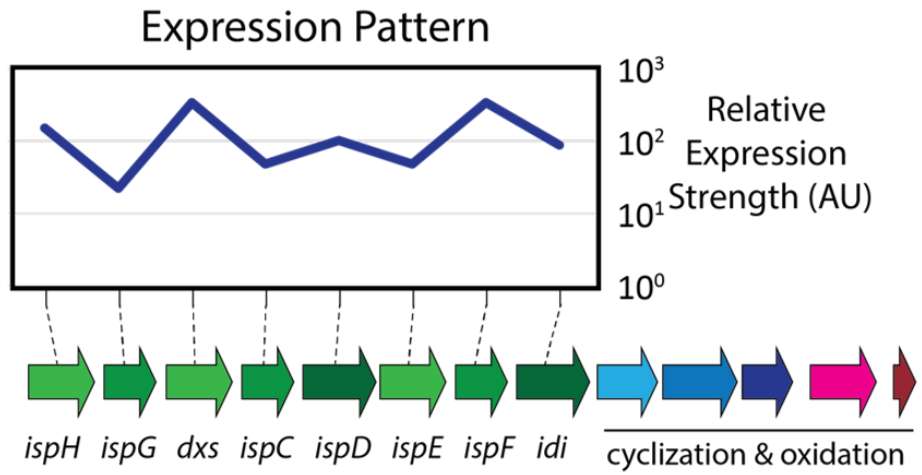


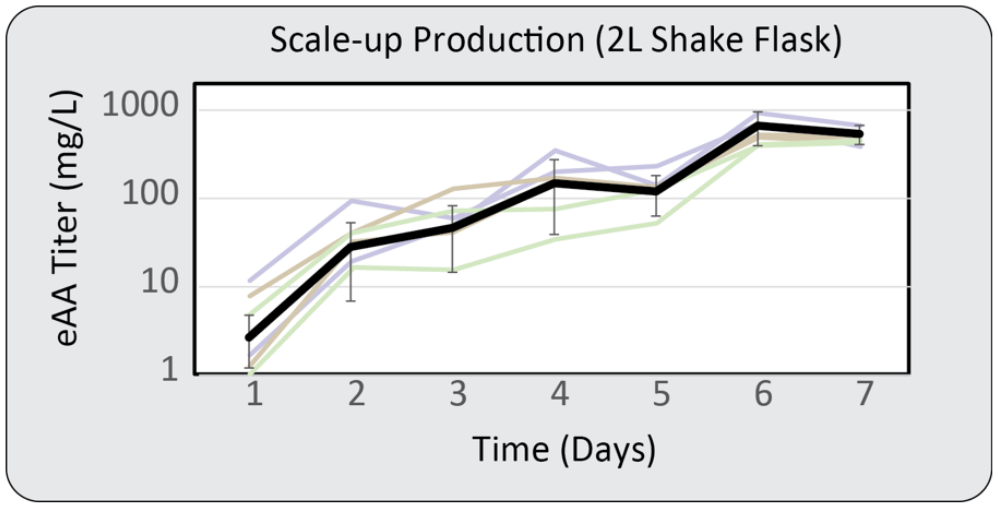
Design Rule

- 8 independent variables (gene expression)
- 5 expression levels
- unique terminator paired with each rCDS
- 4 cistrons per partial cluster (PC)
- 2 PCs per full cluster (FC)

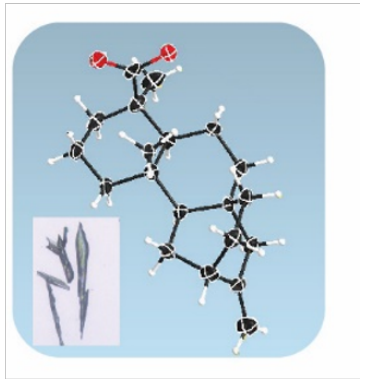
Preliminary Results Show Several Improved Strains



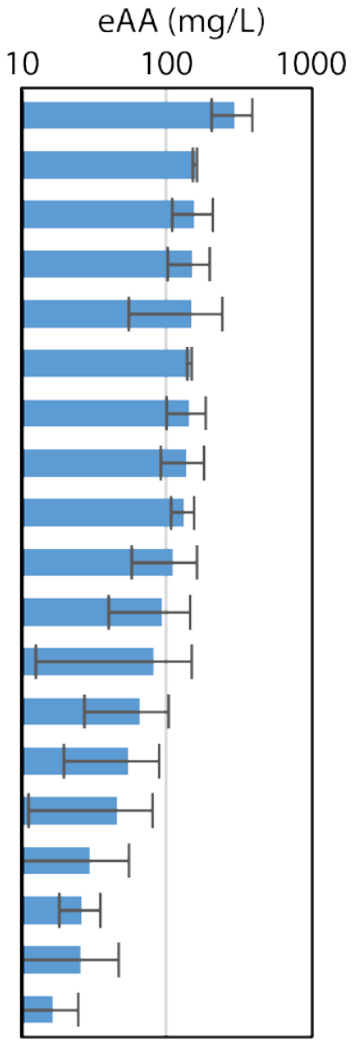


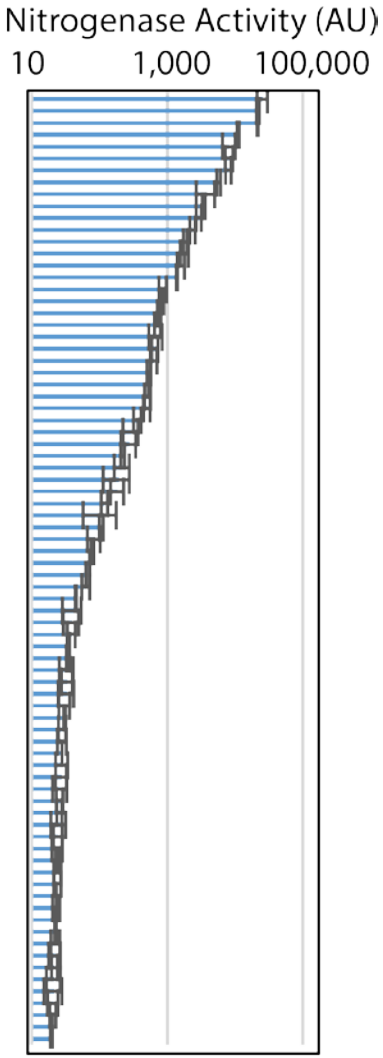


Recent titers
>500 mg/L

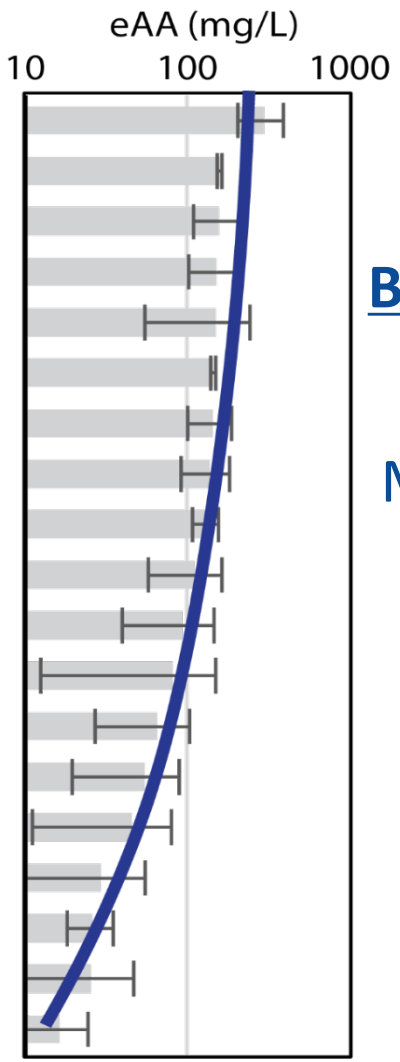


Preliminary Results Show Several Improved Strains





Robustness to Varying Expression Levels Will Not Be the Same for Every System



Diterpene Biosynthesis

Secondary Metabolism

vs.

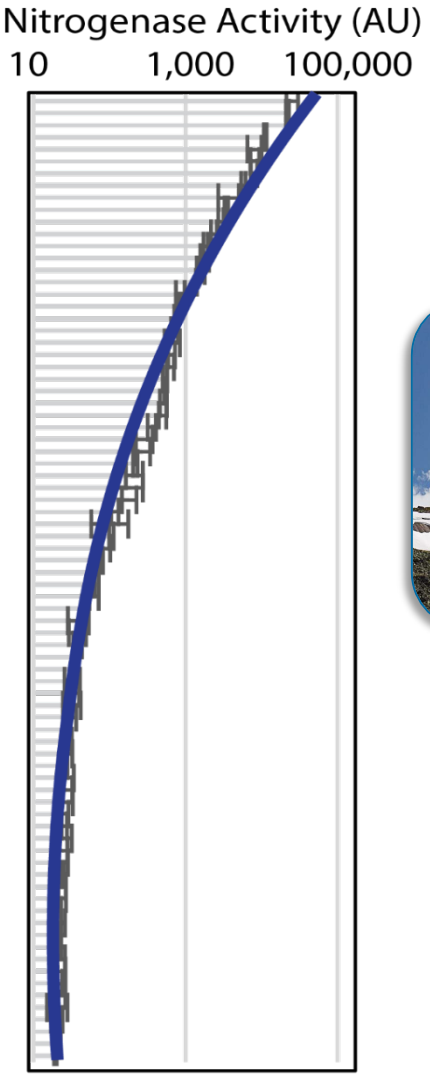
Discrete Enzymes

Nitrogen Fixation

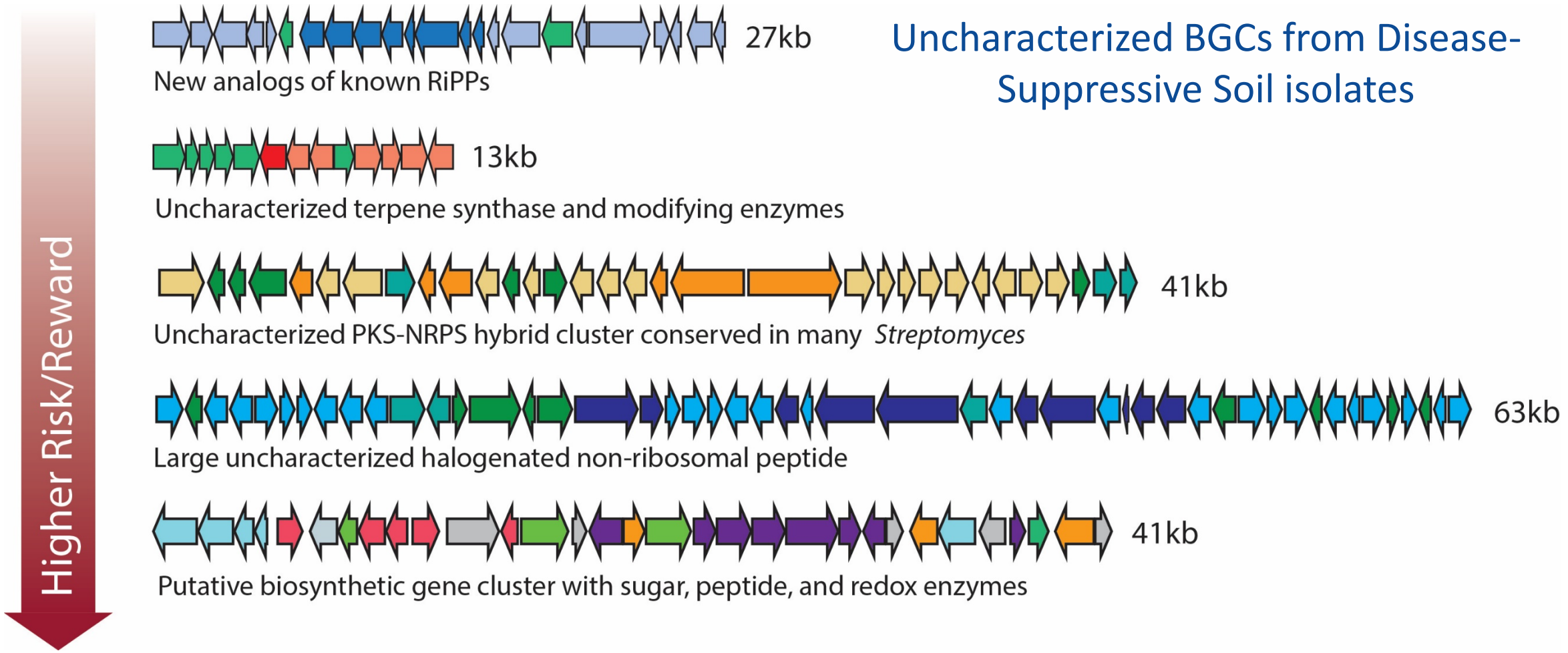
Primary Metabolism

vs.

Multi-protein Complexes

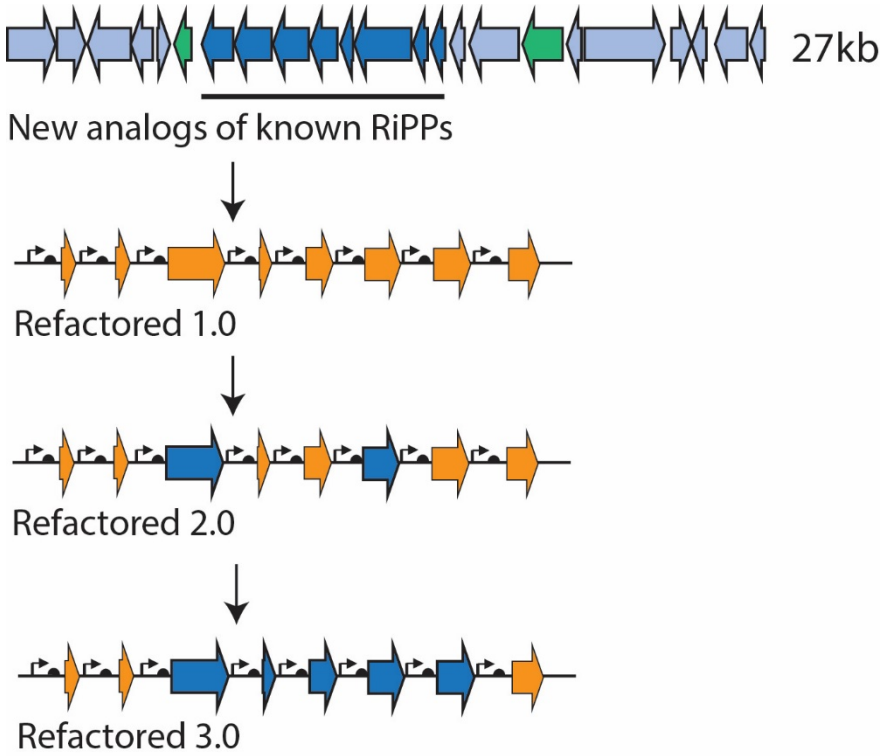


Extending the DNA Assembly Pipeline for New Compound Discovery



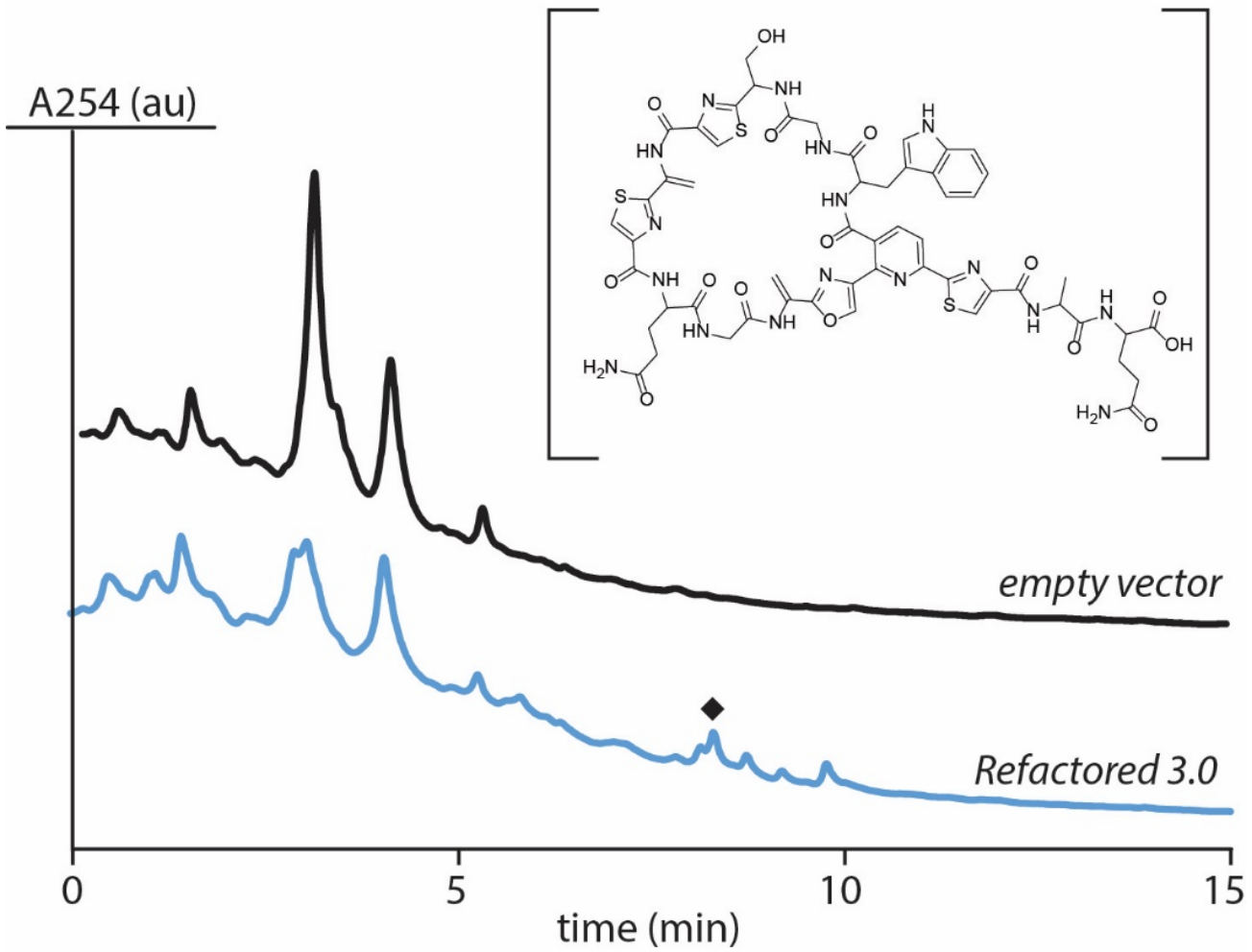
Uncharacterized BGCs from Disease-Suppressive Soil isolates

Family of Putative Thiazole-Containing Molecules



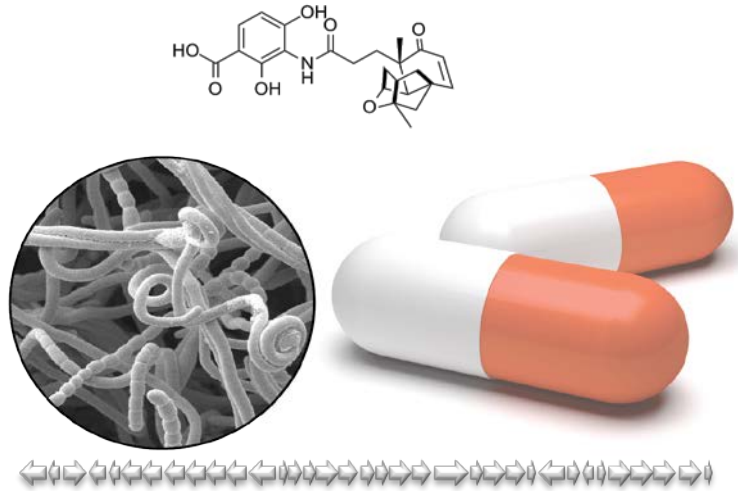
Wildtype CDS
Codon altered CDS

Thiazole BGC with proposed role in interspecies signaling.

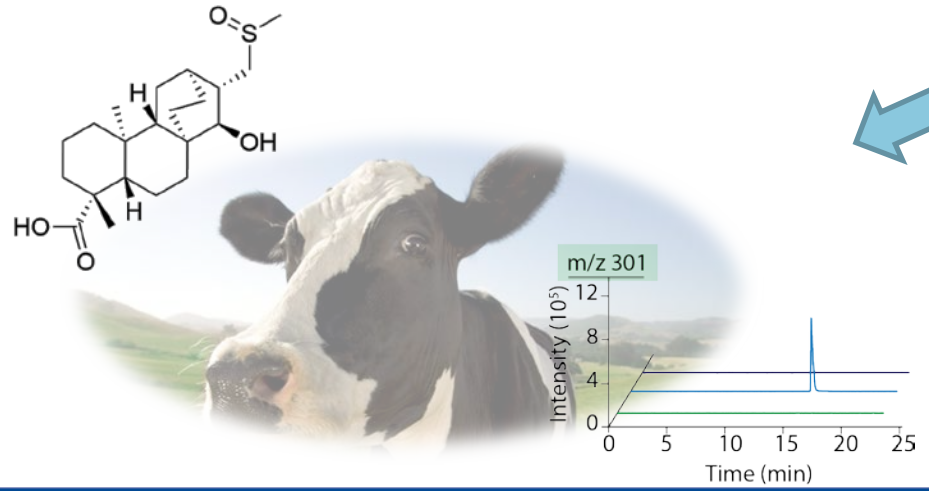


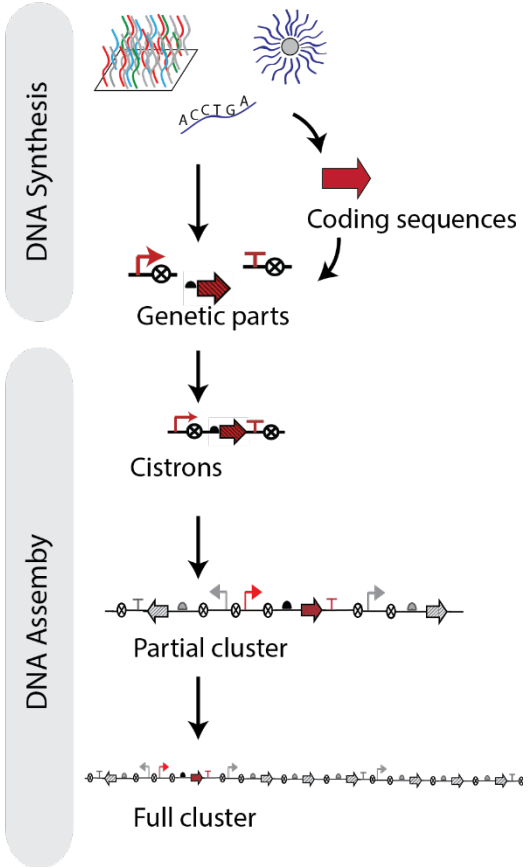
Summary: DNA Assembly for Engineering Natural Product Biosynthesis

- Independent control of gene expression is important for high-titer heterologous production
- Developed an algorithmic DNA assembly pipeline compatible with *Streptomyces*
- Demonstrated a new sustainable route to Serofendic acid, a diterpenoid natural product of unknown origin



Natural Product Biosynthesis





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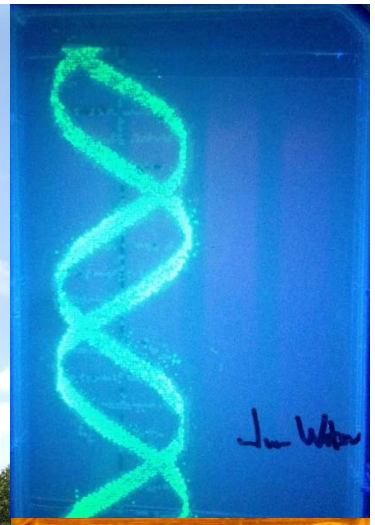
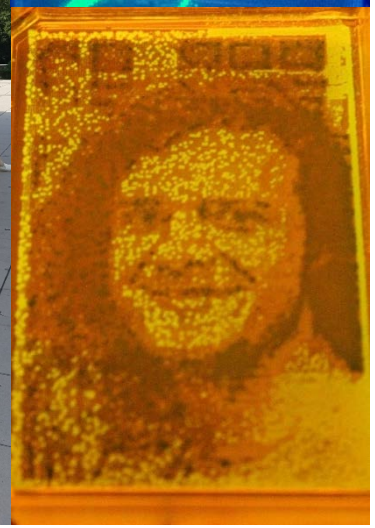
Dr. Lindsey Hansen
Dr. Zewei Song
Dan Schlatter





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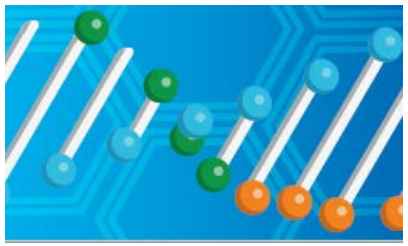
Synthetic Biology Portfolio		
Gene Synthesis	DNA Fragments	Combinatorial DNA Libraries



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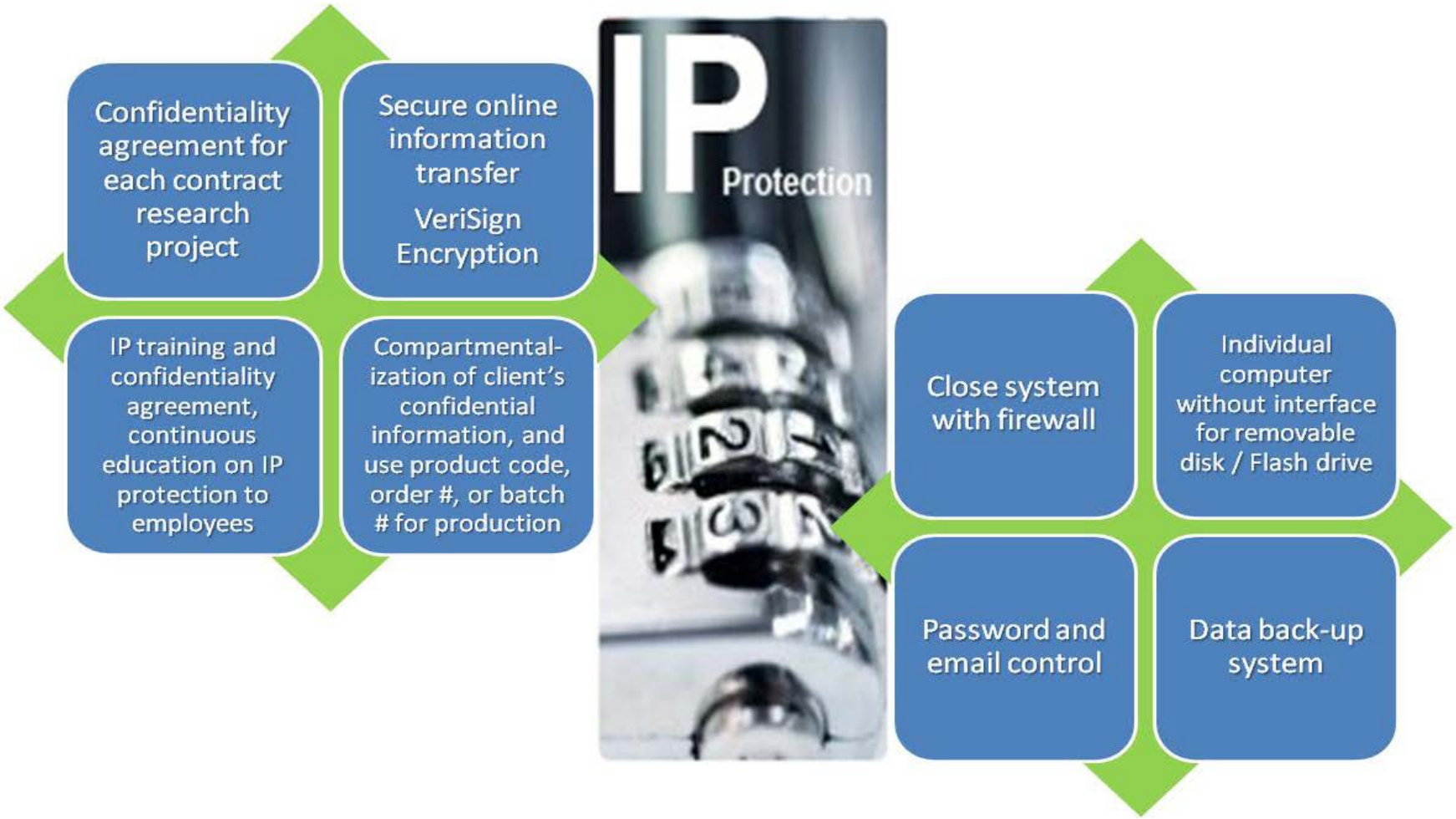

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