Complete biosynthesis of opioids in yeast

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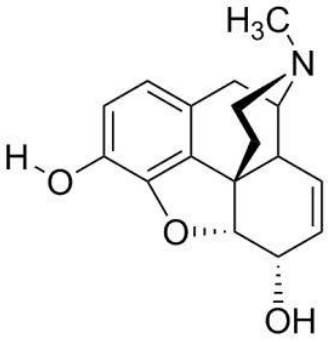
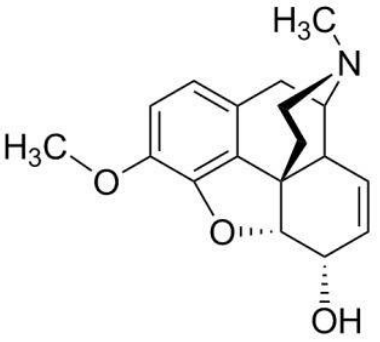
Introduction - Opioids

- WHO classifies opioids as essential medicine for pain management
- The total chemical synthesis of opioides is not commercially competitive
- Currently opioids are derived from opium poppy (*Papaver somniferum*)
- Morphine and thebaine are chemically converted into higher-value compounds, including codeine, oxycodone, and hydrocodone.



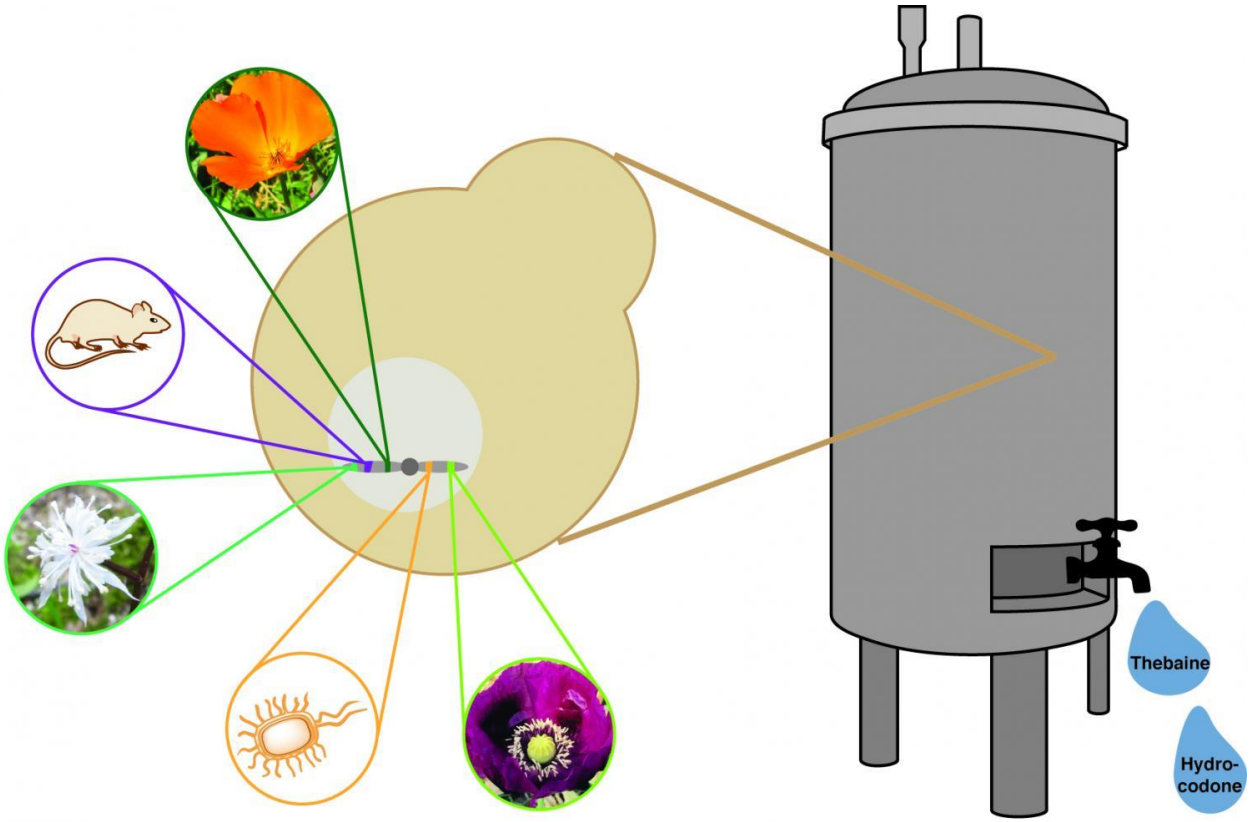
Morphine



Codeine

Introduction – Production in Yeast

- Industrial poppy farming is susceptible to environmental factors → instability and variability
- Microbes are grown in closed fermentation vessels → process is not susceptible to external environmental factors → provide greater consistency in product composition
- Poppies are annuals – idustrial cultivation of microorganisms occurs over days



Introduction - Addiction

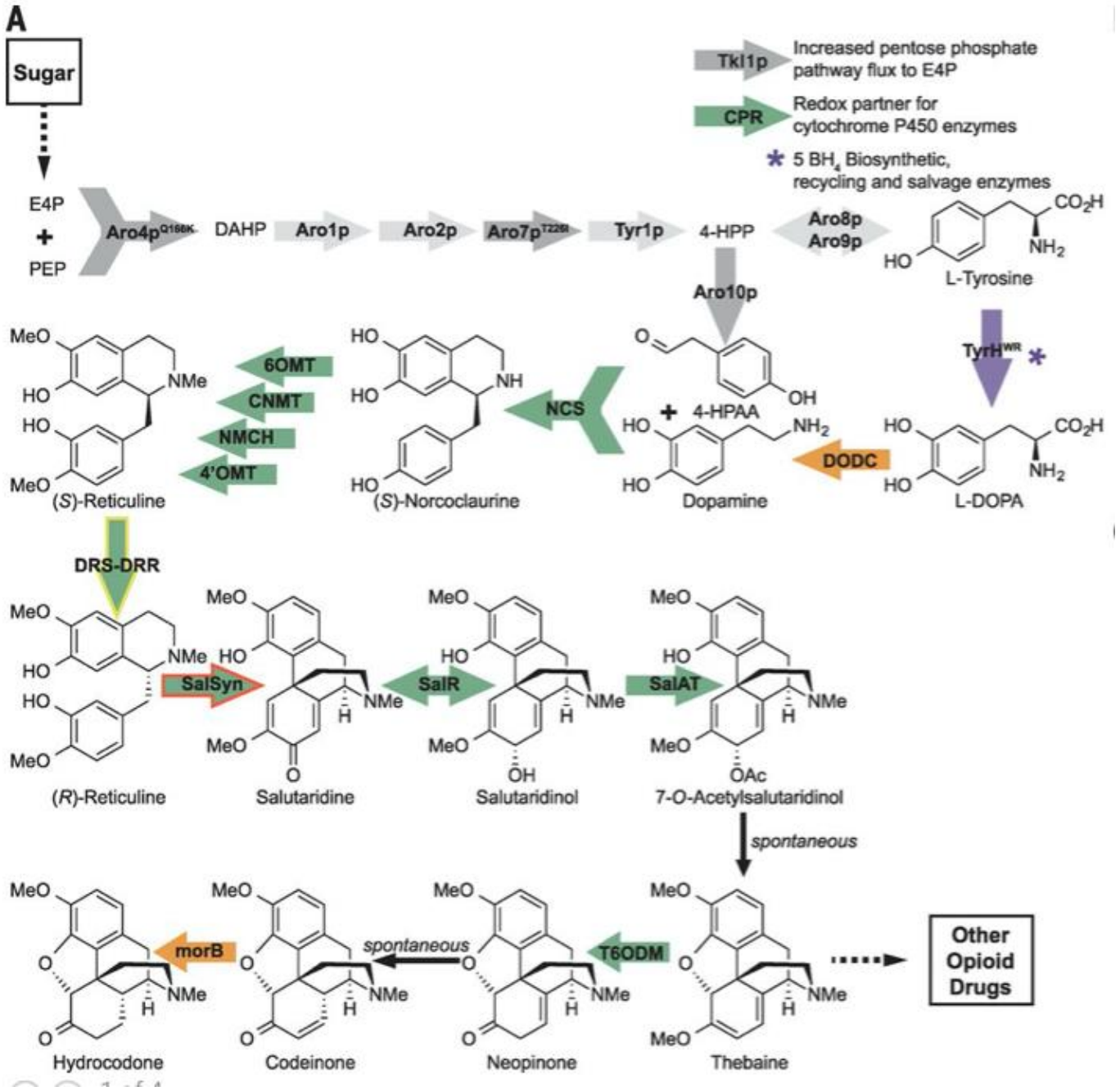
- Opioids are highly addictive.
- Roughly 21-29% of patients prescribed opioids for chronic pain misuse them.[1]
- Researchers were aware of possible negative impacts.
- Gained permission via Stanford University's institutional research registration with the U.S. Drug Enforcement Agency (DEA).

[1] https://www.drugabuse.gov/drug-topics/opioids/opioid-overdose-crisis

Biosynthetic scheme

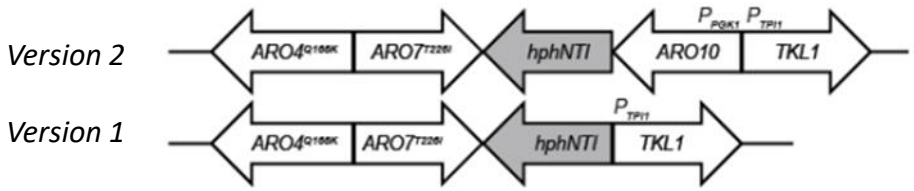
- Main objective:
  - Production of thebaine and hydrocodone from sugar
- Main methods:
  - 7 modules
  - Enzyme discovery
  - Enzyme engineering
  - Pathway and strain optimization

Light gray arrows → unmodified yeast
Dark gray arrows → overexpressed and modified yeast
Purple arrows → mammalian
Orange arrows → bacterial
Green arrows → plant

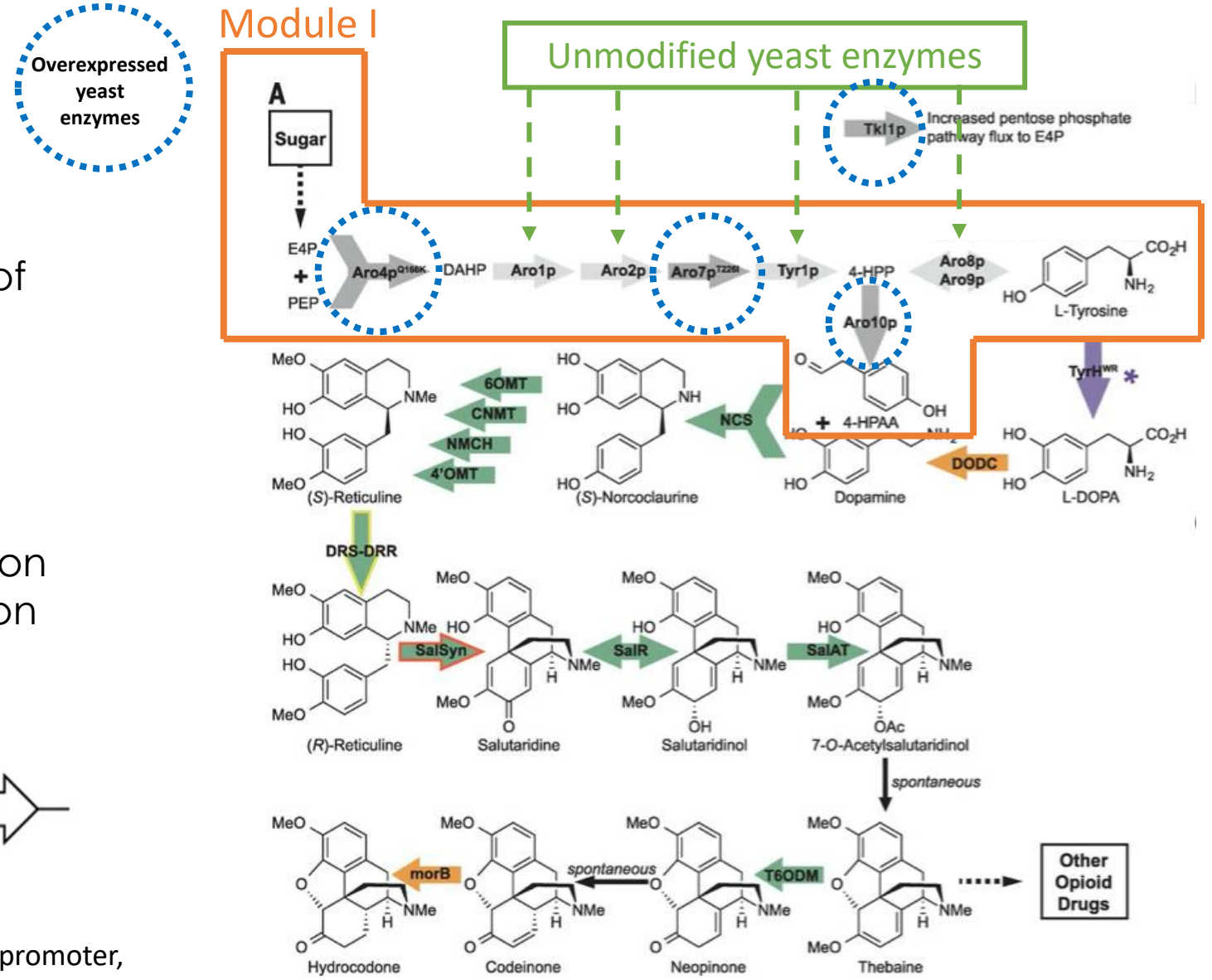


Module I

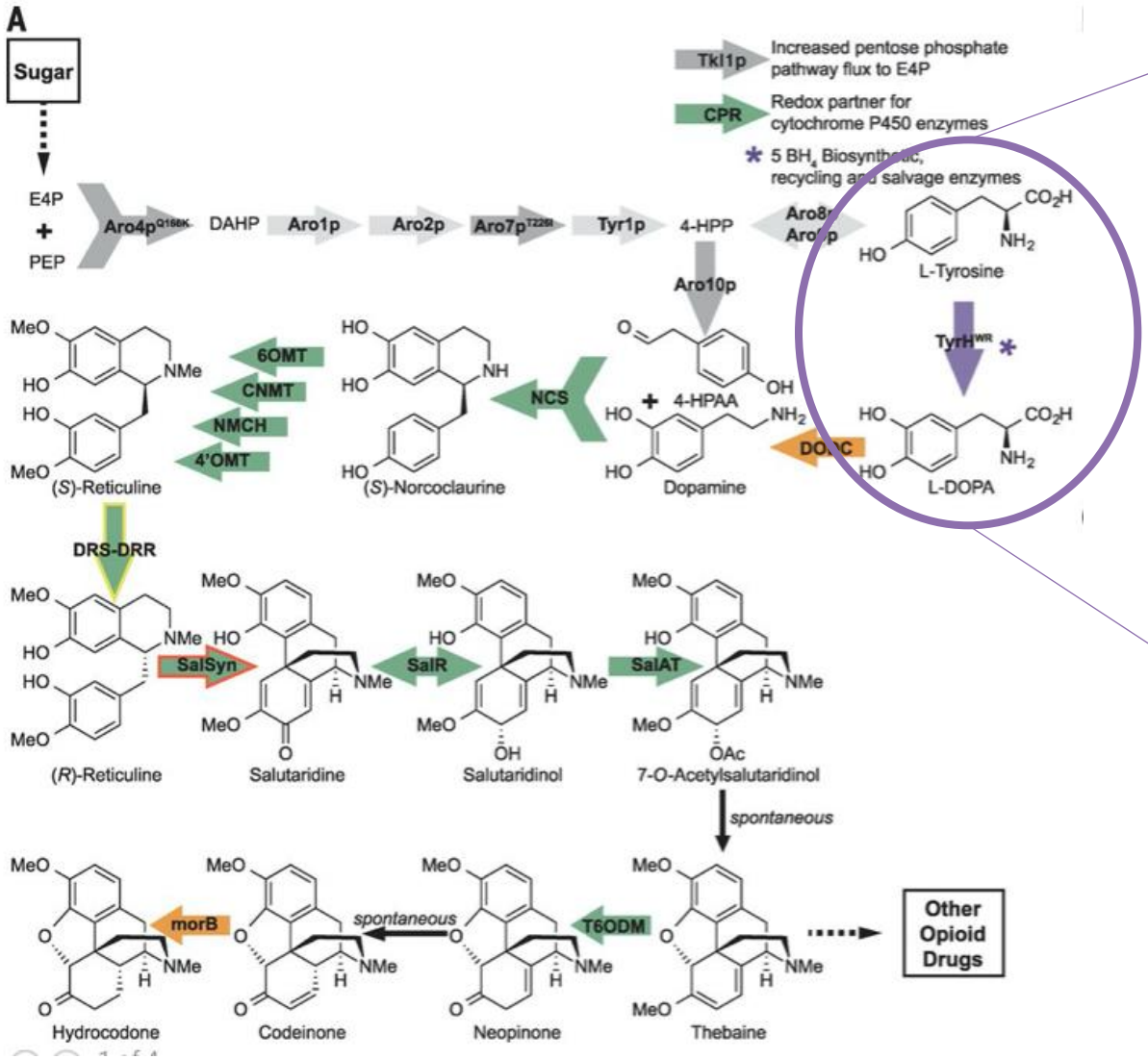
- Objective:
  - increases accumulation of 4- hydroxyphenyl acetaldehyde (4-HPAA) and L-tyrosine
- Method:
  - A precursor overproduction encoded in overexpression of three or four yeast proteins

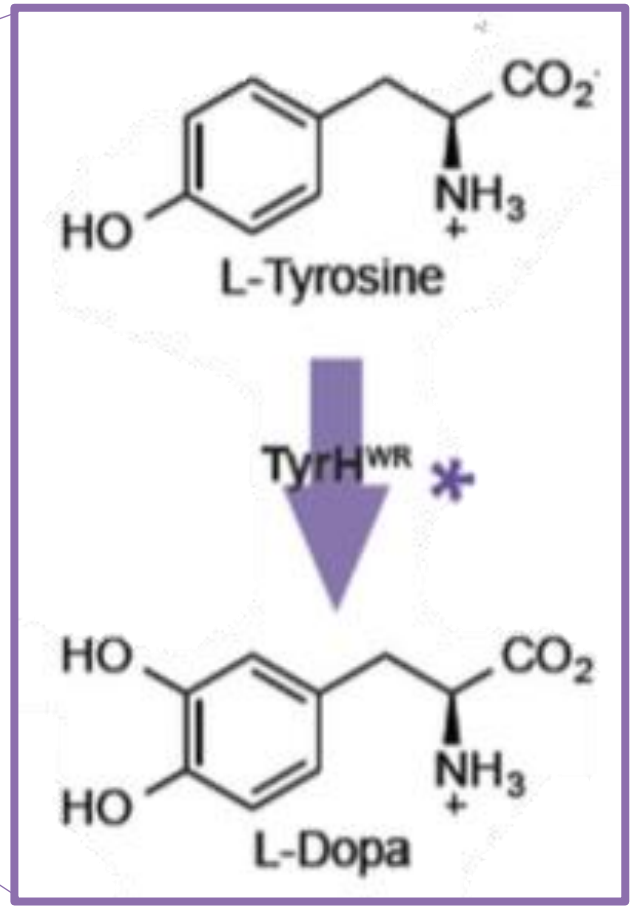


White arrows = gene expression cassette with a promoter, coding sequence and terminator
Grey arrows = loxP-flanked selection markers



Module II





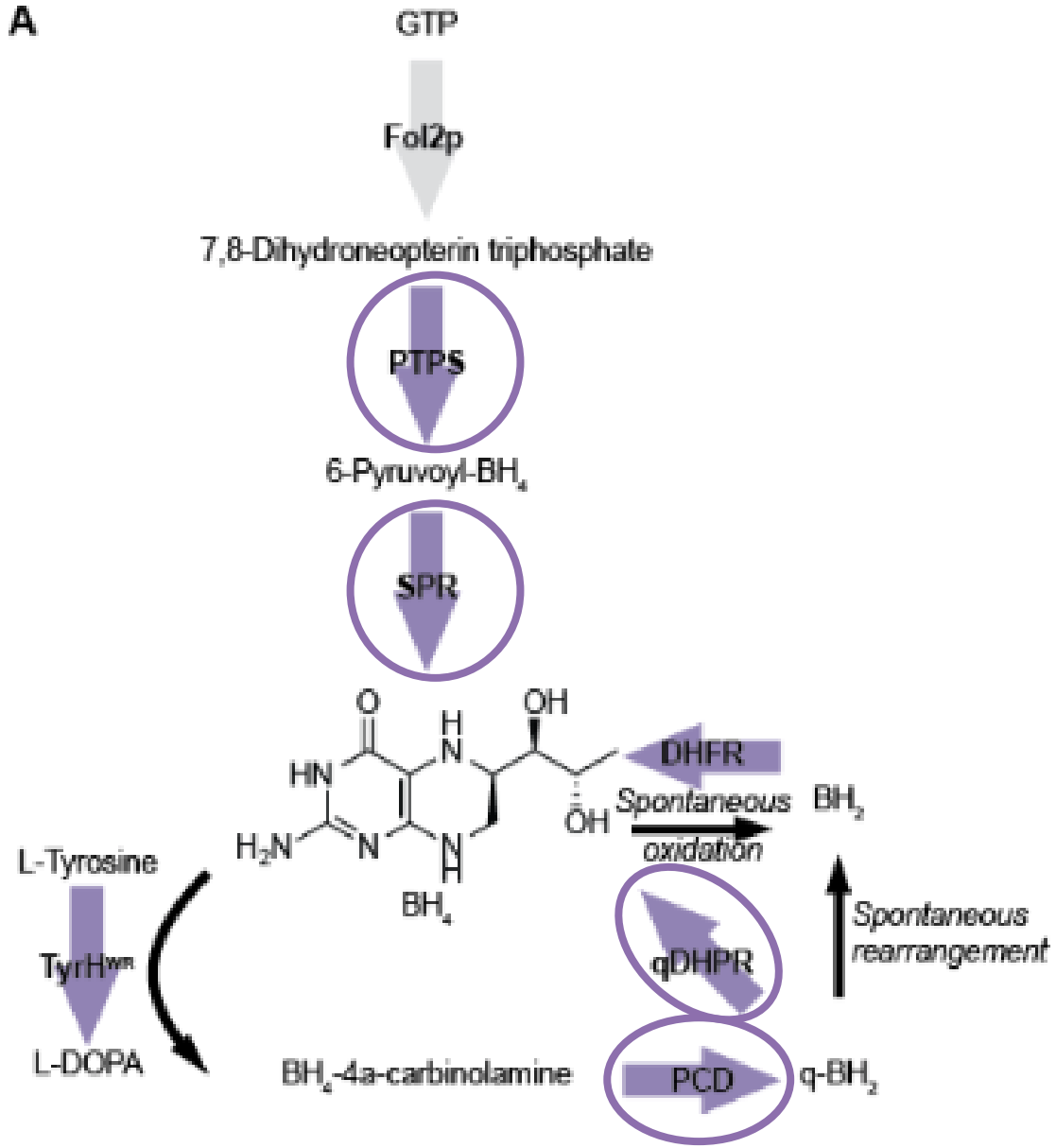
* A BH_4 cofactor is needed for conversion of tyrosine to dopa.

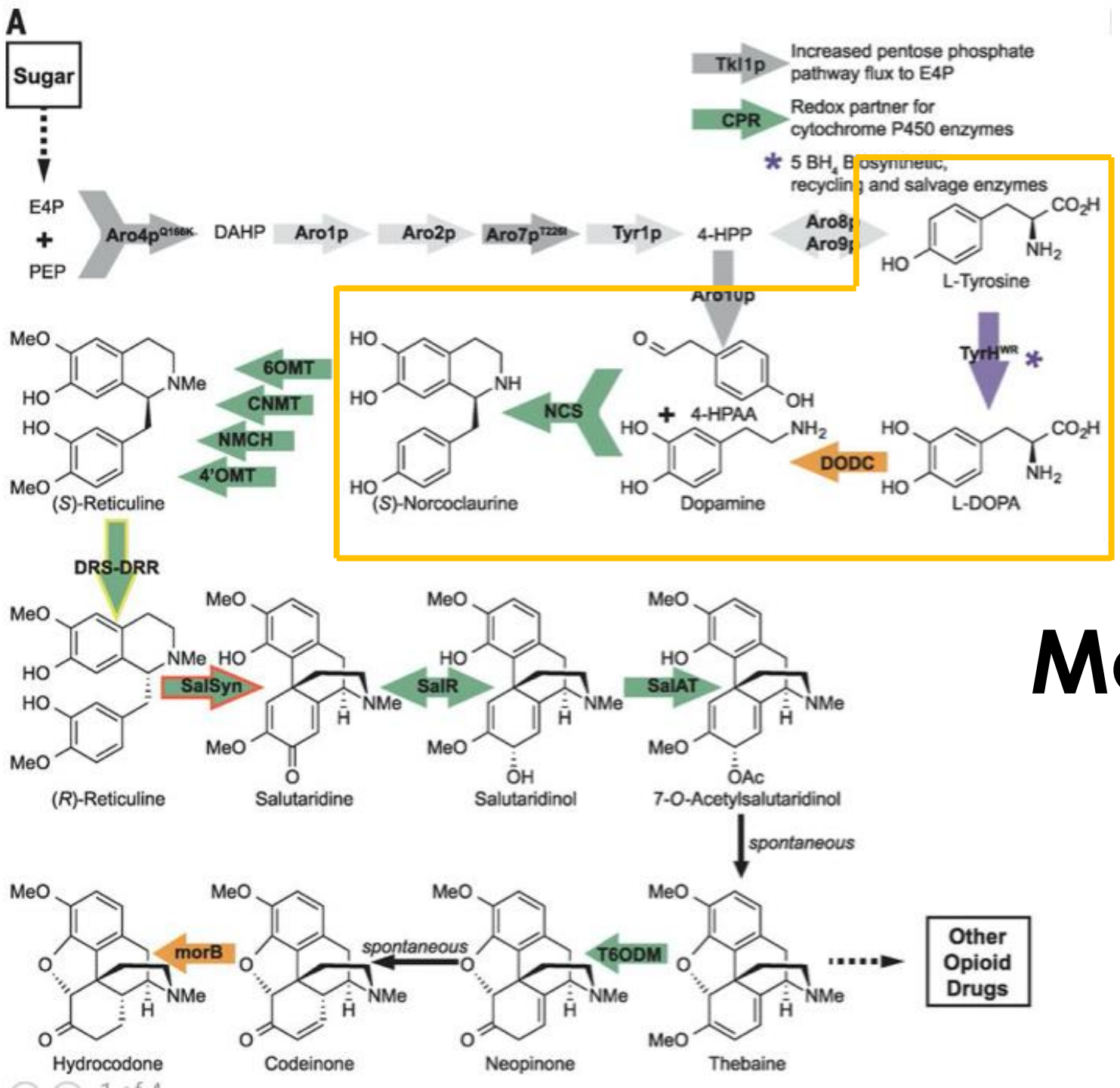
Module II

Objective:

- Synthesize and recycle mammalian cofactor BH_4

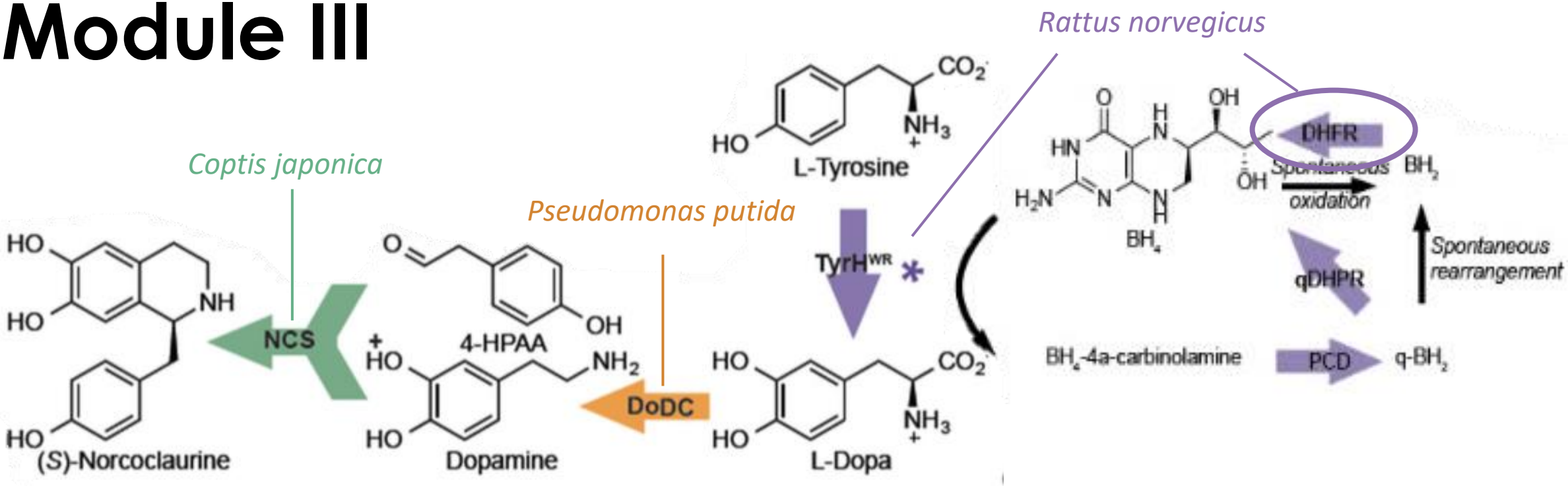
- Method:
  - Introduction of 4 genes from *Rattus norvegicus*
    - SPR: Sepiapterin reductase
    - PTPS: 6-pyruvoyl tetrahydrobiopterin synthase
    - QDHPR: Quinonoid dihydropteridine reductase
    - PCD: Pterin carbinolamine dehydratase




A
Sugar
E4P
+
PEP
Aro4p^Q166K
DAHP
Aro1p
Aro2p
Aro7p^T226I
Tyr1p
4-HPP
Tkl1p
Increased pentose phosphate pathway flux to E4P
CPR
Redox partner for cytochrome P450 enzymes
* 5 BH4 Biosynthetic, recycling and salvage enzymes
Aro8p
Aro9p
L-Tyrosine
TyrH^WR *
L-DOPA
DODC
Dopamine
4-HPAA
Aro10p
NCS
(S)-Norcoclaurine
6OMT
CNMT
NMCH
4'OMT
(S)-Reticuline
DRS-DRR
(R)-Reticuline
SalSyn
Salutaridine
SalR
Salutaridinol
SalAT
7-O-Acetylsalutaridinol
spontaneous
Thebaine
T6ODM
spontaneous
Neopinone
morB
Codeinone
Hydrocodone
Other Opioid Drugs

Module III

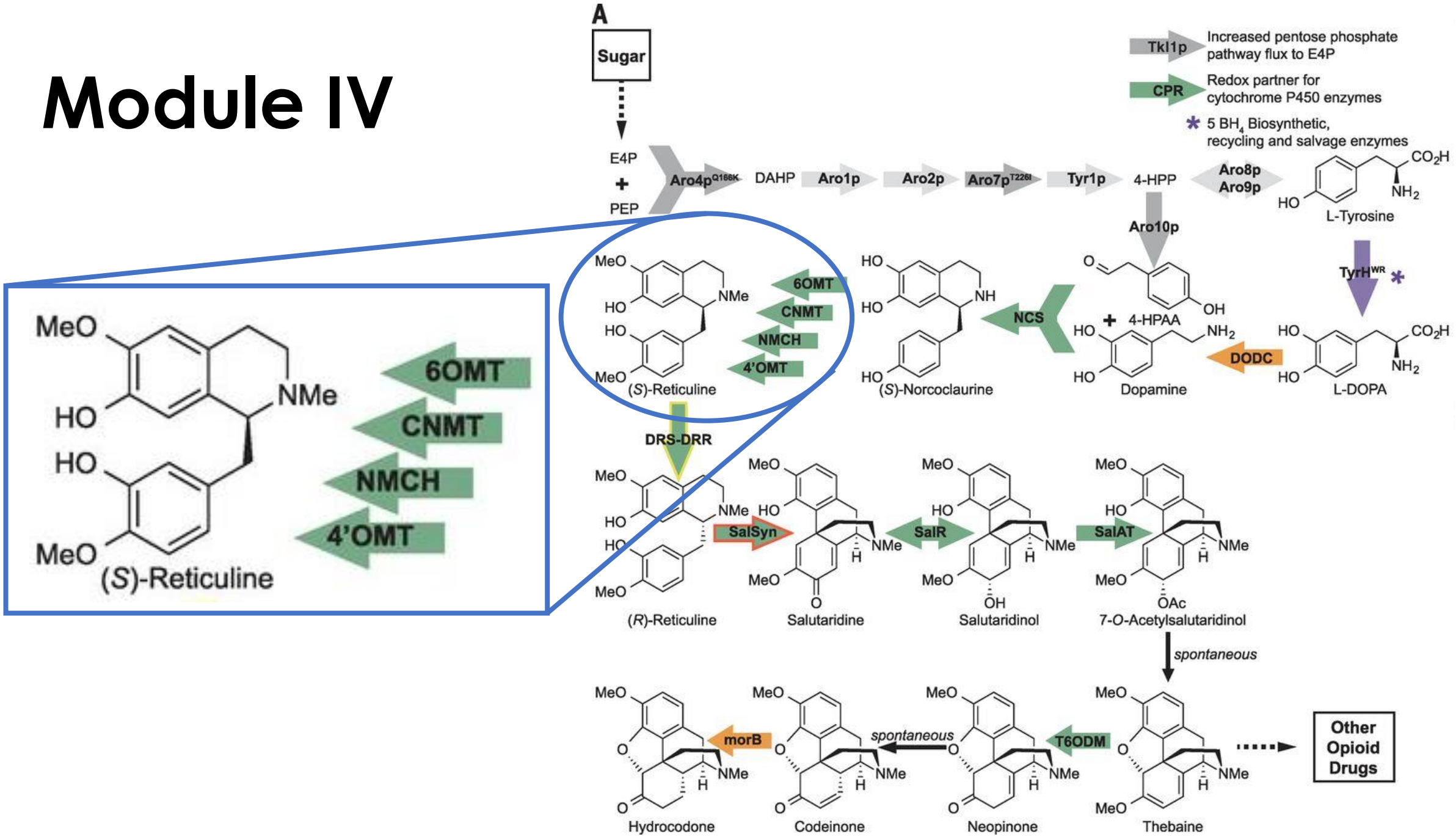
Module III



- Objective:
  - Synthesize the first backbone molecule (S)-Norcoclaurine
- Method:
  - Introduction of 4 genes from bacteria, plant and mammal

- Genes introduced
  - $TyrH^{WR}$ Tyrsosine hydroxylase
  - DHFR: Dihydrofolate reductase
  - DoDC: DOPA decarboxylase
  - NCS: Norcoclaurine

Module IV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6OMT
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(R)-Reticuline
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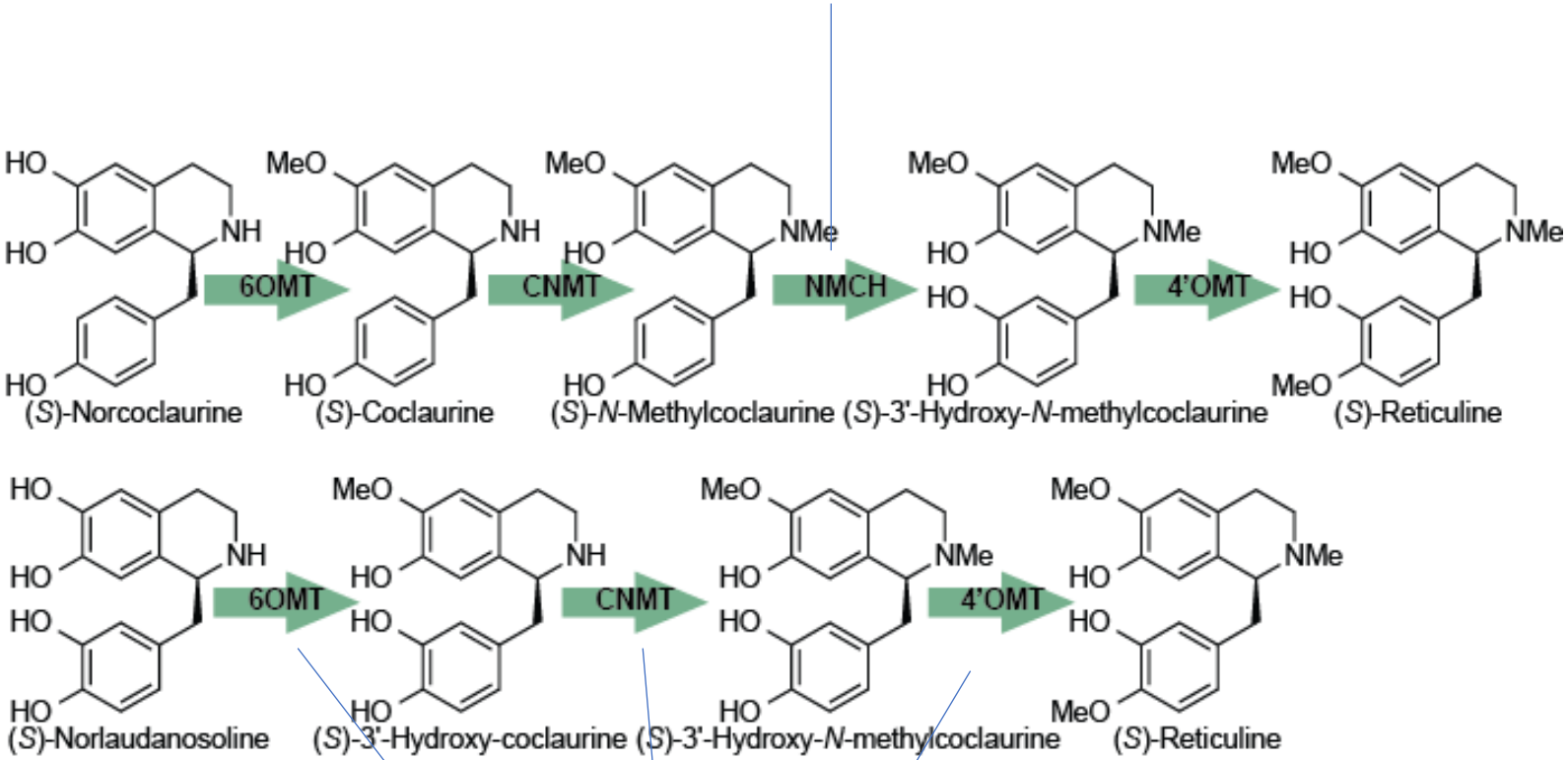
Salutaridine
SalR
Salutaridinol
SalAT
7-O-Acetylsalutaridinol
spontaneous
morB
Hydrocodone
spontaneous
Codeinone
T6ODM
Neopinone
Thebaine
Other Opioid Drugs

(S)-reticuline module IV

Eschscholzia californica

- Objective
  - Synthesization of the key BIA branchpoint molecule
- Method
  - Encoding the expression of five plant proteins
    - Norcoclaurine 6-O-methyltransferase (6OMT)
    - Coclaurine-N-methyltransferase (CNMT)
    - 4'-O-methyltransferase (4'OMT)
    - Cytochrome P450 reductase (CPR)
    - N-methylcoclaurine hydroxylase (NMCH)



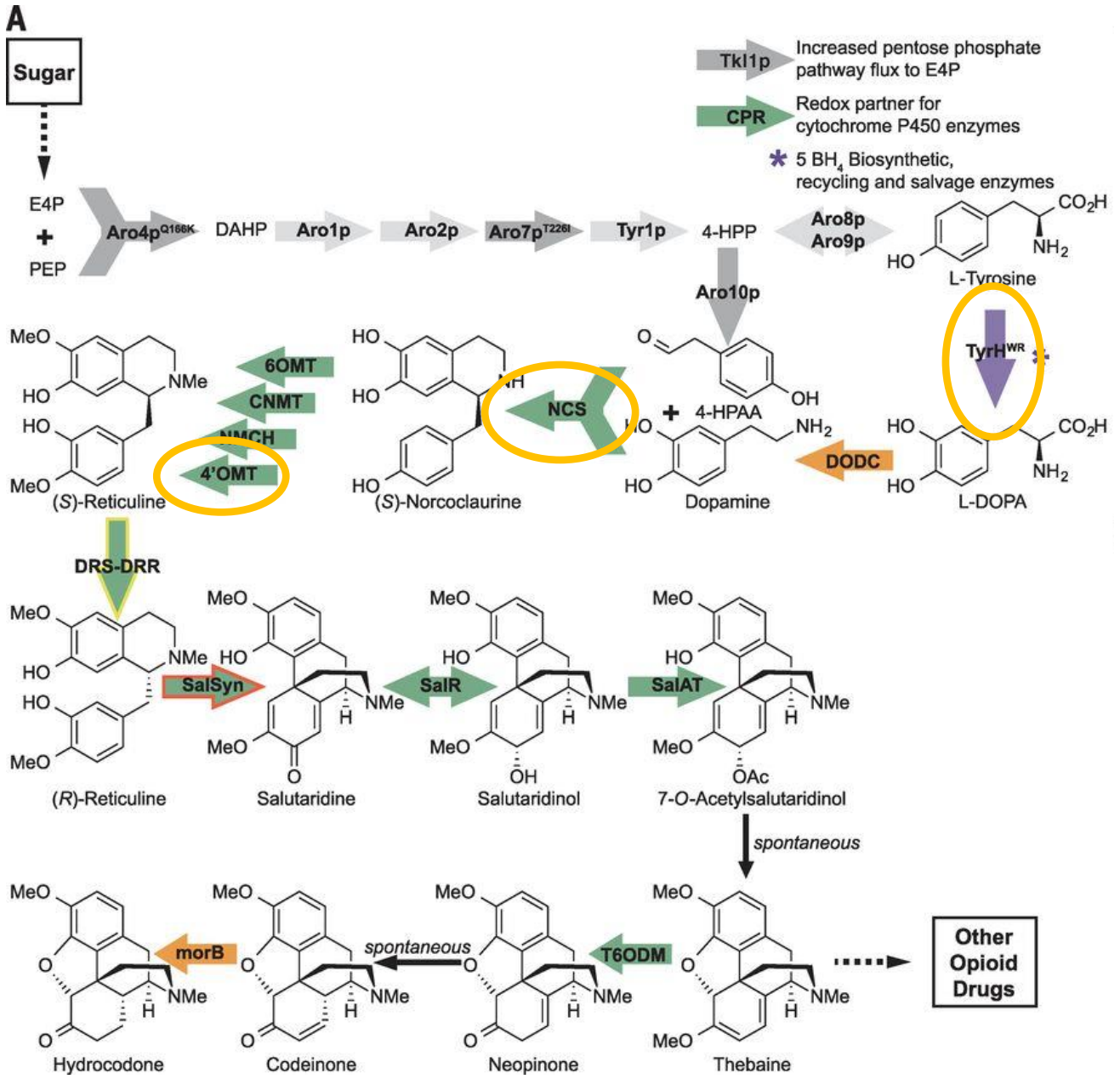
P. somniferum

Experimentation

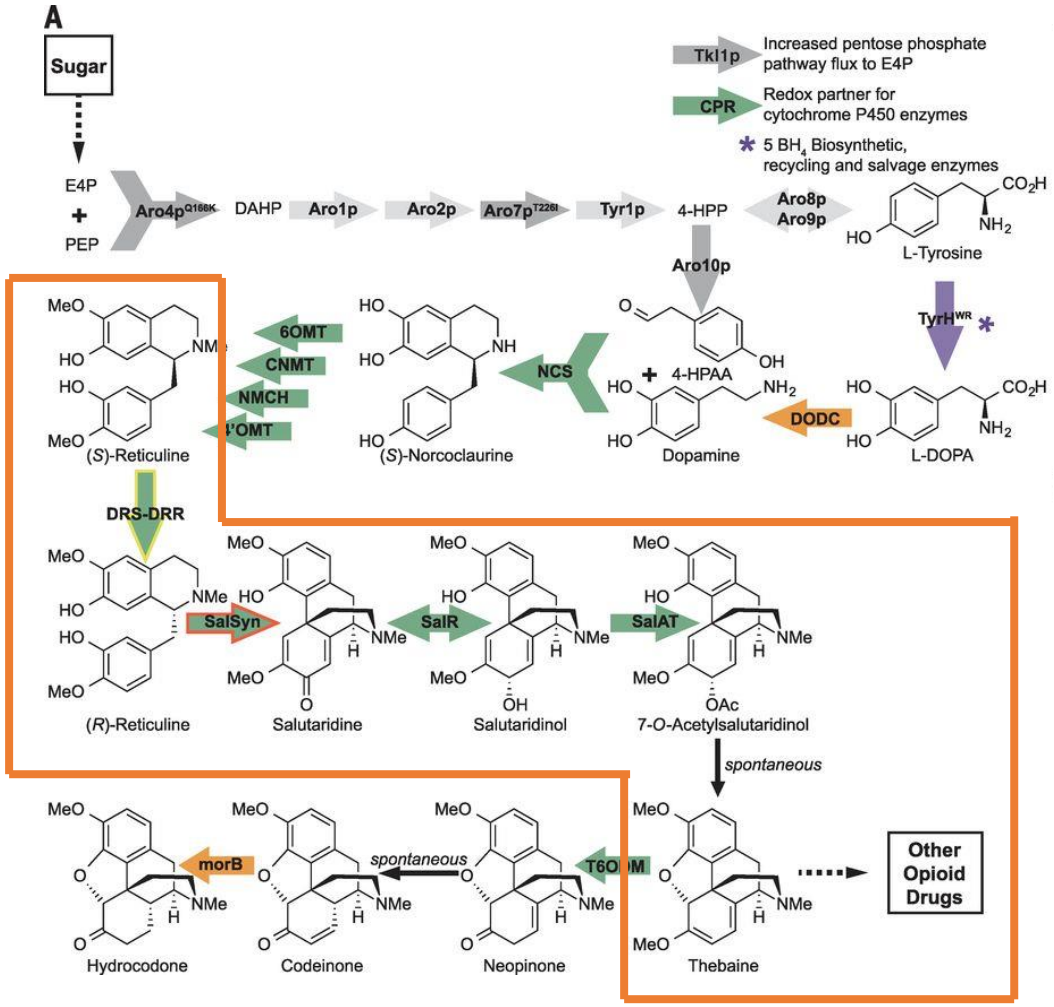
- BIA modules integrated into haploid CEN.PK2 strain, reticuline production assayed
- Strain with modules II to IV produced 12.3 µg/L of reticulin
  - Factor of 1.6 improvement with addition of module I
- Nearly complete consumption of L-DOPA, accumulation of dopamine and 3'hydroxy-N-methylcoclaurine
- Hypotheses:
  I. Increased NCS increases conversion of dopamine and 4-HPAA to noroclaurine
  II. Increased expression of $TyrH^{WR}$ replenishes dopamine
  III. Increased expression of 4'OMT reduces accumulation of 3'hydroxy-N-methylcoclaurine and enhances reticulin flux

Bottleneck module

- Objective
  - Designed to end the overexpression of three proteins
    - TyrH^{WR}, 4'OMT, NCS
- Method
  - Module integrated into CSY1059
    - Native ZWF1 gene knocked out, or integration into separate locus
- Factor of 4 improvement in reticuline accumulation
- Factor 2 decrease in accumulated 3'-hydroxy-N-methylcoclaurine



Thebaine Module VI

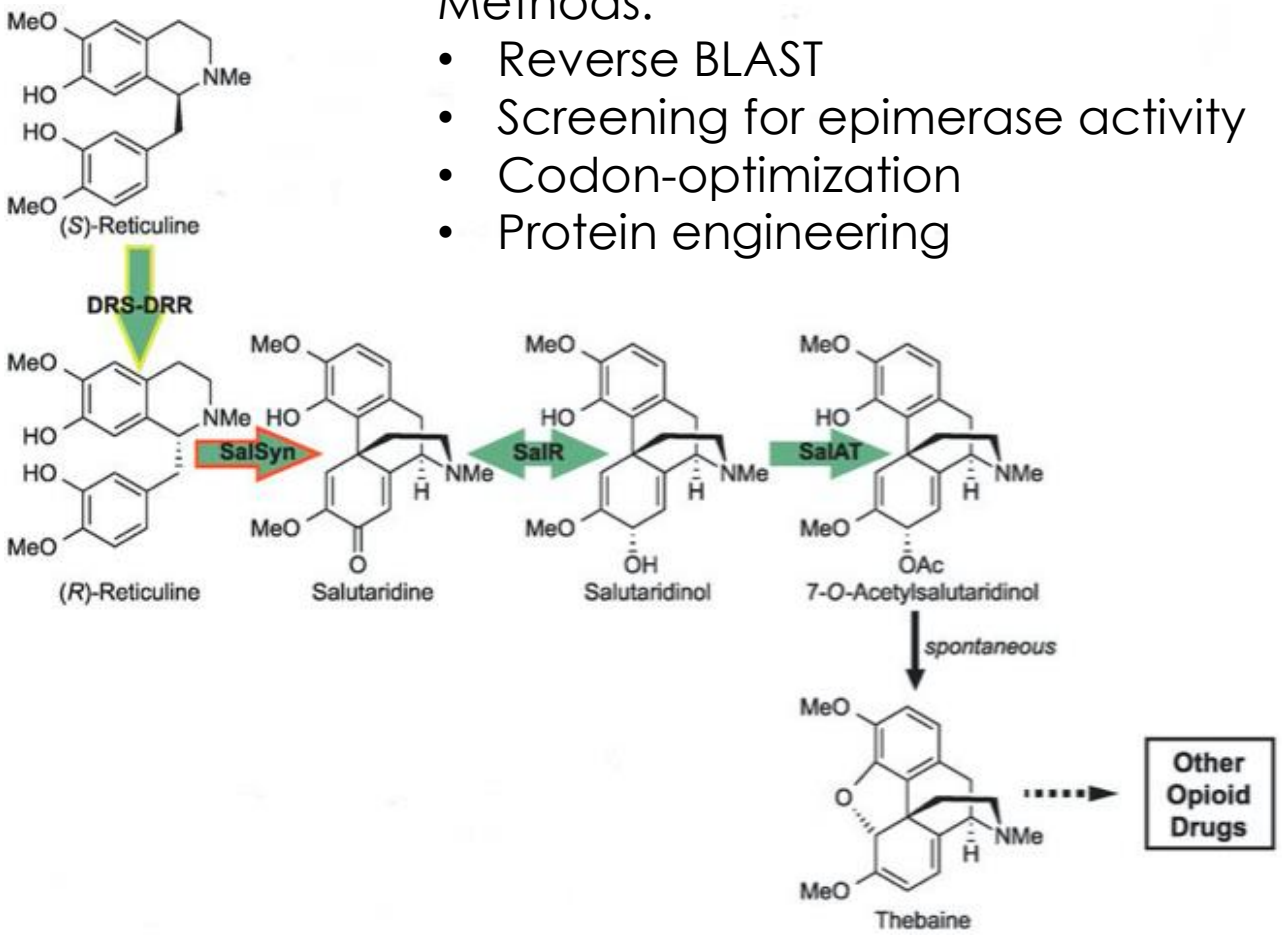


Objective:

- (S)- to (R)-epimerization of reticuline
- Optimization of salutaridine synthase

Methods:

- Reverse BLAST
- Screening for epimerase activity
- Codon-optimization
- Protein engineering



(*S*)- to (*R*)-epimerization of reticuline

- Objective:
  - Search for coding sequences for appropriate enzymes for converting (S) to (R)

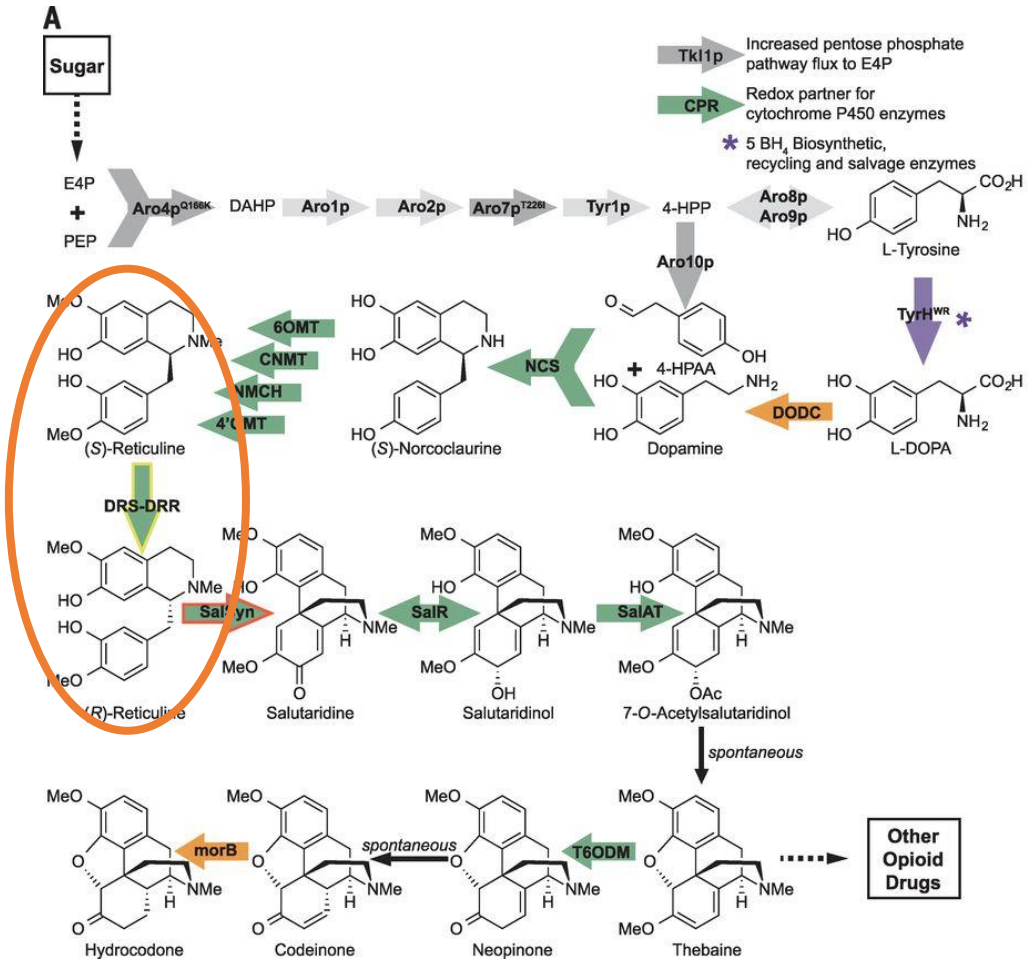
- COR-like enzyme may catalyze the stereospecific reduction

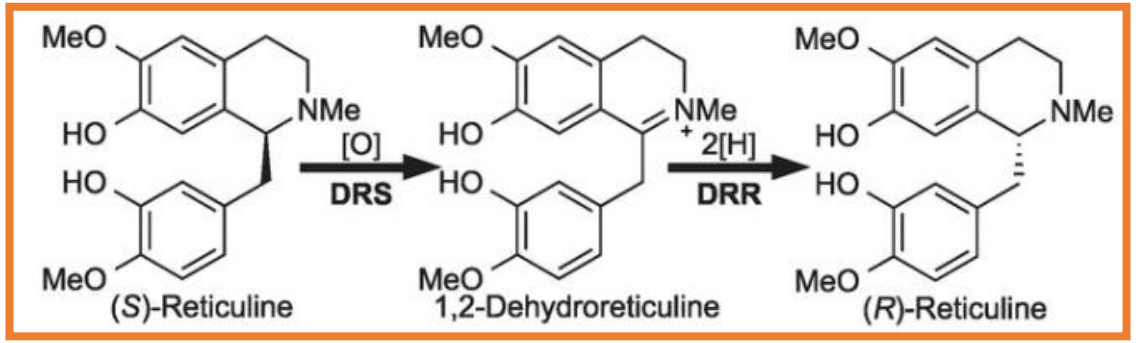
→ BLAST search against Papaver species

→ 4 species had COR + cytochrome P450 oxidase (CYP)

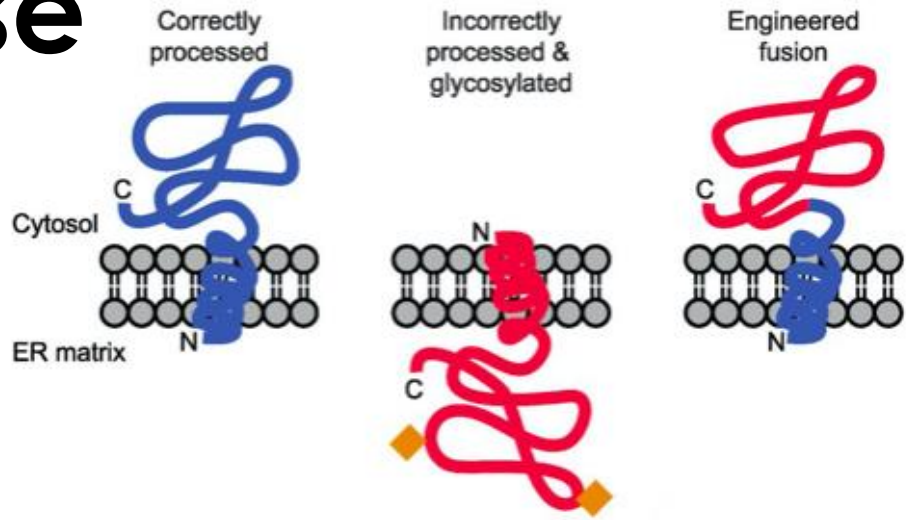
→ *P. bracteatum* DRS-DRR sequence Pbr.89405

- DRS-DRR sequence of interest, Pso.2062398 identified with another BLAST





Salutaridine synthase



- Strain with optimized *P. somniferum* salutaridine synthase (yPsSalSyn) succeeded to produce Thebaine with other downstream enzymes but reticuline intermediate was accumulated

→ Conversion of (*R*)-reticuline to salutaridine, catalyzed by SalSyn, warranted further optimization

- In yeast incorrect N-terminal sorting of the nascent SalSyn polypeptide to the ER lumen→ N-glycosylated rather than anchoring the N terminus in the outer ER membrane and maintaining the catalytic domain in the cytosol

→ misprocessing reduced SalSyn activity in yeast

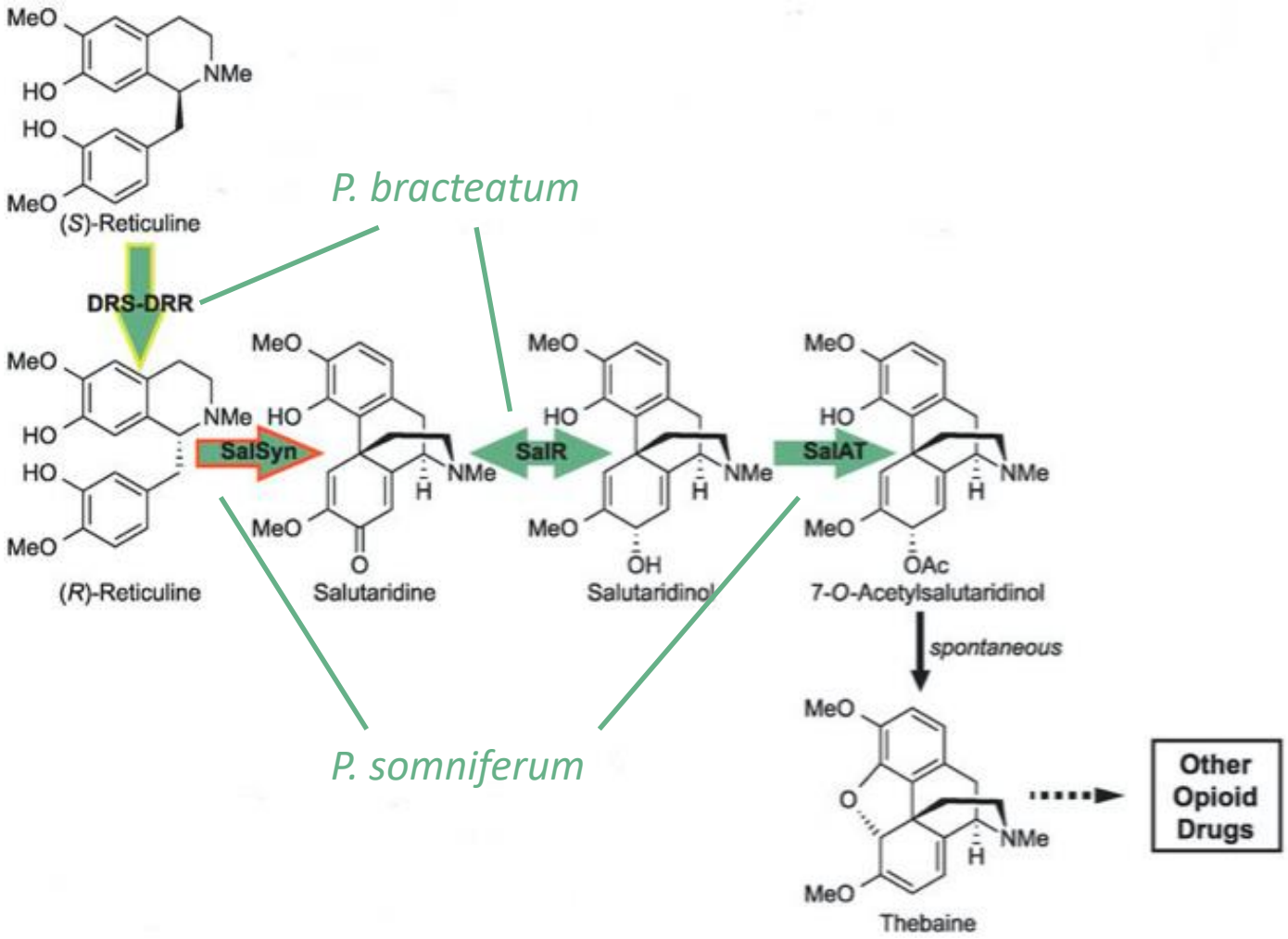
→ repaired by the engineered fusions.

- Multiple engineered variants compared

→ yEcCFS$^{1\text{-}83}$-yPbSalSyn$^{92\text{-}50}$ highest activity

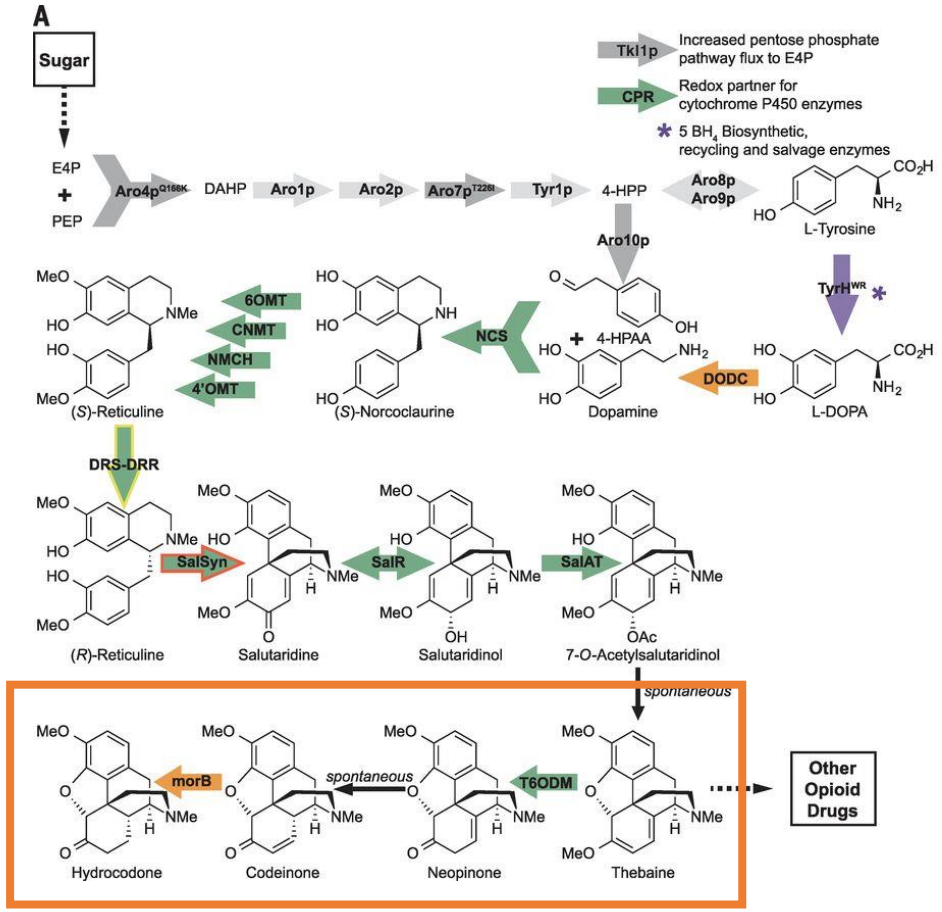
A thebaine Module VI

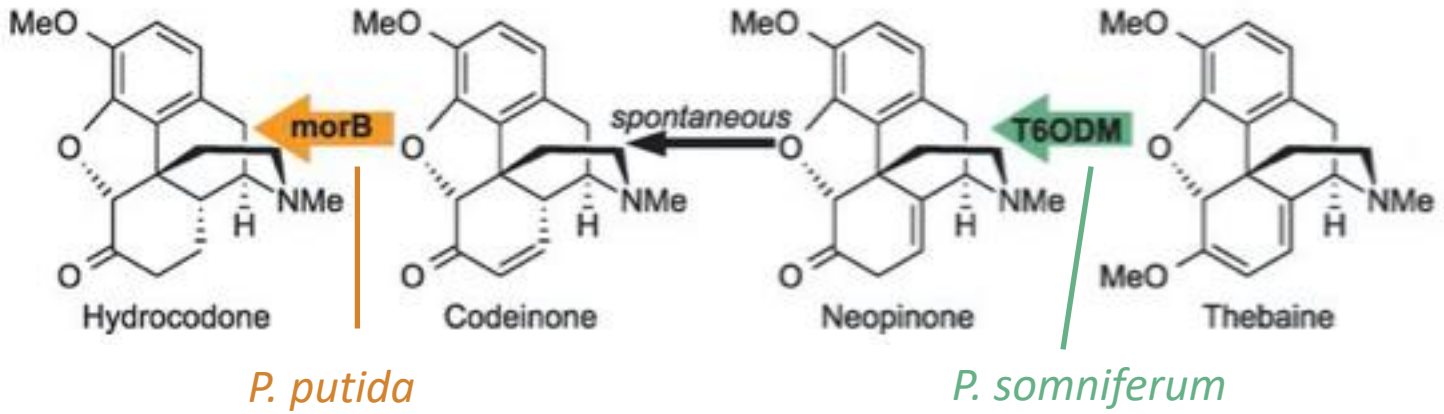
- Converts (*S*)-reticuline to the morphinan alkaloid thebaine
- Encodes the expression of the best enzyme variants:
  - PbDRS-DRR
  - yEcCFS$^{1\text{-}83}$-yPbSalSyn$^{92\text{-}504}$
  - PbSalR
  - PsSalAT
- Added to the reticuline-producing platform strain (CSY1060) as a chromosomal integration → CSY1064
- I-VI produced thebaine at concentrations of 6.4 ± 0.3 µg/liter



Hydrocodone module VII

- Objective:
  - Introduce genes morphinone reductase and hebaine 6-O-demethylase to existing CSY1064 strain
- Method:
  - YAC pCS2765 including enzymes T6ODM and morB enconding genes





Summary

- Module I-VI: Production of thebaine from simple carbon and nitrogen sources
  - 21 heterologous enzymes from bacteria, plants and mammals
  - Overexpression of 2 native enzymes
  - Inactivation of 1 native enzyme
  - 6.4 ± 0.3 µg/L of thebaine
- Module VII: Production of hydrocodone
  - 23 heterologous enzymes from bacteria, plants and mammals
  - Cultured with 50 mM 2-oxoglutarate for additional enzyme support
  - ~0.3 µg/L hydrocodone
- Over 100,000 fold improvement in titer needed for feasibility of commercial production

Further Applications

- In recent years, researchers are engineering yeast to produce a variety of plant-based products.
- Substantially improved production of opioids via yeast should be expected
- Nevertheless, this article highlights the potential of yeast as a chassis for complex chemicals