

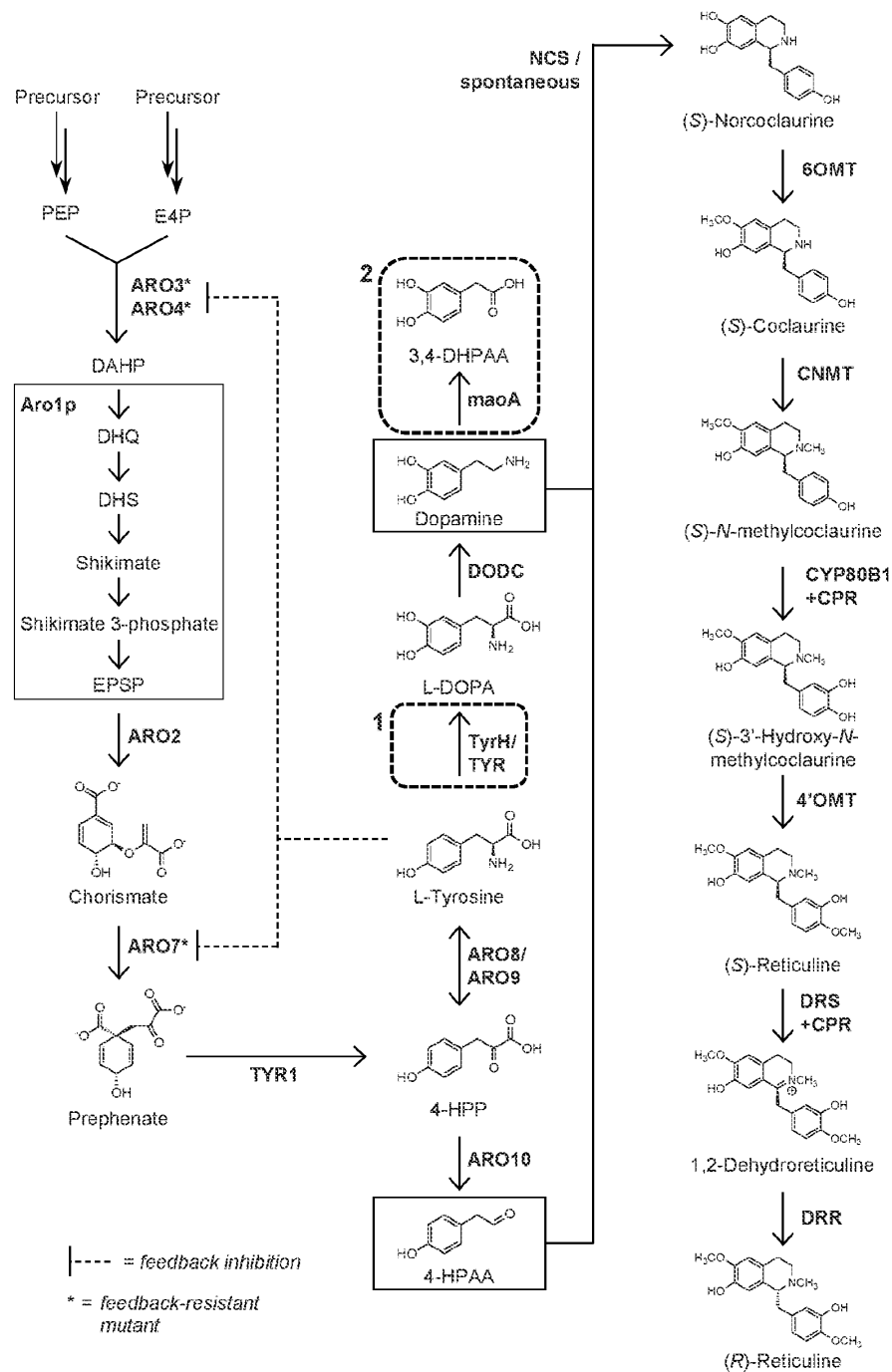


FIGURE 1

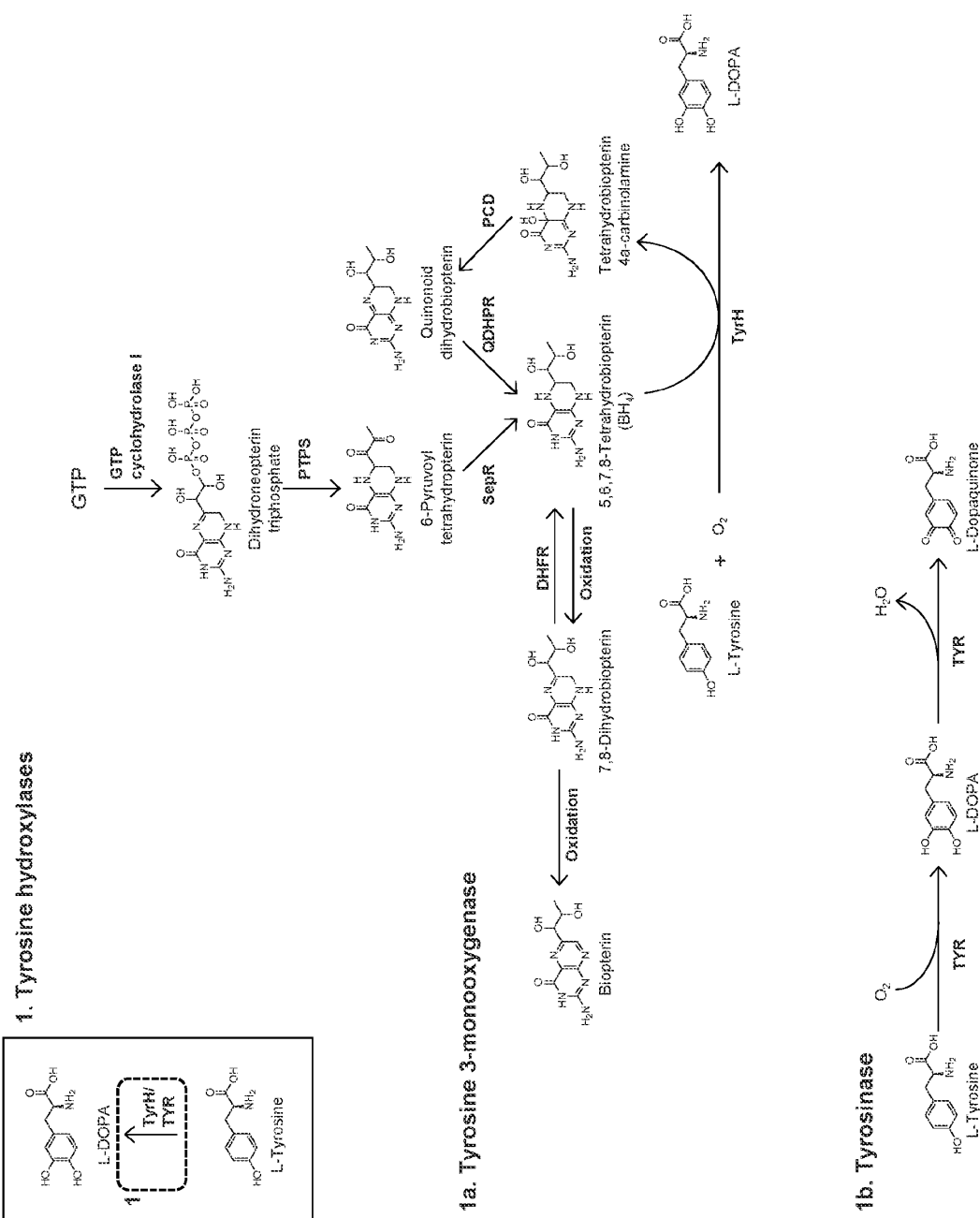


FIGURE 2

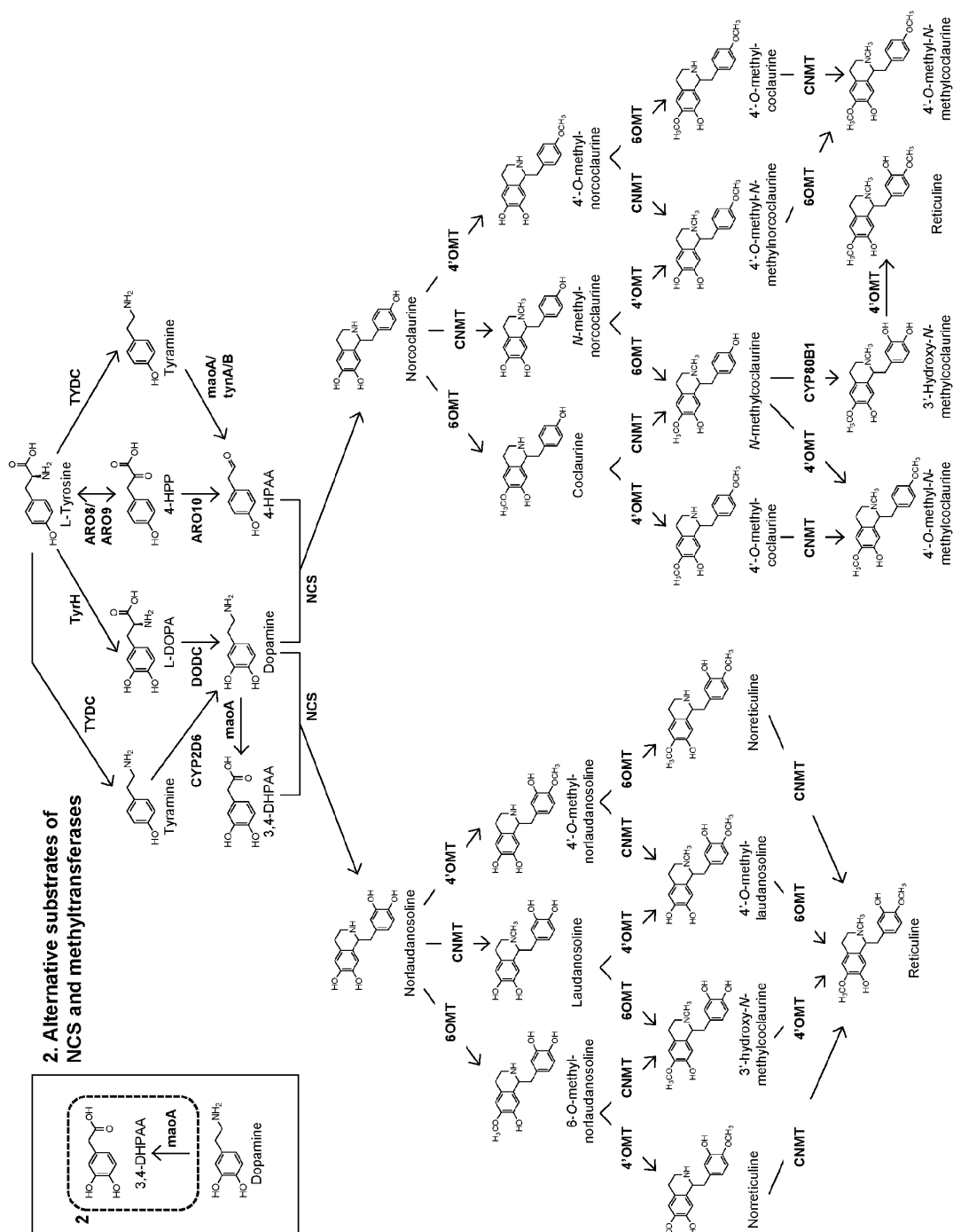


Figure 3



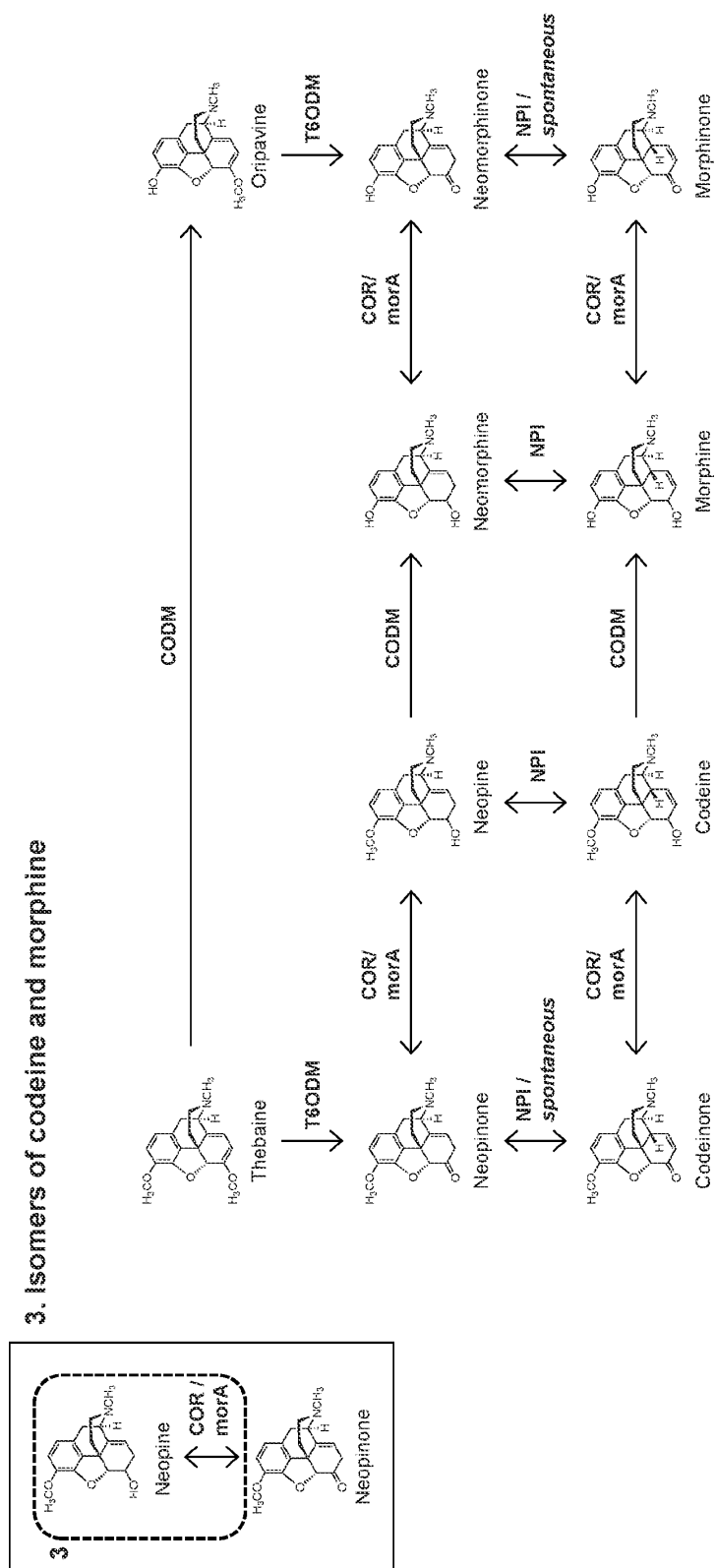


FIGURE 5

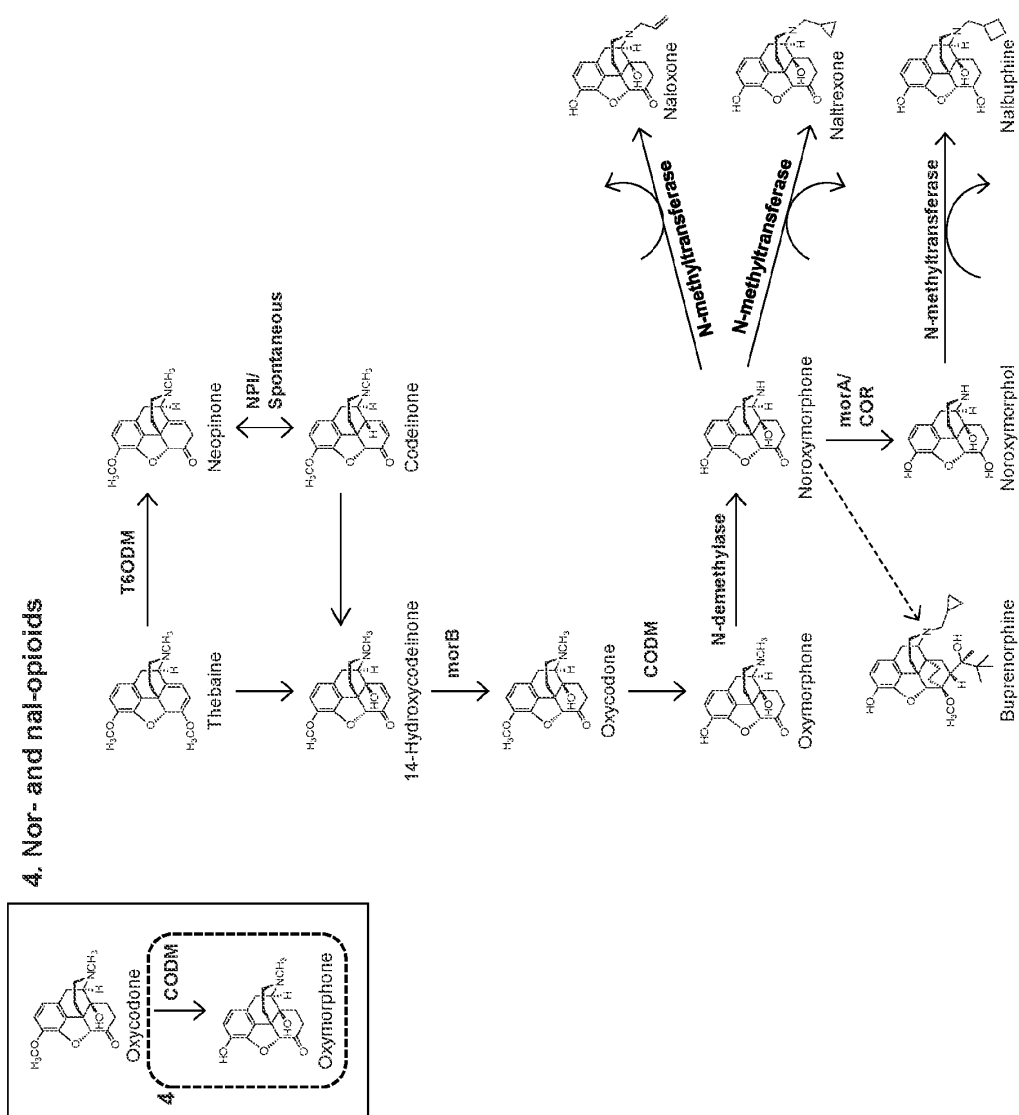


FIGURE 6

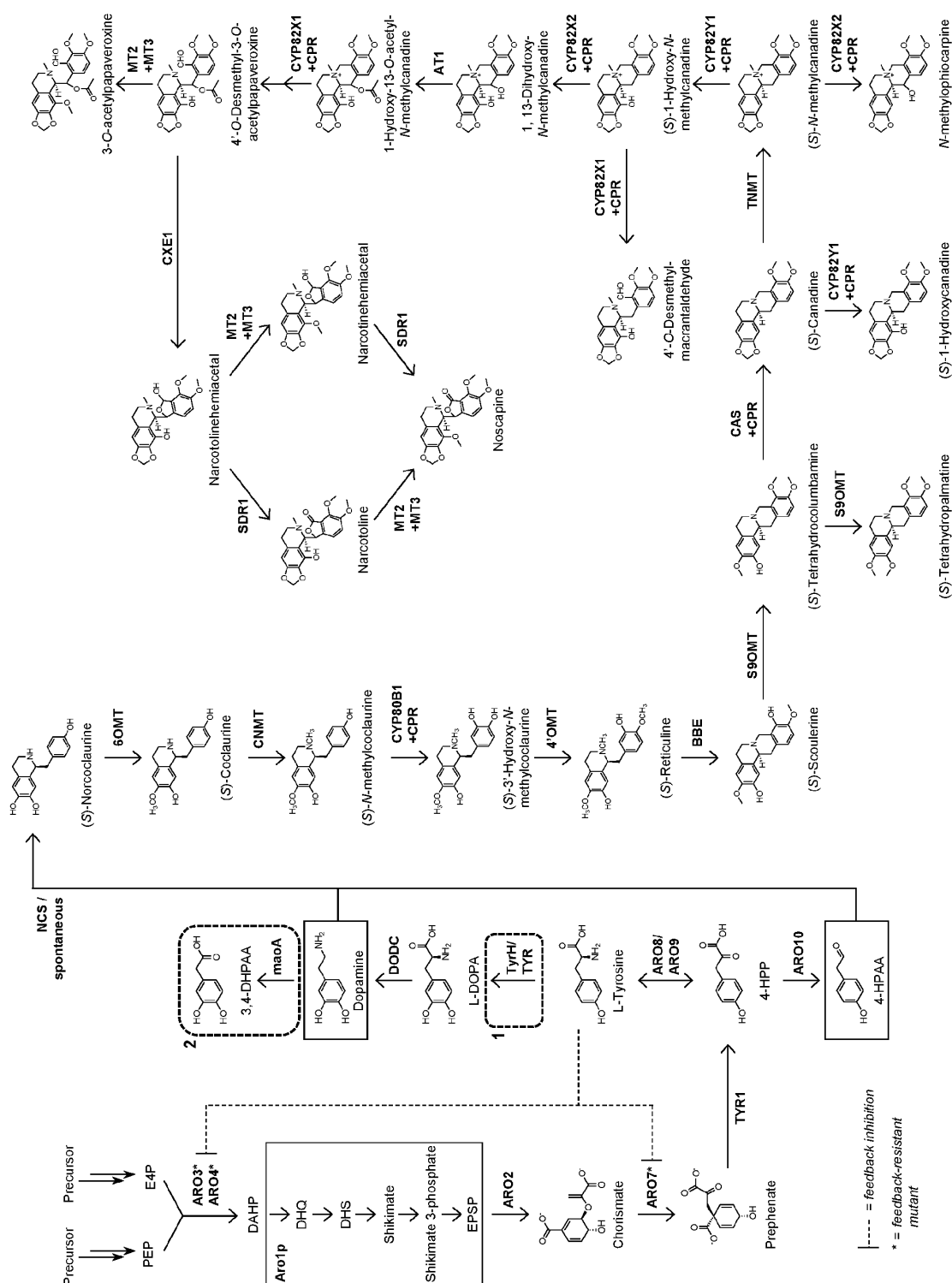


Figure 7

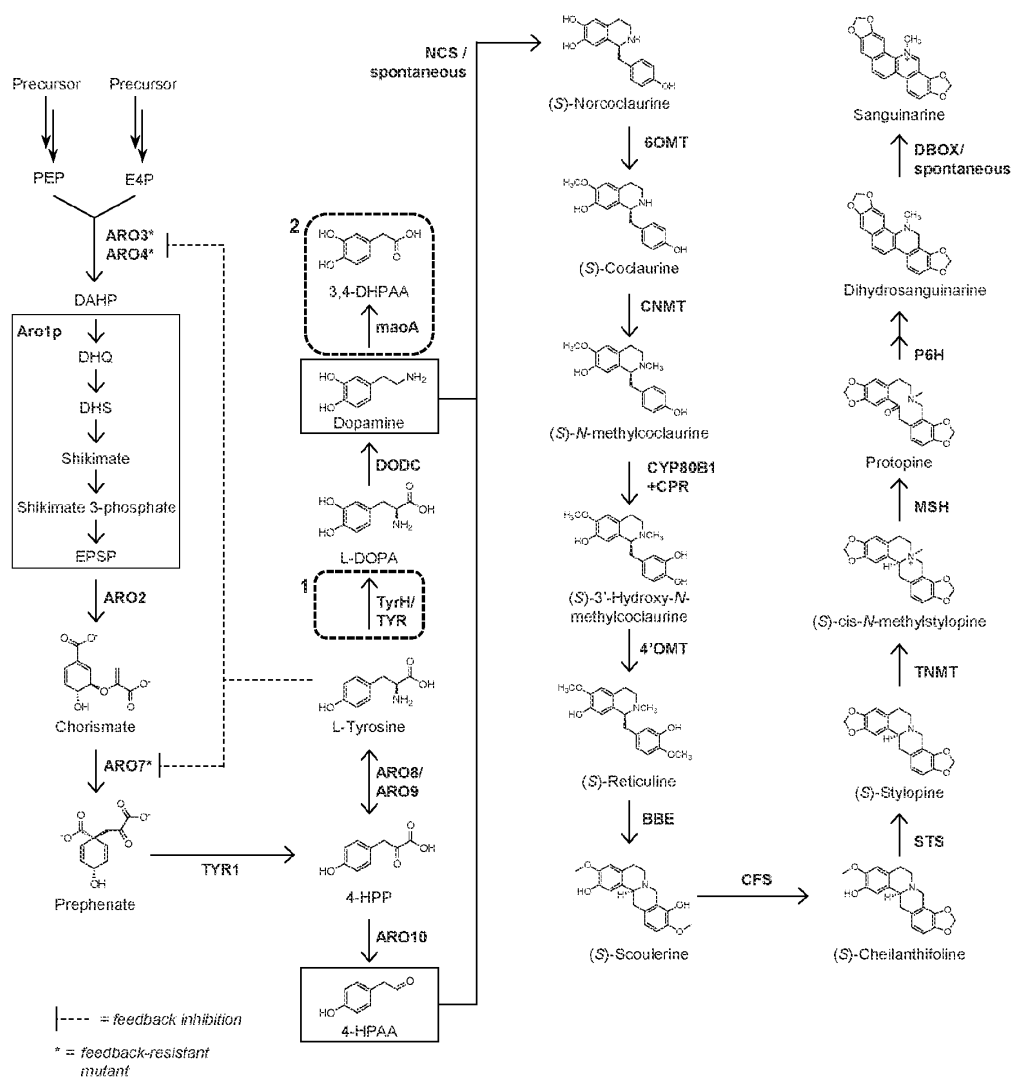


FIGURE 8
 SUBSTITUTE SHEET (RULE 26)

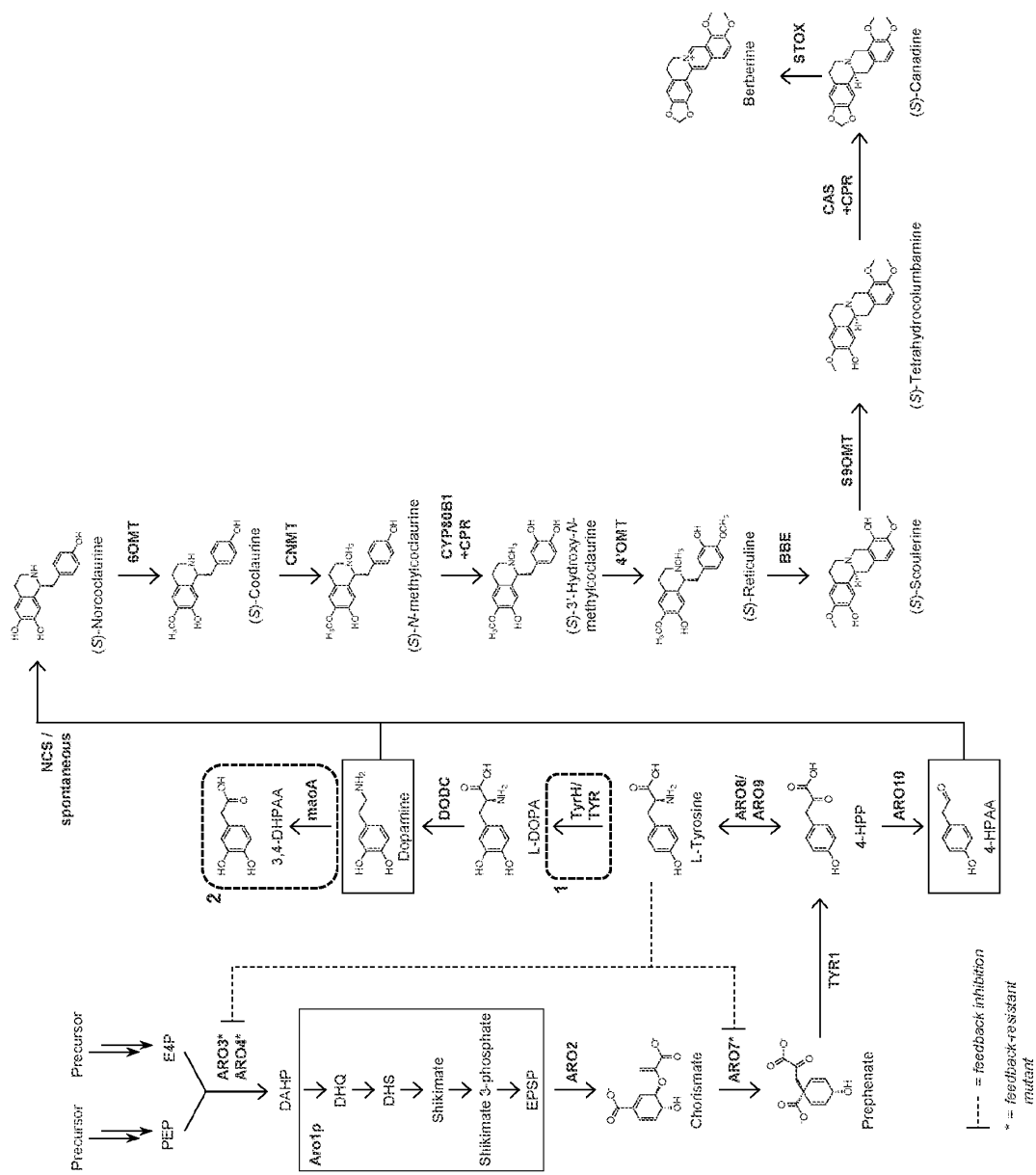


Figure 9

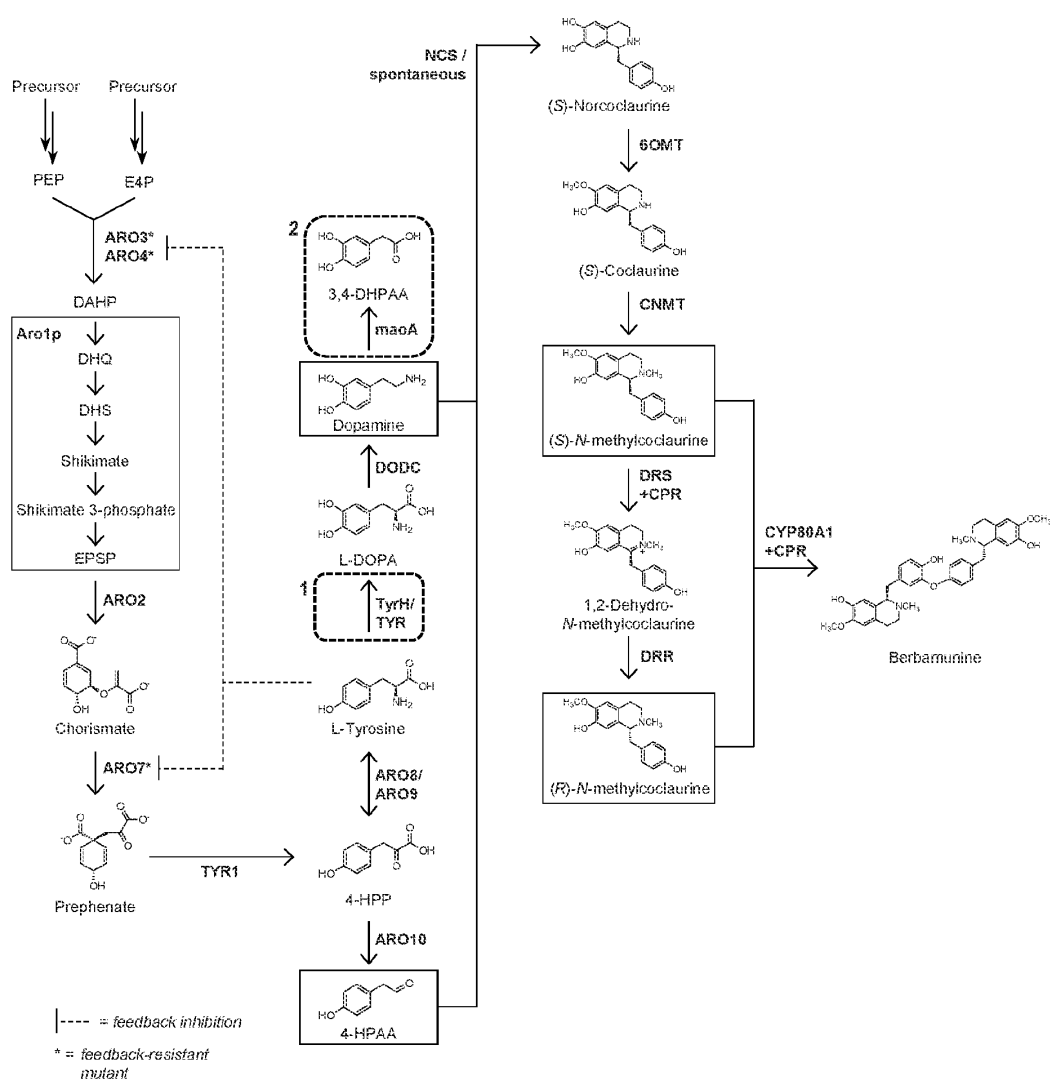
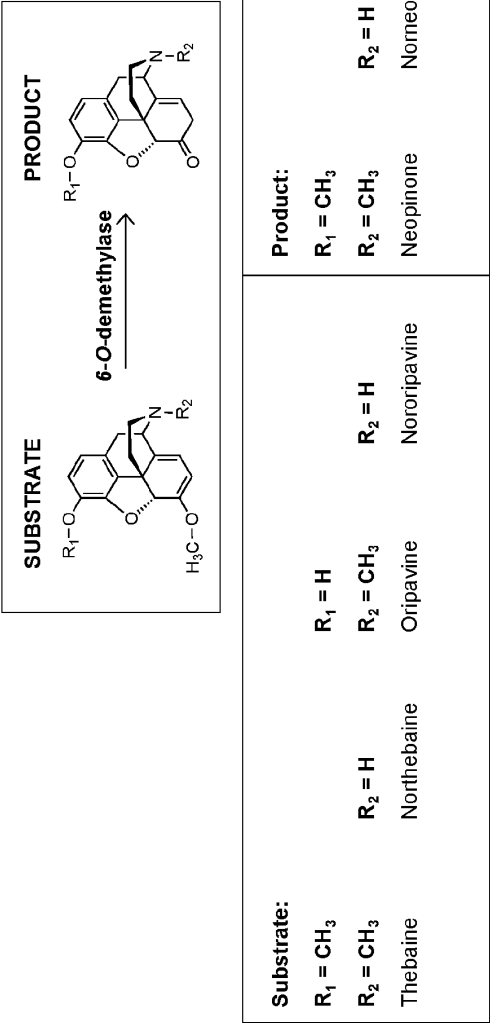
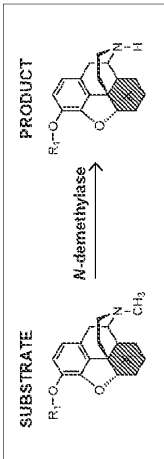


FIGURE 10



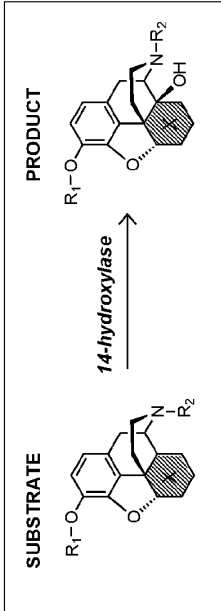
SUBSTRATE 3-O-demethylase PRODUCT		
Substrate: $R_1 = CH_3$ Thebaine Codaine Neopine Codaine Neopine Hydrocodone Dihydrocodaine Oxycodone 14-Hydroxycodaine 14-Hydroxycodaine Oxycodol	Product: $R_1 = CH_3$ Oripavine Morphine Neomorphine Morphine Neomorphine Hydromorphone Dihydromorphine Oxymorphone 14-Hydroxymorphine 14-Hydroxymorphine Oxymorphone	Product: $R_1 = H$ Nortibaine Norcodeine Norcodeine Norcodeine Norcodeine Norhydrocodone Norhydrocodone Noroxycodone Nor-14-Hydroxycodaine Nor-14-Hydroxycodaine Noroxycodol

Figure 12



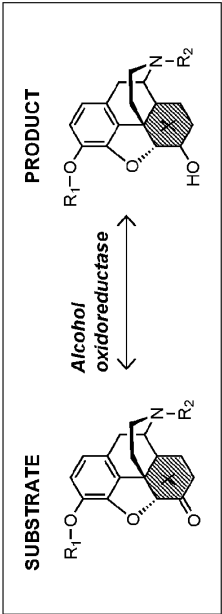
Substrate: $R_1 = CH_3$		Product: $R_1 = CH_3$	
Thebaine	Critavine	Northebaine	Nororipavine
Codeine	Morphine	Narcodeine	Normorphine
Neopine	Neomorphine	Neomorphine	Neomorphine
Codaine	Morphine	Narcodeine	Normorphine
Neopine	Neomorphine	Neomorphine	Neomorphine
Hydrocodone	Hydromorphine	Narhydrocodone	Narhydrocodone
Dihydrocodaine	Dihydromorphine	Nardihydrocodaine	Nardihydrocodone
Oxycodone	Oxymorphine	Naroxycodone	Naroxycodone
14-Hydrodihydroxycodone	14-Hydrooxymorphine	Nar-14-Hydrodihydroxycodone	Nar-14-Hydrooxymorphine
14-Hydroxycodone	14-Hydrooxymorphine	Nar-14-Hydroxycodone	Nar-14-Hydrooxymorphine
Oxycodol	Oxymorphol	Naroxycodol	Naroxymorphol

Figure 13



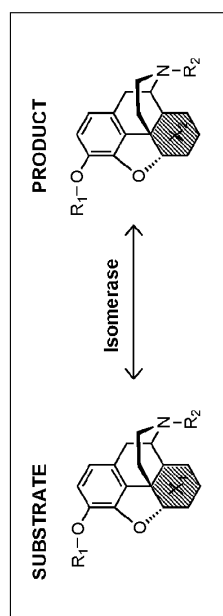
			Product: $R_1 = \text{CH}_3$ $R_2 = \text{CH}_3$ 14-Hydroxythebaine	$R_1 = \text{H}$ $R_2 = \text{CH}_3$ 14-Hydroxythebaine	$R_2 = \text{H}$ Nor-14-hydroxythebaine	$R_1 = \text{H}$ $R_2 = \text{CH}_3$ 14-Hydroxythebaine	$R_2 = \text{H}$ Nor-14-hydroxythebaine
	$R_1 = \text{H}$ $R_2 = \text{CH}_3$ Thebaine	$R_2 = \text{H}$ Northebaine	$R_1 = \text{H}$ $R_2 = \text{CH}_3$ Oripavine	$R_2 = \text{H}$ Nor-14-hydroxythebaine	$R_2 = \text{H}$ Nor-14-hydroxythebaine	$R_1 = \text{H}$ $R_2 = \text{CH}_3$ 14-Hydroxythebaine	$R_2 = \text{H}$ Nor-14-hydroxythebaine
	Codeinone	Norcodeinone	Morphinone	14-Hydroxycodeinone	14-Hydroxycodeinone	14-Hydroxycodeinone	14-Hydroxycodeinone
	Neopinone	Nomeopinone	Neomorphinone	14-Hydroxyneopinone	14-Hydroxyneopinone	14-Hydroxyneopinone	14-Hydroxyneopinone
	Codeine	Norcodeine	Morphine	14-Hydroxycodeine	14-Hydroxycodeine	14-Hydroxycodeine	14-Hydroxycodeine
	Neopine	Nomeopine	Neomorphine	14-Hydroxynepine	14-Hydroxynepine	14-Hydroxynepine	14-Hydroxynepine
	Hydrocodone	Norhydrocodone	Hydromorphone	Oxycodone	Oxycodone	Oxycodone	Oxycodone
	Dihydrocodeine	Nor-dihydrocodeine	Dihydromorphine	Oxycodol	Oxycodol	Oxycodol	Oxycodol

FIGURE 14



	Substrate: $R_1 = CH_3$ $R_2 = H$ Codeinone $R_1 = H$ $R_2 = CH_3$ Morphinone $R_1 = H$ $R_2 = H$ Neopinone $R_1 = H$ $R_2 = CH_3$ Hydrocodone $R_1 = H$ $R_2 = H$ Oxycodone $R_1 = CH_3$ $R_2 = H$ 14-Hydroxy-codeinone	$R_1 = H$ $R_2 = H$ Normorphinone $R_1 = H$ $R_2 = H$ Neomorphinone $R_1 = H$ $R_2 = H$ Hydromorphone $R_1 = H$ $R_2 = H$ Oxymorphone $R_1 = H$ $R_2 = H$ 14-Hydroxy-morphinone	Product: $R_1 = CH_3$ $R_2 = CH_3$ Codeine $R_1 = H$ $R_2 = H$ Neopine $R_1 = H$ $R_2 = H$ Dihydrocodeine $R_1 = H$ $R_2 = H$ Oxycodol $R_1 = CH_3$ $R_2 = CH_3$ 14-Hydroxy-codeine	$R_2 = H$ Normorphine $R_2 = H$ Neomorphine $R_2 = H$ Dihydro-morphine $R_2 = H$ Oxymorphol $R_2 = H$ 14-Hydroxy-morphine
---	---	--	---	--

FIGURE 15



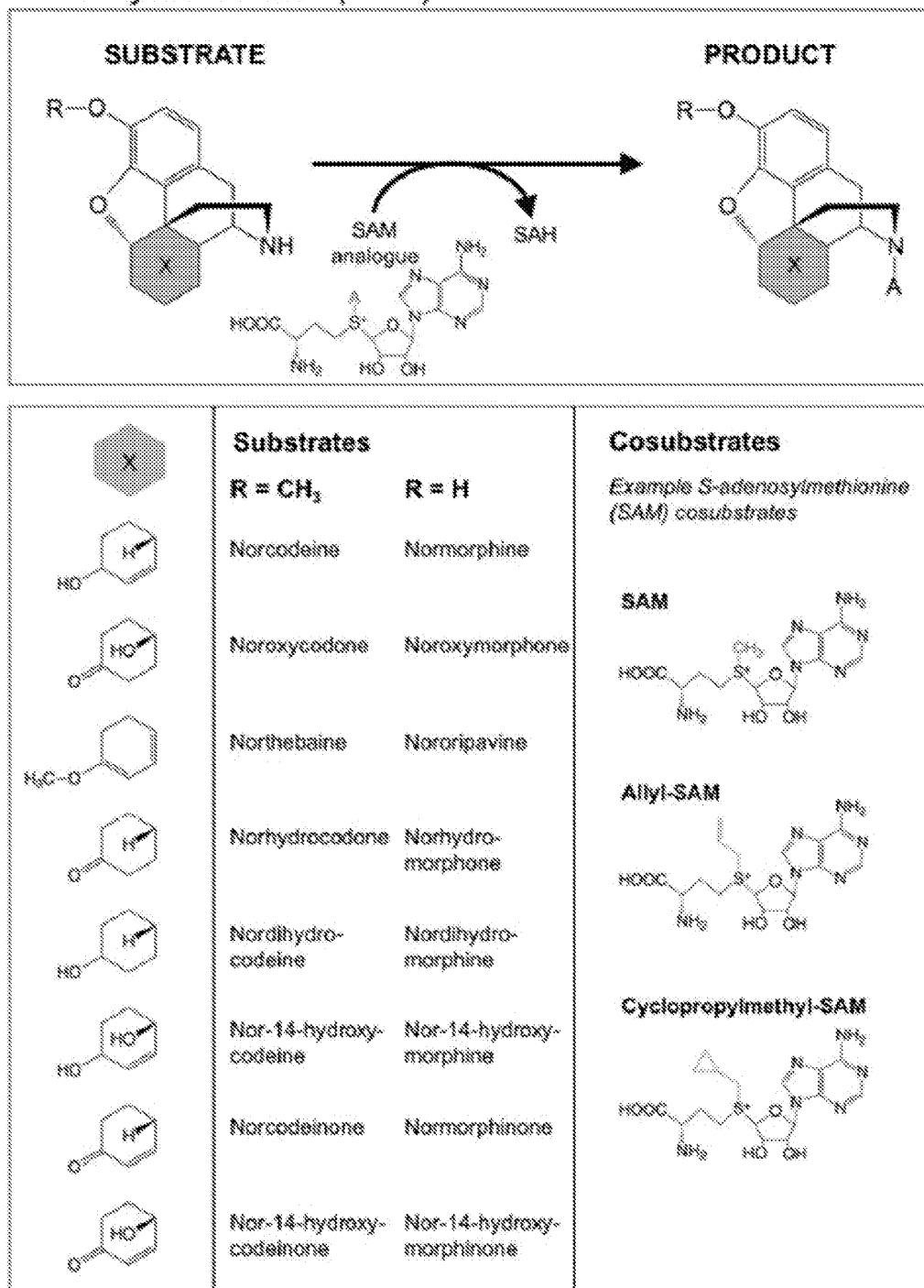
N-methyltransferase (NMT):

Figure 18

Four genetic constructs integrated into the yeast genome:

1. YBR197CΔ::P_{TPH1}-RnSepR-T_{STE2}, P_{TEF1}-RnPTPS-T_{CYC1}, *KanMX* marker, P_{GPD}-RnQDHPR-T_{ADH1}, P_{PGK1}-RnPCD-T_{PHO5}
2. HIS3 Δ::P_{GPD}-RnTyH-T_{ADH1}, P_{TPH1}-PpDODC-T_{STE2}, *HIS5* marker, P_{TEF1}-RnDHR-T_{CYC1}, P_{PGK1}-CJNCS-T_{PHO5}
3. YDR514CΔ::P_{PGK1}-PSCNMT-T_{MF31}, P_{TPH1}-Pp6OMT-T_{PHO5}, P_{GPD}-EcCYP80B1-T_{ADH1}, *LEU2* marker, P_{TEF1}-PSCPR-T_{CYC1}, P_{TPH1}-Pp4OMT-T_{STE2}
4. ARO4Δ::P_{TEF1}-ARO4^{FBR}-T_{CYC1}, P_{GPD}-ARO7^{FBR}-T_{ADH1}, *HygR* marker, P_{PGK1}-TKL1-T_{PHO5}, P_{TPH1}-ARO10-T_{STE2}

Schematic of the integrated constructs indicating the orientation of each expression cassette:

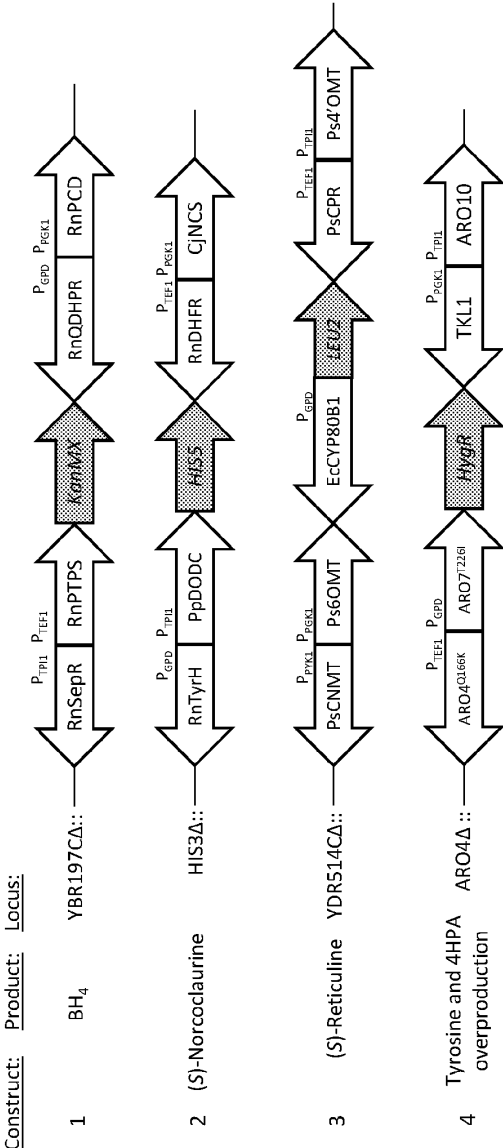


Figure 19

Example genetic constructs:

5. YPL250CA::P_{GPD}-PbDRS-T_{ADH1}, P_{TEF1}-PbDRR-T_{CYC1}, P_{PGK1}-PstS-T_{PHO5}, bleR marker

6. TRP1::P_{PGK1}-PsSalAT-T_{PHO5S}, P_{TRP1}-PbSalR-T_{STE2}, URA3 marker, P_{GPD}-EcCFS¹⁻⁸³-PbSalSyn⁹²⁻⁵⁰⁴-T_{ADH1}

7. HOΔ::P_{GPD}-Pst6ODM-T_{ADH1}, P_{PGK1}-morB-T_{PHO5}, P_{TP1}-PsnPI-T_{STE2}

** Note that morB can be replaced with COR to create codeine-producing strain; CODM can be added to construct 7 to create morphine-producing strain*

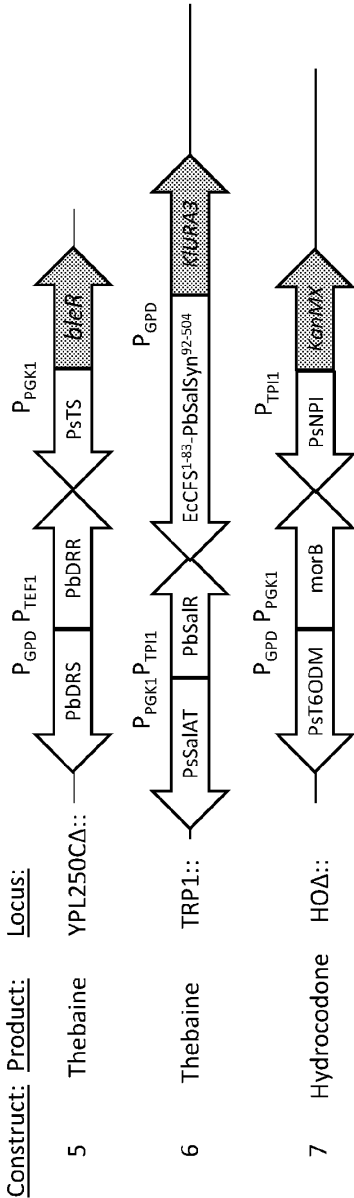
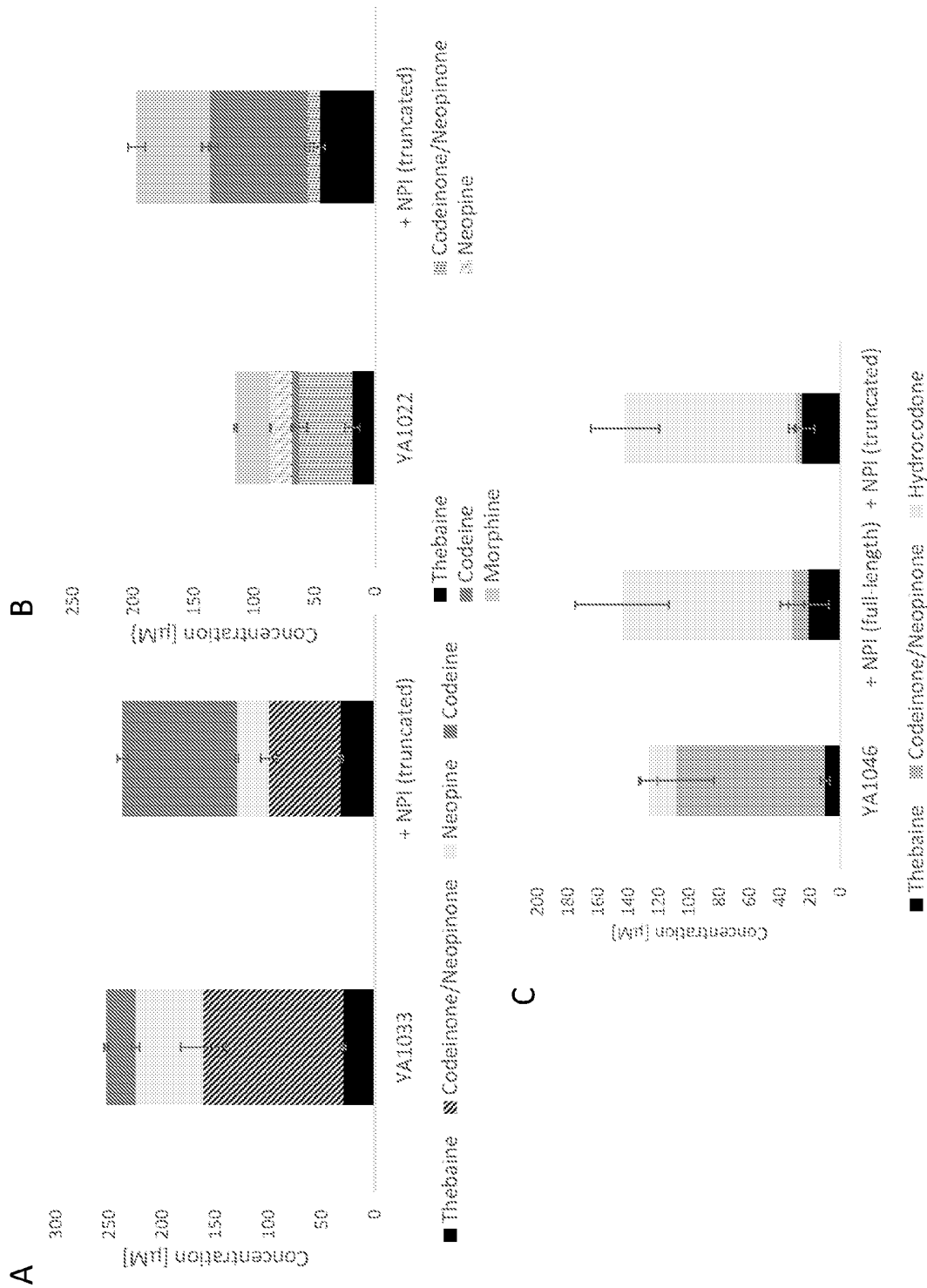


Figure 20



Figures 21A-C

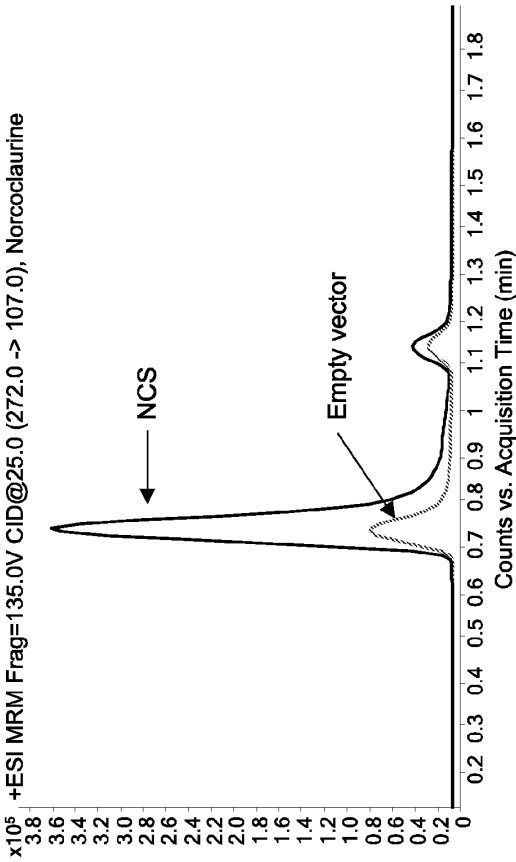


FIGURE 22

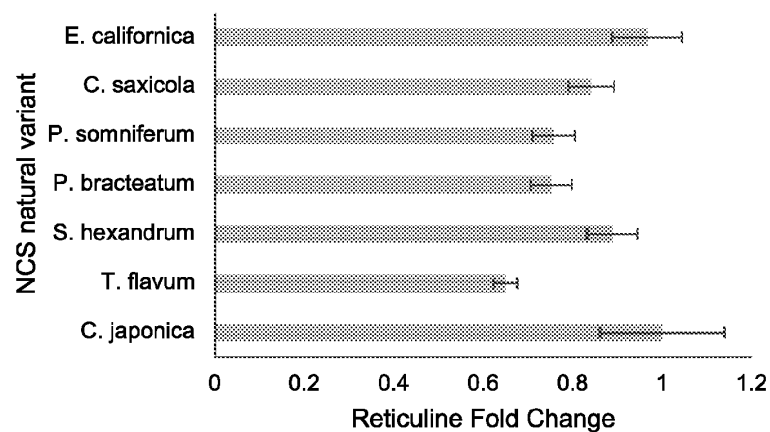
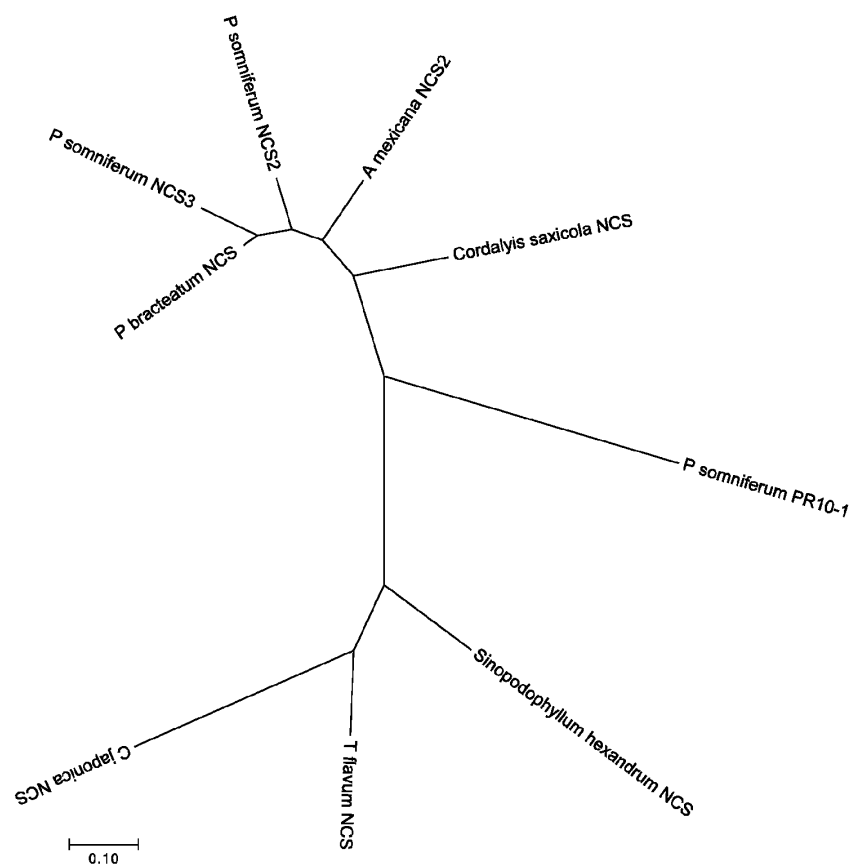


FIGURE 23

SUBSTITUTE SHEET (RULE 26)

CjNCS full length (SEQ ID NO:69)

12

15

22

24

26

28

32

34

36

40

↓

↓

↓

↓

↓

↓

↓

↓

↓

↓

1

61

121

181

MRMEVVLVVF

ELMFIGTINCERLI

FNGRPLLHRVTKEETV

MLYHELEVAASADEVWS

VEGS

PELGLHLPDLLPAGI

FAKFEITGDGGE

GSILDMTFPPGQ

FPHHYREKEVFFDHKNRY

KL

V

EQIDGDFDLGV

TYMDTIRVVATGPD

SCVIKSTTEYHVKPE

FAKIVKPLIDTV

PLA

IMS

EAI

AKVVLENKHK

SSE

60

120

180

196

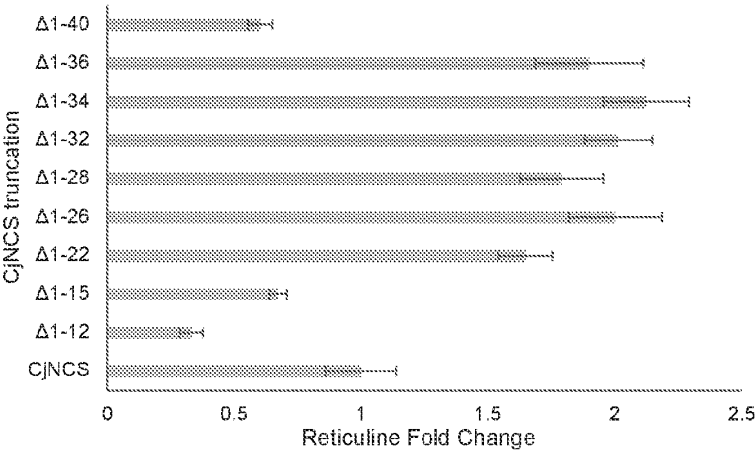


FIGURE 24

SEQ ID NO: 70

SEQ ID NO: 74

SEQ ID NO: 70

SEQ ID NO: 168

NCS parent

TNCS, PDB: 5N8Q

MNGRPLLHRYTKBETVMLYHELEVAASADEVWSVBGSPELGLHLPDLLPAGIFAKFEITG

-----TKVTHHELEVAASADDLWTVVSWPGLAKHLPDLLP-GAFKLEIIG

DGGEISILDMTFPPGQFFHHYREKFVFDHKNRYKLVEQIDGDFFDLGVTTYMDTIRVVA

DGGVGTILDMTFVPGEFPEYKEKFFILVDNEHRLKKVQMIEGGYLDLGVTTYMDTIHVVP

TGPDSCVIKSTTTEYHVKPEFAKIVKPLIDITVPLAINSEAIKVVLENKHKSSSE

TGKDSCVIKSTTEYHVKPEFVRIVEPLITTGFLAAMADAI SKLVLEHK

60

81

120

121

173

164

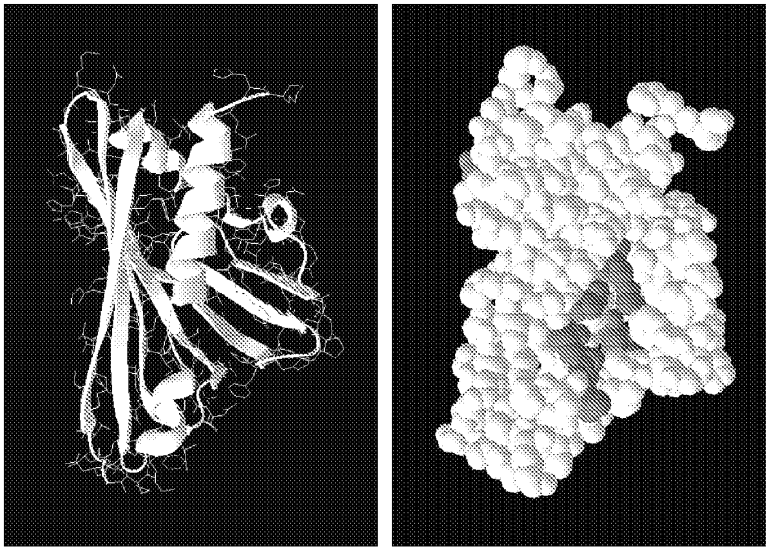


Figure 25

[illegible]

Figure 26

	X91	X101	X104
NCS parent	87	FFL	KNRRLV
CjNCS	110	FFL	KNRRLV
TfNCS	114	L	ENHRLK
AmNCS	102	K	INNEKRLREV
ShNCS	106	L	IDE
PbNCS	76	N	LVRLREV
PnNCS2	113	N	ENKRLREV
PnNCS3	75	N	ENKRLREV
CsNCS	88	K	ENHRLK
PSPR10-1	74	T	ENHRLK
		X147	X149
		X151	X155
NCS parent	147	DD	VPIATMS
CjNCS	170	DD	VPIATMS
TfNCS	174	DD	GPATMA
AmNCS	162	DD	VPIATMS
ShNCS	166	DD	VPIATMS
PbNCS	136	DD	TEPLOSMA
PnNCS2	173	DD	TEPLOSMA
PnNCS3	135	DD	TEPLOSMA
CsNCS	148	DD	TEPLOSMA
PSPR10-1	134	H	VESE

FIGURE 26 cont.

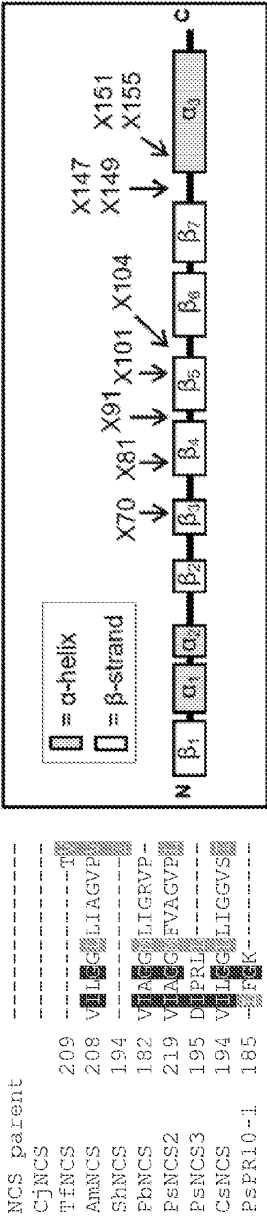


FIGURE 26 cont.

SEQ ID NO: 69 CjNCS full length
 SEQ ID NO: 70 NCS parent
 SEQ ID NO: 73 NCS3

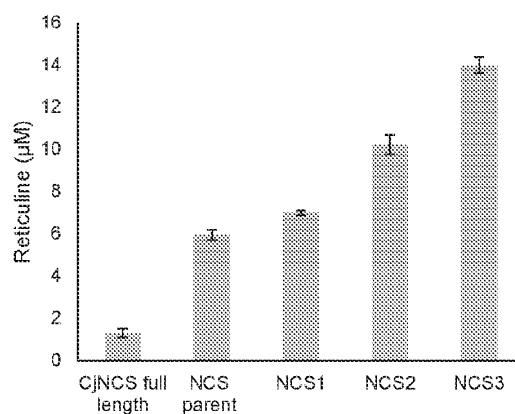
```

SEQ ID NO: 69 MRMEVVLVVFLMFIGTINCERLIFNGRPLLHRVTKEETVMLYHELEVAASADEVWSVEGS 60
SEQ ID NO: 70 M-----NGRPLLHRVTKEETVMLYHELEVAASADEVWSVEGS 37
SEQ ID NO: 73 M-----NGRPLLHRVTKEETVMLYHELEVAASADEVWSVEGS 37

61 PELGLHLPDLLPAGIFAKFEITGDGGEGSILDITFPPGQFPHHYREKFVFFDHKNRYKLV 120
38 PELGLHLPDLLPAGIFAKFEITGDGGEGSILDITFPPGQFPHHYREKFVFFDHKNRYKLV 97
38 PELGLHLPDLLPAGIFAKFEITGDGGEGSILDITFPPGQFPHHYREKFVFFDHKNRYKLV 97

121 EQIDGDFFDLGVTYMDTIRVVATGPDSCVIKSTTEYHVKPEFAKIVKPLIDTVPLAIMS 180
98 EQIDGDFFDLGVTYMDTIRVVATGPDSCVIKSTTEYHVKPEFAKIVKPLIDTVPLAIMS 157
98 EQIDGDFFDLGVTYMDTIRVVATGPDSCVIKSTTEYHVKPEFAKIVKPLIDTVPLAIMS 157

181 EAIKVVLENKHKSS 196
158 EAIKVVLENKHKSS 173
158 EAIKVVLENKHKSS 173
  
```



	NCS variant	Description
SEQ ID NO: 69	CjNCS full length	Native sequence, yeast codon optimized
SEQ ID NO: 70	NCS parent	Δ1-24
SEQ ID NO: 71	NCS1	Δ1-24, I155N
SEQ ID NO: 72	NCS2	Δ1-24, I155N, D149T
SEQ ID NO: 73	NCS3	Δ1-24, I155N, D149T, M70I

FIGURE 27

SUBSTITUTE SHEET (RULE 26)

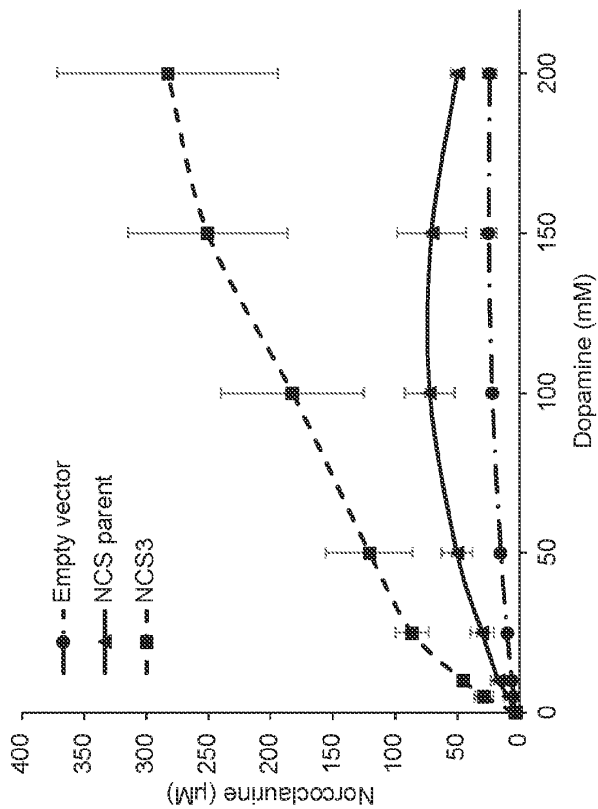


Figure 28

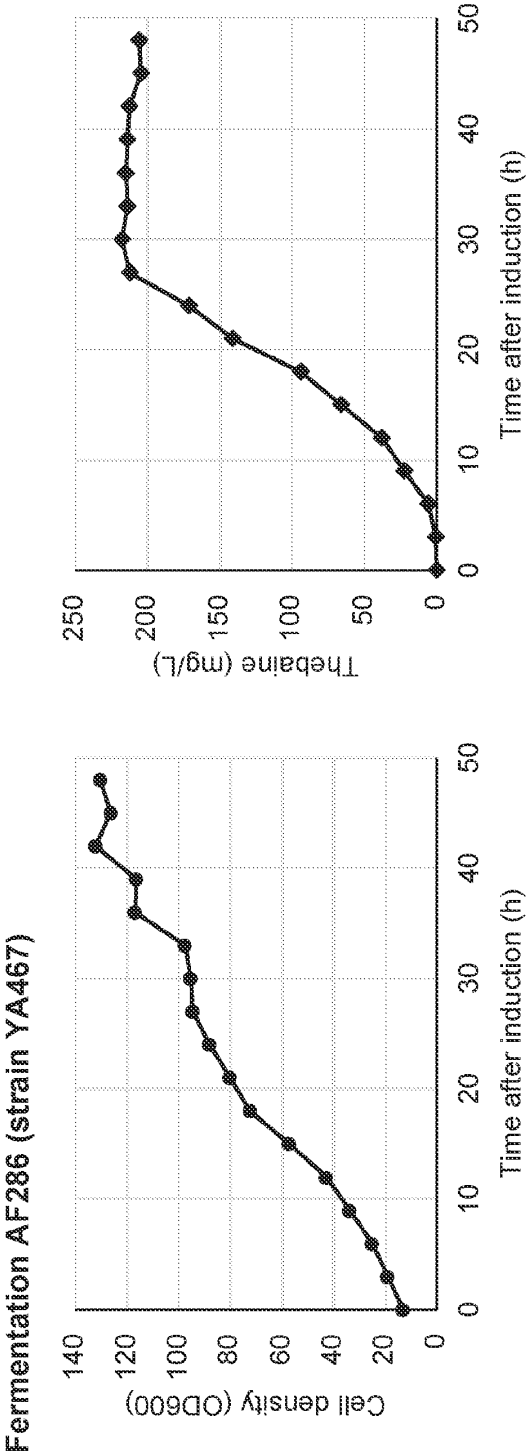
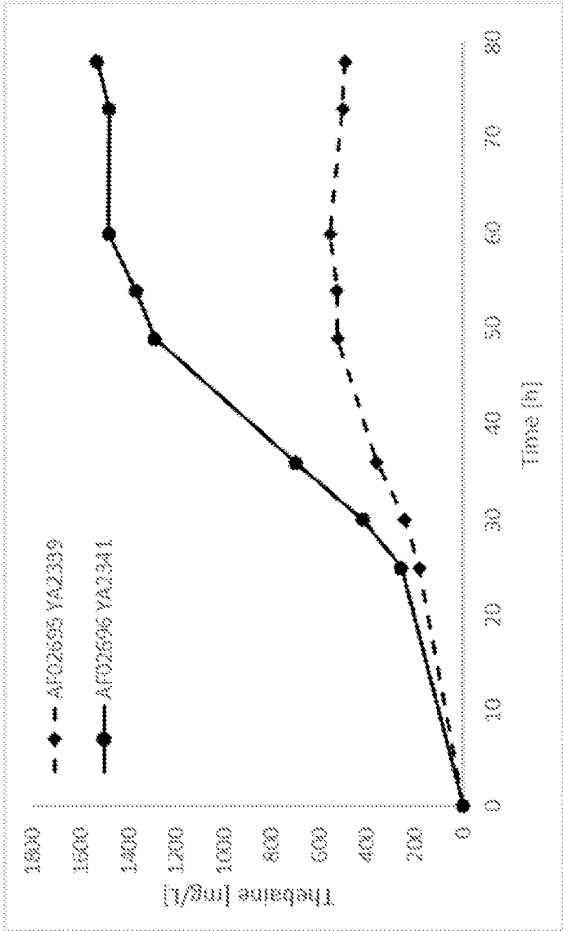


Figure 29



Thebaine titer of fermentation runs of isogenic strains containing one copy of the wild-type *C. japonica* NCSΔ24 (YA2339, AF02695) or the mutant NCS3 (YA2341, AF02696).

Figure 30

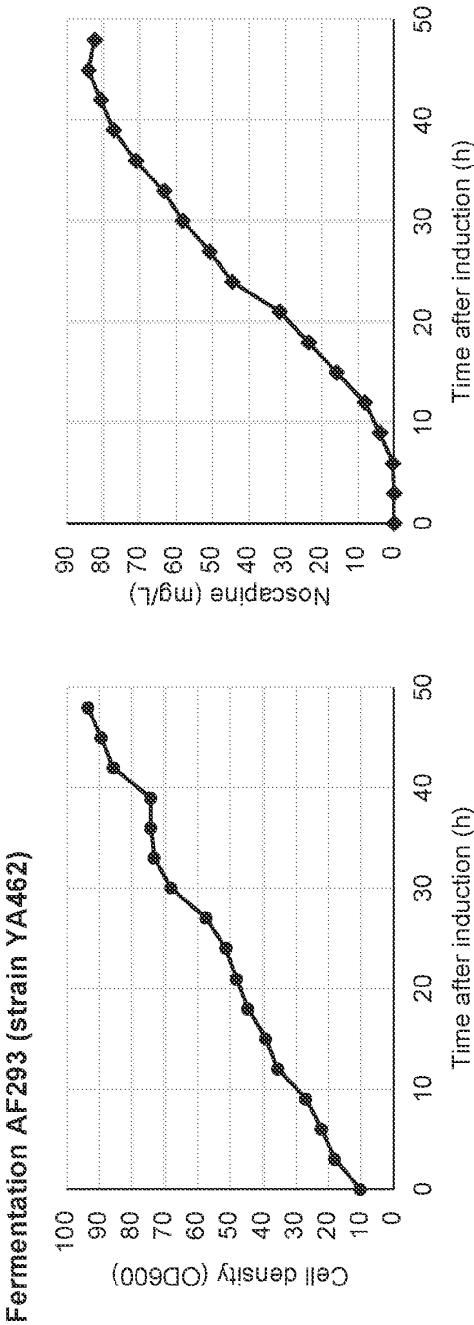


Figure 31

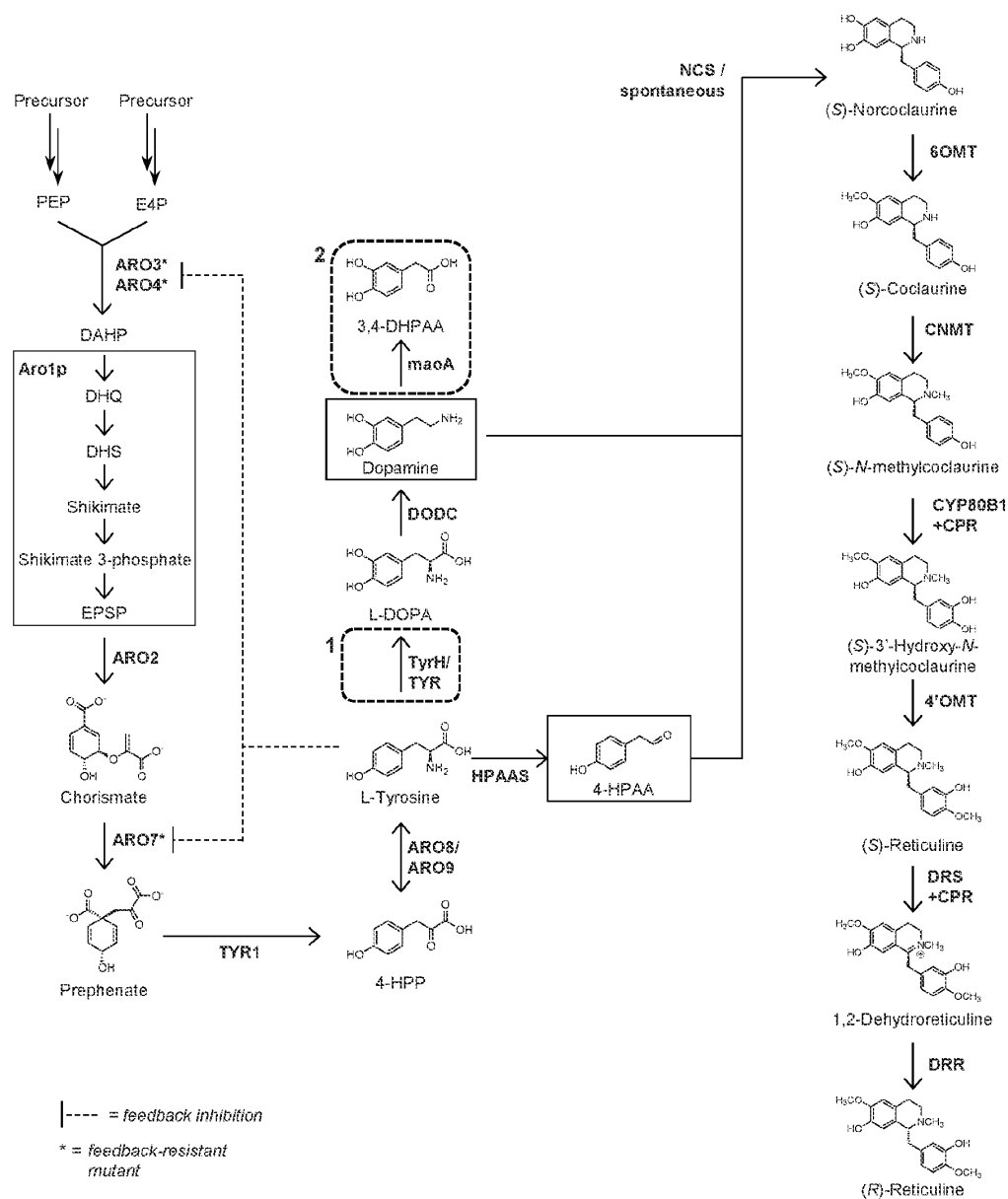
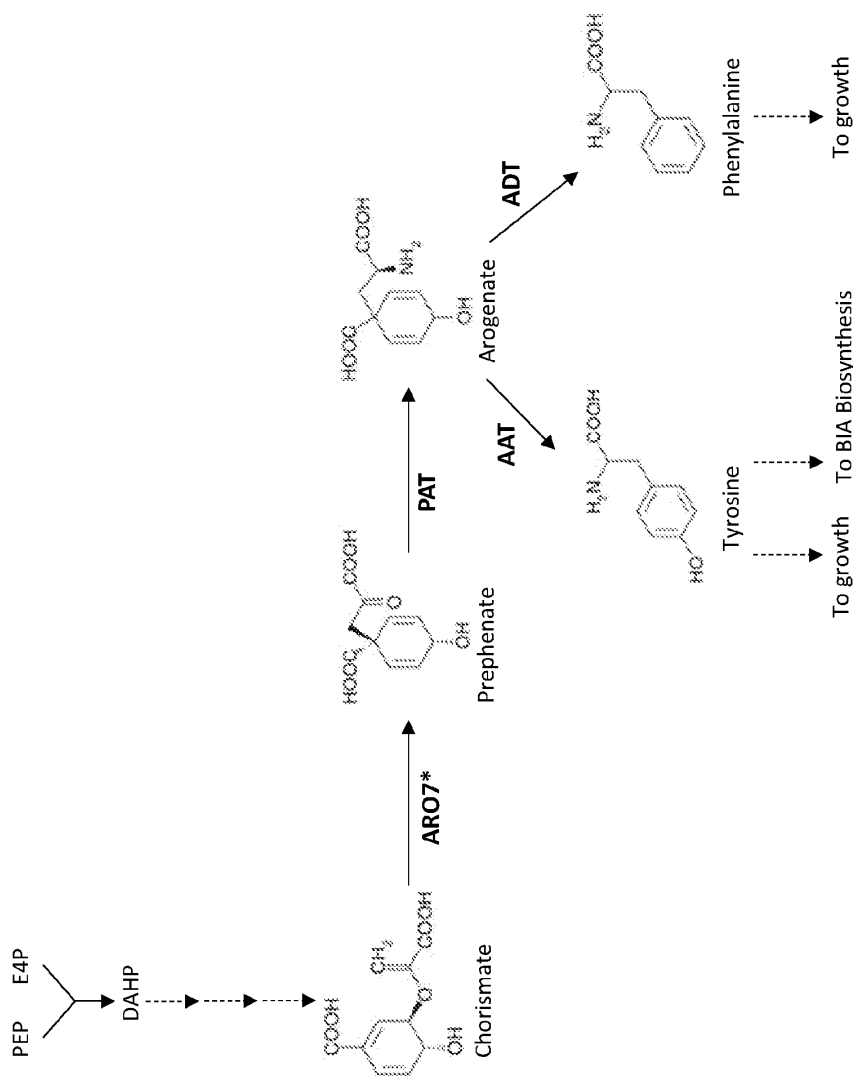


Figure 1 with
aro10^Δ::HPAAS

FIGURE 32

SUBSTITUTE SHEET (RULE 26)



The heterologous pathway for tyrosine (and phenylalanine) that goes through the arogenate intermediate and allows deletion of the native yeast genes TYR1, PHA2, ARO8, and ARO9.

Figure 33

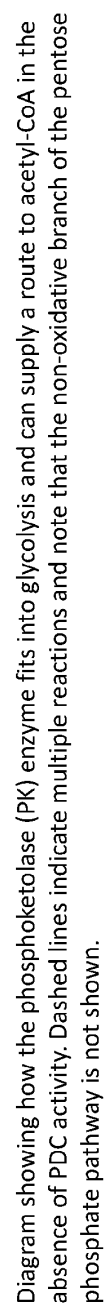


Figure 34

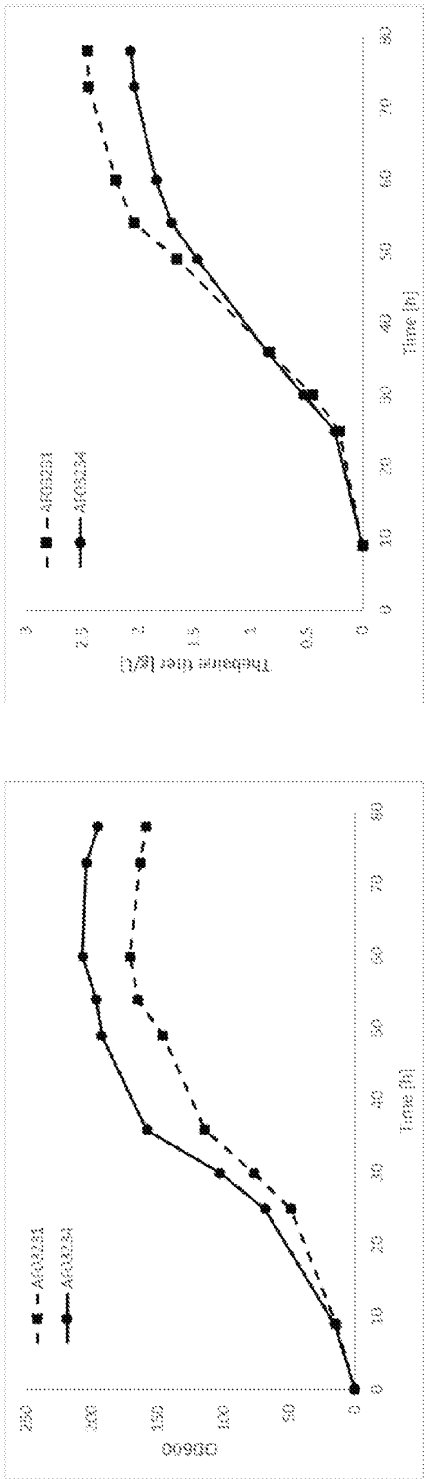


FIGURE 35

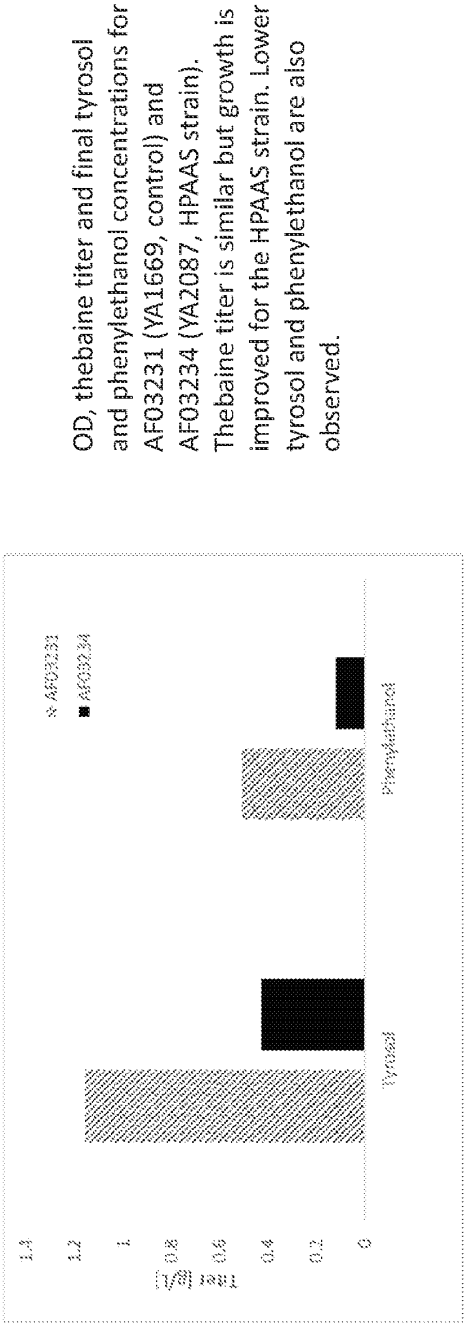
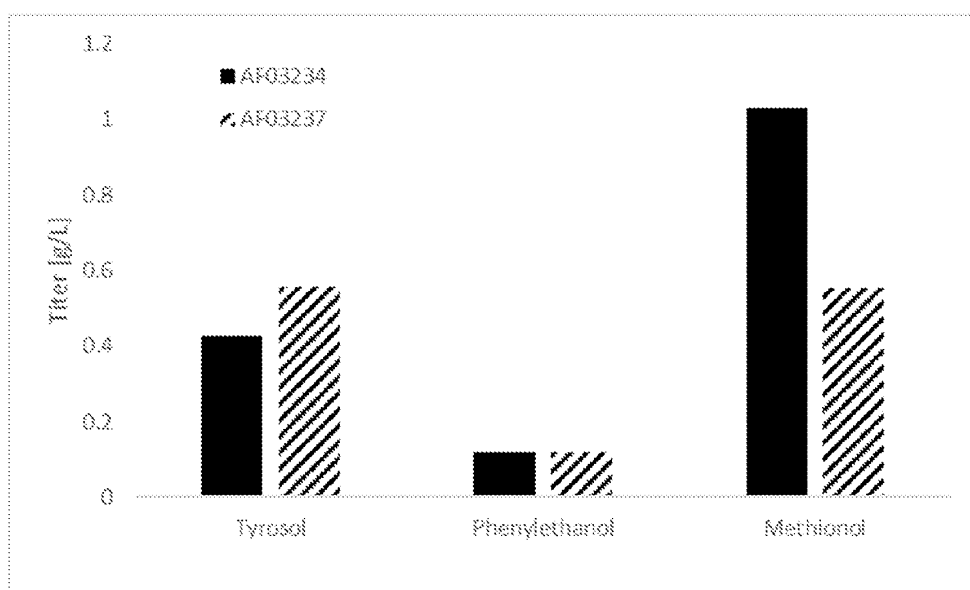
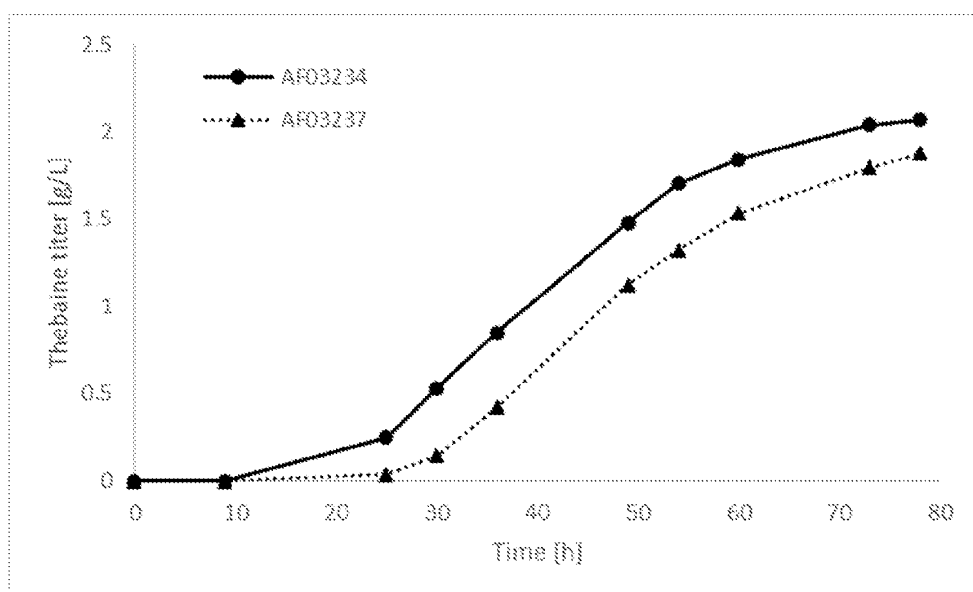
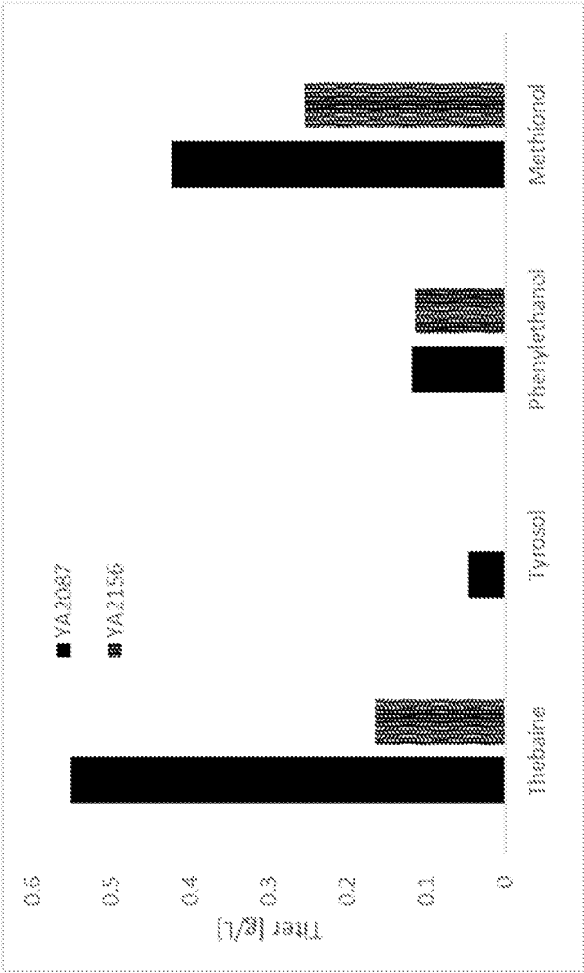


FIGURE 35 cont.



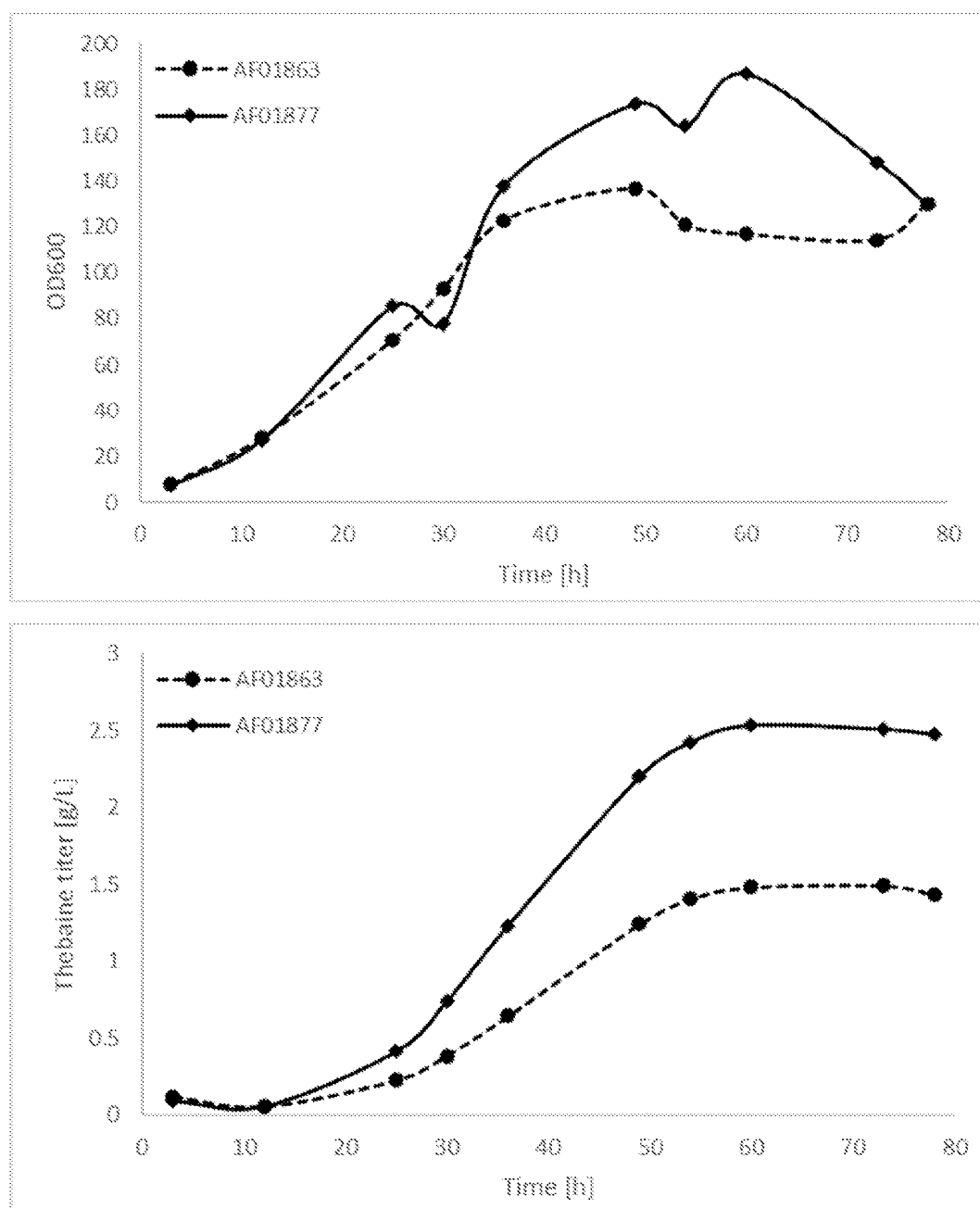
Thebaine titer and final tyrosol, phenylethanol, and methionol concentrations of AF03234 (YA2087, parent) and AF03237 (YA2696, arogenate strain) in a fed-batch fermentation process. Thebaine production was similar but methionol was significantly lower for the arogenate strain due to deletion of ARO8 and ARO9.

FIGURE 36



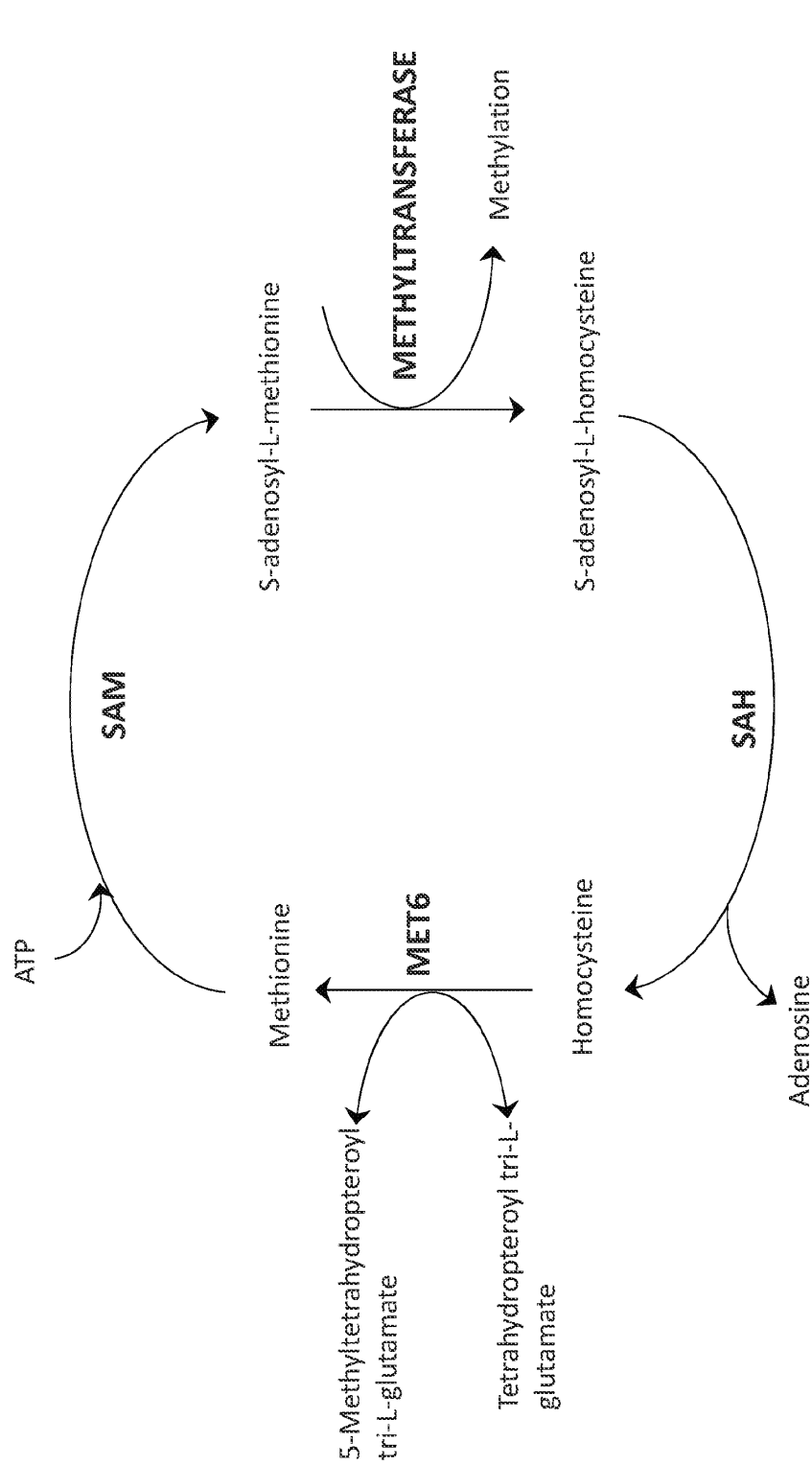
Final thebaine titer, tyrosol, phenylethanol, and methionol from a shake flask experiment comparing YA2087 (parent) to YA2156 (*pdc4* + PK).

Figure 37



OD and thebaine titer of a fed-batch fermentation process comparing the parent strain YA1158 (AF01863) to the improved strain with additional copies of SAH1 and SAM2 YA1669 (AF01877).

FIGURE 38



The methionine cycle illustrating the reactions performed by SAM and SAH and how they relate to the methyltransferases in the BIA pathway.

FIGURE 39

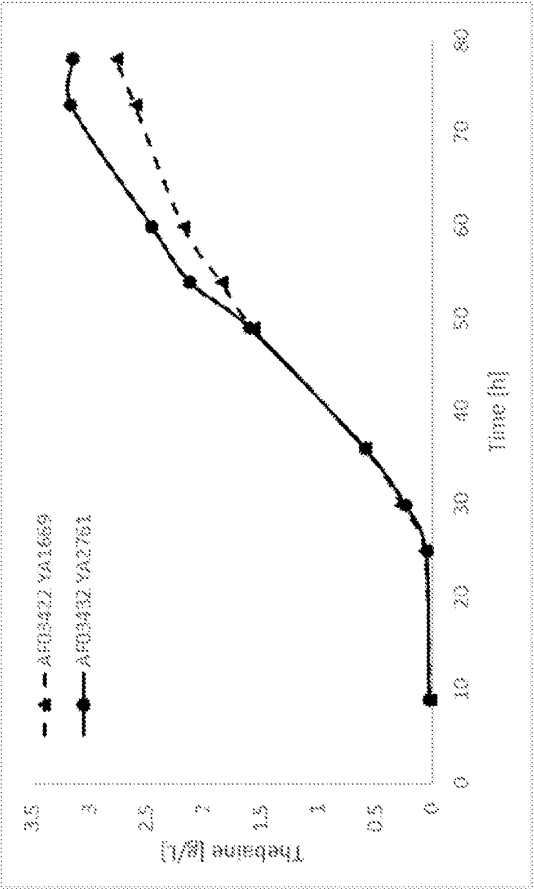
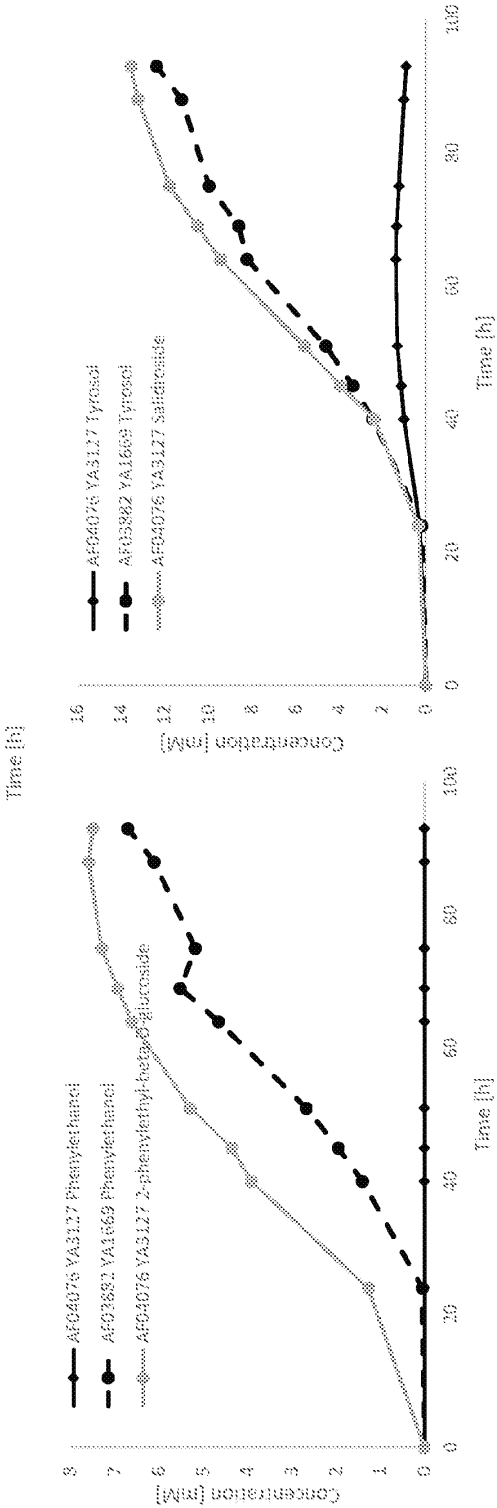


Figure 40



Top: Thebaine titer of YA1669 (parent) and YA2761 expressing UGT33. Bottom left: Phenylethanol and phenylethyl-beta-D-glucoside (PG) concentrations of YA1669 and YA2761. Bottom right: Tyrosol and salidroside concentrations of YA1669 and YA2761. The UGT enzyme converts ~1/2 of the phenylethanol to PG resulting in a 20% increase in thebaine titer.

FIGURE 40 cont.