Supporting Information for:

Traveling two diverging roads, cytochrome-P450 catalyzed demethylation and γ-lactone formation in bacterial gibberellin biosynthesis

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Materials and Methods

Cloning of EtCYP112 and EtFdR

*Et*CYP112 was amplified from genomic DNA of *E. tracheiphila* with Q5 Hot Start High-Fidelity DNA polymerase (NEB), using the primers given in Table S2. The forward primer was designed to add an alanine, six histidines, and a glycine after the initial methionine to the N-terminus for purification of the protein. The reverse primer included a stop codon at the 3'-end so that the His-Tag of pET101 (Invitrogen) is omitted after cloning the PCR product into the vector according to the manual. Clones were verified by full-gene sequencing. The ferredoxin reductase of *E. tracheiphila* (*Et*FdR) was amplified with Q5 Hot Start High-Fidelity DNA polymerase, using the primers given in Table S2, and the amplified fragment was then cloned into pET100 (Invitrogen).

Expression and Purification of EtCYP112 and EtFdR

*Et*CYP112 or *Et*FdR were transformed into *E. coli* BL21* (Invitrogen) for expression. The starter culture of 50 ml liquid NZY medium (10 g/L NaCl, 10 g /L casein, 5 g/L yeast extract, 1 g/L $MgSO_4$ (anhydrous), pH 7.0) with 50 µg/mL carbenicillin was inoculated from 3 individual colonies and grown for 2 days at 18°C under constant shaking at 200 rpm. 25 mL were used to inoculate 1 L of NZY including 50 µg/mL carbenicillin at 18°C and constant shaking at 200 rpm until an OD_{600} of 0.8 was reached. At this point the culture was induced with 1 mM IPTG and, for cultures expressing *Et*CYP112, 1 mM aminolevulinic acid, 1 mM riboflavin and 0.1 mM $FeCl_3$ were added. After 36 hours, the cells were harvested by centrifugation at 5000 x *g* for 20 min. The *Et*CYP112 cell pellet was re-suspended in 10 mL buffer 1 (25 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), pH 7.2, 10% (v/v) glycerol), while the *Et*FdR cell pellet was re-suspended in 10 mL of buffer 2 (50 mM phosphate, pH 7.5, 10% glycerol). Both cell suspensions were then homogenized using an EmulsiFlex C-3 (Avestin, Canada). The lysate was centrifuged at 17,000 x *g* for 30 min. The cleared lysate was then added to 1 mL of Ni-NTA Agarose (Qiagen), buffer 1 or 2 with 1 M imidazole was added to a final concentration of 20 mM imidazole and

incubated with gentle shaking at 4°C for 1 h. The column was washed with 5 mL buffer 1 or 2 containing 20 mM imidazole and 60 mM imidazole each, before being eluted with buffer 1 or 2 with 250 mM imidazole. The protein was then transferred to dialysis tubing with a molecular weight cutoff of 15 kDa and dialyzed 3 times against buffer 3 (10 mM MOPSO, pH 7.2, 5% (v/v) glycerol) in the case of *Et*CYP112, or buffer 4 (25 mM phosphate, pH 7.5, 10% glycerol) in the case of *Et*FdR. The resulting enzymes were either used fresh or stored at -80°C after flash freezing by brief immersion in liquid nitrogen of 0.5 mL aliquots in 1.5 mL Eppendorf tubes.

Enzyme assays

For activity assays 0.01 ml each of purified *Et*CYP112, spinach ferredoxin (Sigma-Aldrich), and *Et*FdR (all at 1 mg/mL in buffer 1) were diluted with 1.5 mL of buffer 3 in 4 mL clear glass vials, and 20 µM of the specified substrate and 60 µM NADPH were added. The vial was sealed with a Teflon septum and incubated for 12 h at 25 °C. For $^{18}O_2$ labeling experiments clear 4 mL glass vials with a Teflon seal were flushed with nitrogen for 5 min then 0.05 mL each of purified *Et*CYP112, spinach ferredoxin (Sigma-Aldrich), and *Et*FdR (all at 1 mg/mL in buffer 1), and 1.7 mL degassed buffer 1 were added using a syringe. $^{18}O_2$ (Sigma-Aldrich, 97% labeled; max loaded pressure less than 2.4 bar) was added from the gas cylinder via a needle puncturing the septum until the pressure equilibrated between the tank and the vial. The reaction was started with the addition of substrates to a final concentration of 40 µM. NADPH was added in 3- or 10- fold excess relative to substrate in assays with GA_{12}, with 2-fold excess in assays with GA_{15} and at equal concentrations in assays with GA_{24}. The assay was incubated at 25 °C for 16 h. When H_2O_2 or *tert*-butyl hydroperoxide was used as the reduction agent and oxygen donor, no NADPH, *Et*FdR, or Fd were added, instead H_2O_2 was added to a final concentration of 60 µM or *tert*-butyl hydroperoxide to a final concentration of 100 µM. The products of all assays were extracted three times with 2 ml ethyl acetate, the pooled extract evaporated to dryness, and the residue methylated with diazomethane and, after re-evaporation, dissolved in hexane for GC-MS analysis as described in (4). Assays from $^{18}O_2$ labeling experiments were analyzed with an Agilent 6540 GC coupled to a Waters GCT Premier mass spectrometer, separation was achieved on a DB-5MS column (30 m, 250 µm, 0.25 µm) with the same gradient as described in (4) . When GA_{12}-aldehyde was used as a substrate, two-thirds of the extracted products were separated and, after evaporation, dissolved in 300 µL of methanol:DMSO (1:1, v/v) without methylation. This extract was added to a culture expressing the SDR from the GA operon in *E. tracheiphila* and, after incubation for 3 days, extracted and analyzed with a 3900 Saturn GC coupled to a 2100T ion trap mass spectrometer (Varian, Palo Alto, CA, USA) as described (4). GC-MS spectra of GA_9 differed in the abundance of higher molecular weight fragments between the GC-MS ion trap and GC-MS time of flight.

$C^{18}O_2$ measurement by GC-MS

The reaction was carried out as described under enzyme assays with NADPH added in 10-fold excess, and the assay incubated for 4 h at 25°C. 200 µL of the gas phase were manually injected into the inlet of a GC-MS, 3900 Saturn GC coupled to a 2100T ion trap mass spectrometer (Varian, Palo Alto, CA, USA), using a gas-tight syringe. A HP-PLOT/Q column (30 m x 0.32 mm x 20 µm) (Agilent) was used for separation with a constant He flow of 4 mL min^{-1}, the inlet temperature was set at 250 °C and the oven temperature was initially kept at 50 °C for 2 min, the temperature was then increased by 90 °C/min to 240 °C, where it was held for 1 min.

Supplemental Tables

Table 1: Incorporation of $^{18}O_2$ by *Et*CYP112 into various substrates.

% abundance of ^{18}O label

Analyte		Substrates						
		GA_{12}	**GA_{12}**	**GA_{15} (closed)**	**GA_{15} (open)**	**GA_{24}**	**$MeGA_{12}$**	**$MeGA_{15}$**
	[M^+]	**excess NADPH**	**limited NADPH**	**limited NADPH**	**limited NADPH**	**limited NADPH**	**limited NADPH**	**limited NADPH**
GA12	360							
	0	n.d.	99 ± 0.5				99 ± 0.2	
	+2	n.d.	1 ± 0.2				1 ± 0.1	
GA15	344							
	0	n.d.	3± 1	98 ± 1	98 ± 1.5		1 ± 0.5	98 ± 1
	+2	n.d.	96 ± 2	2 ± 1	2 ± 1		98 ± 1	2 ± 0.6
	+4	n.d.	1 ± 0.4				1 ± 0.1	
GA24	374							
	0	n.d.	3 ± 1	3 ± 0.5	32 ± 1.5	99 ± 0.1	0	2 ± 0.3
	+2	n.d.	60 ± 0.4	95 ± 1	67 ± 1.5	1 ± 0.1	1 ± 0.1	96 ± 1
	+4	n.d.	37 ± 0.5	2 ± 1	1 ± 0.4		99 ± 0.6	2 ± 0.4
GA9	330							
	0	56 ± 1	86 ± 0.5	98 ± 0.3	99 ± 0.5	98 ± 1.5	1 ± 0.2	99 ± 0.1
	+2	44 ± 1	14 ± 0.3	2 ± 0.2	1 ± 0.3	2 ± 1	99 ± 0.4	1 ± 0.1

n.d., not detectable

Table S2: Primers used in cloning.

Primer name	Primer Sequence
E. tracheiphila FdR forward	CACCATGGCCGAATGGATTAATGCAAGCATC
E. tracheiphila FdR reverse	TTACCAATAGTGTTCGCTGGTCATATGCC
E. tracheiphila CYP112 forward	CACCATGGCGCATCATCATCATCATCATGGTTTTGAAAATAACCCAGTGCAGC
E. tracheiphila CYP112 reverse	TTACAGATACACGGGAAACTTCTCGAATCCGC

Supplemental Figures

Figure S1

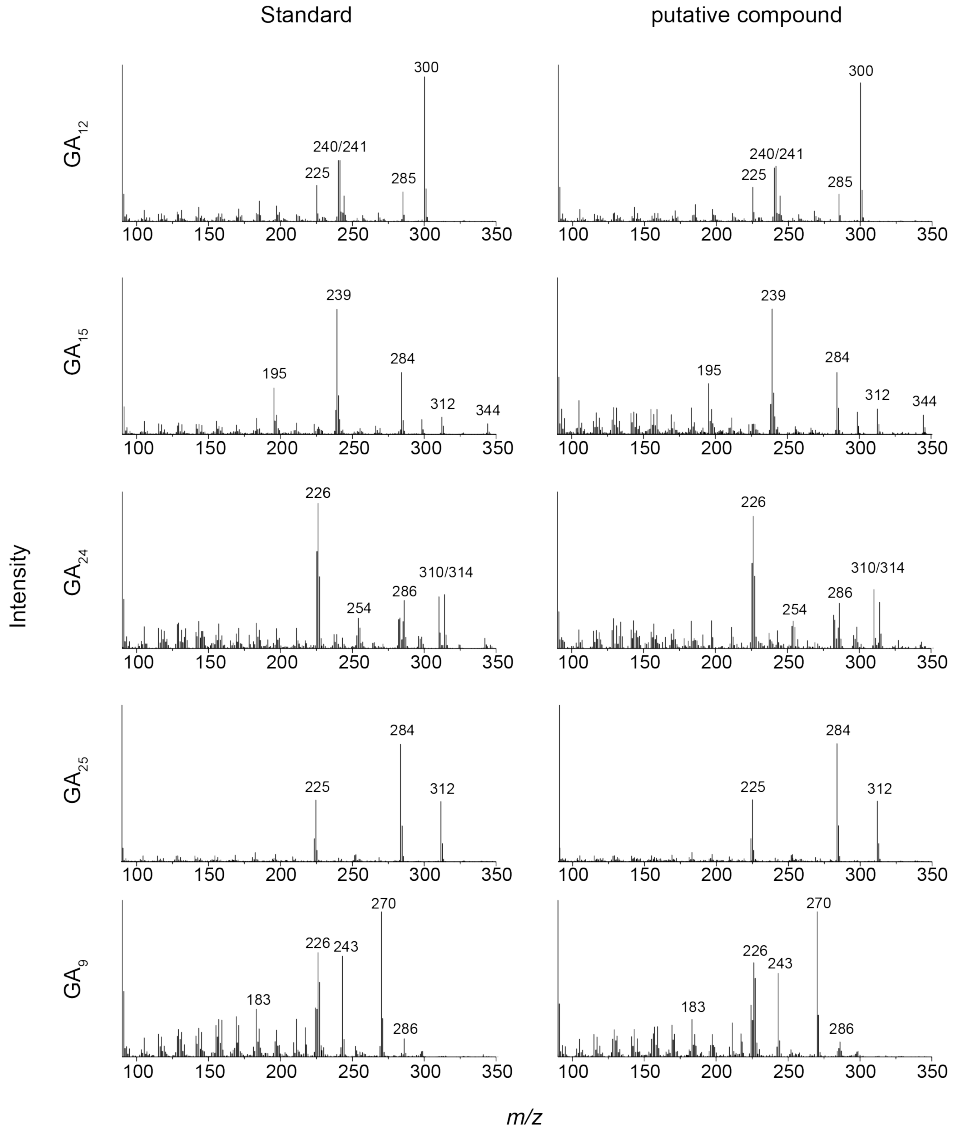


Figure S1: Comparison of MS-Spectra of *Et*CYP112 gibberellin products with authentic standards. MS spectra of authentic standards and compounds produced by *Et*CYP112 in *in vitro* enzyme assays.

Figure S2

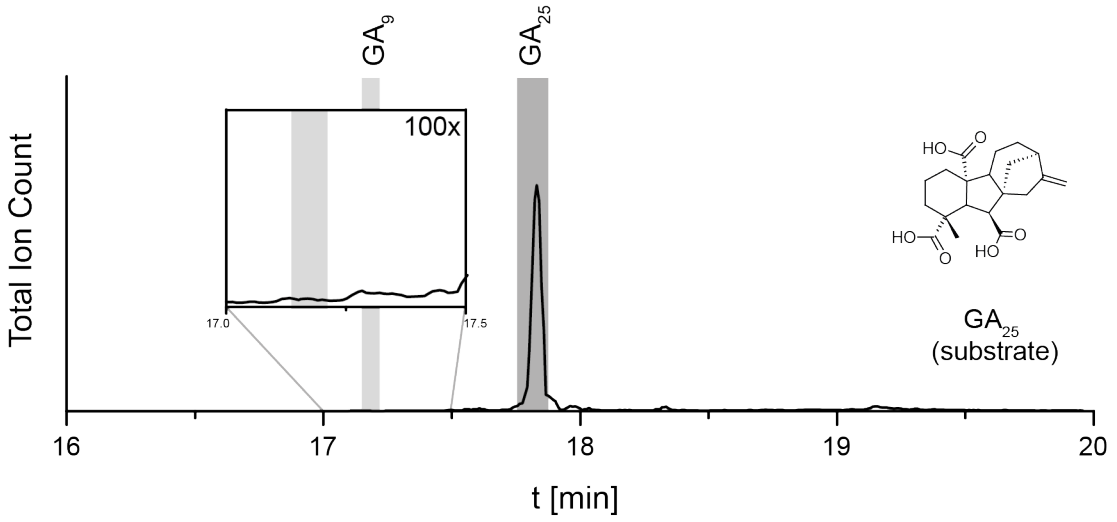


Figure S2: Enzyme activity of *Et*CYP112 with GA_{25}. GC-MS-chromatograms of *in vitro* assays with *Et*CYP112 and GA_{25}, the inset is an enlargements of the y-axis of the chromatogram by 100 fold.

Figure S3

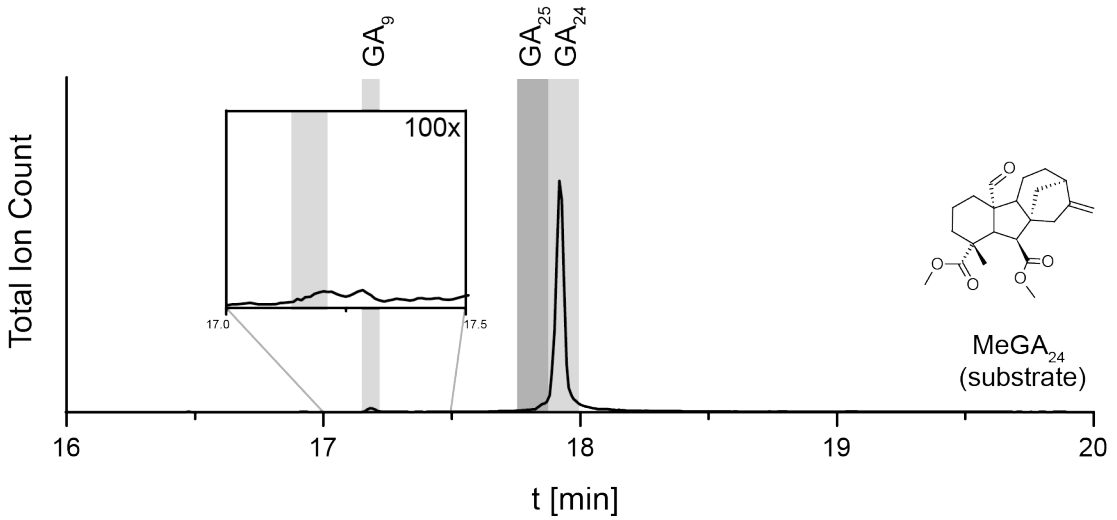


Figure S3: Enzyme activity of *Et*CYP112 with $MeGA_{24}$. GC-MS-chromatograms of *in vitro* assays with *Et*CYP112 and $MeGA_{24}$, the inset is an enlargement of the y-axis of the chromatogram by 100-fold.

Figure S4

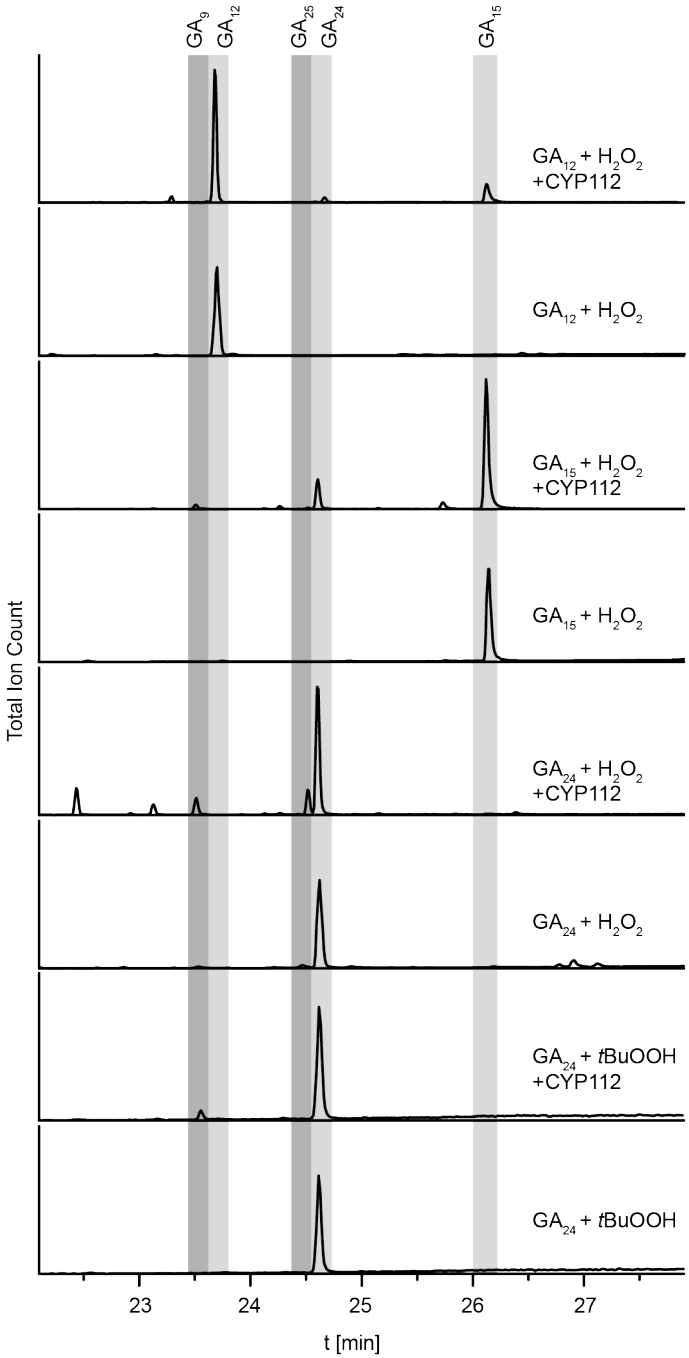
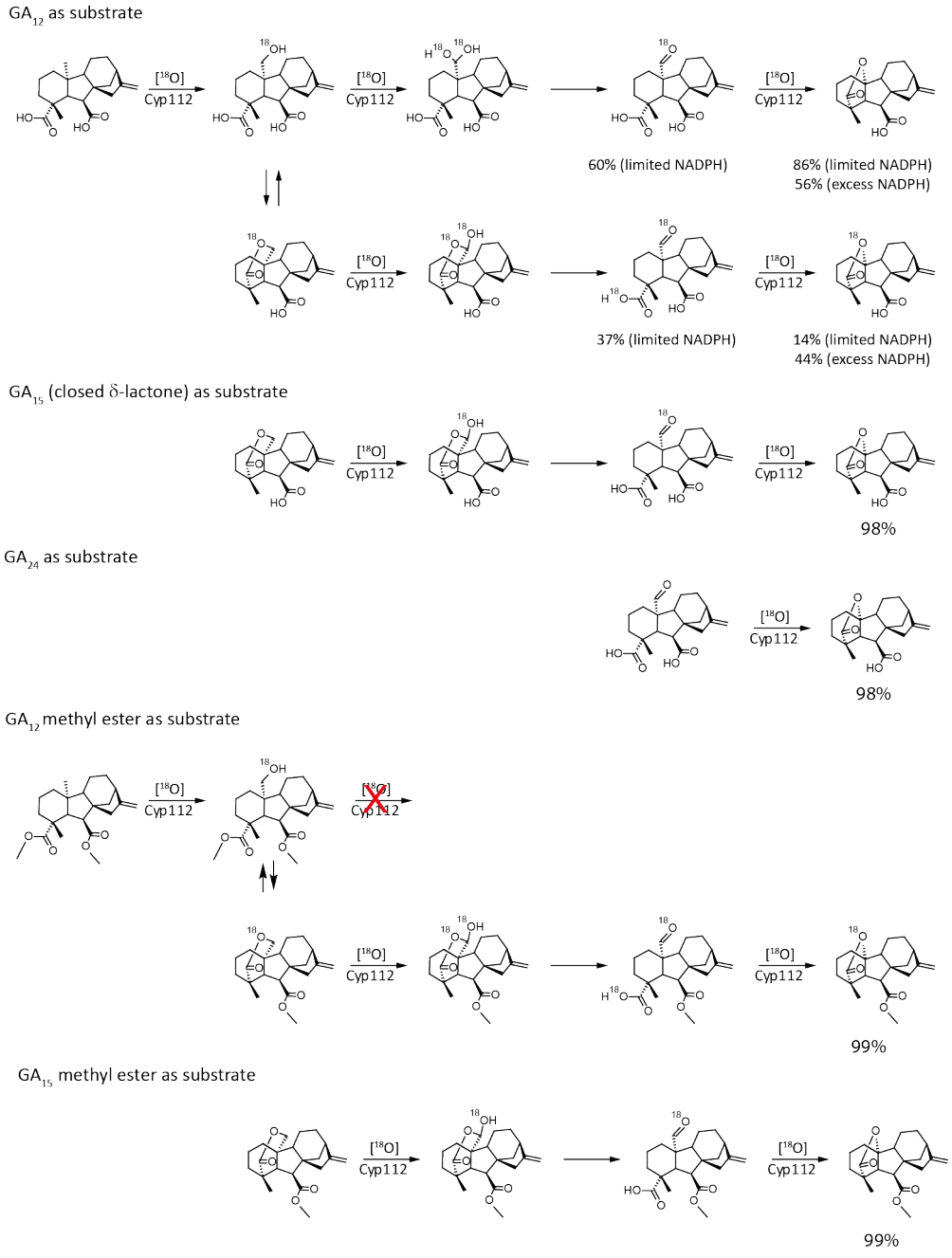


Figure S4: Enzyme activity of *Et*CYP112 employing the H_2O_2-shunt. GC-MS-chromatograms of in vitro assays with *Et*CYP112 and GA_{12}, GA_{15} or GA_{24} or non-enzyme controls with H_2O_2 or *tert*-butyl hydrogenperoxide (*t*BuOOH) ,for GA24 only, as the oxygen and electron donor, instead of O_2 and electrons supplied by a ferredoxin.

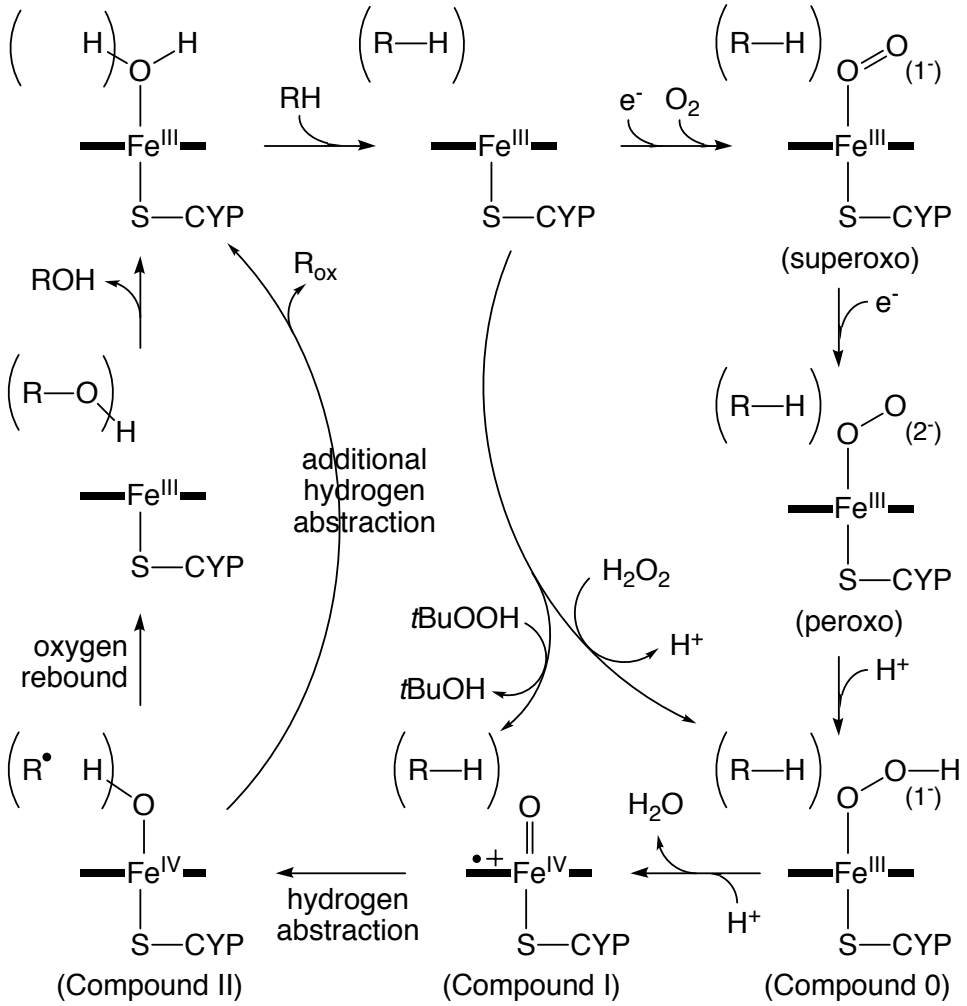
Supplemental Schemes

Scheme S1



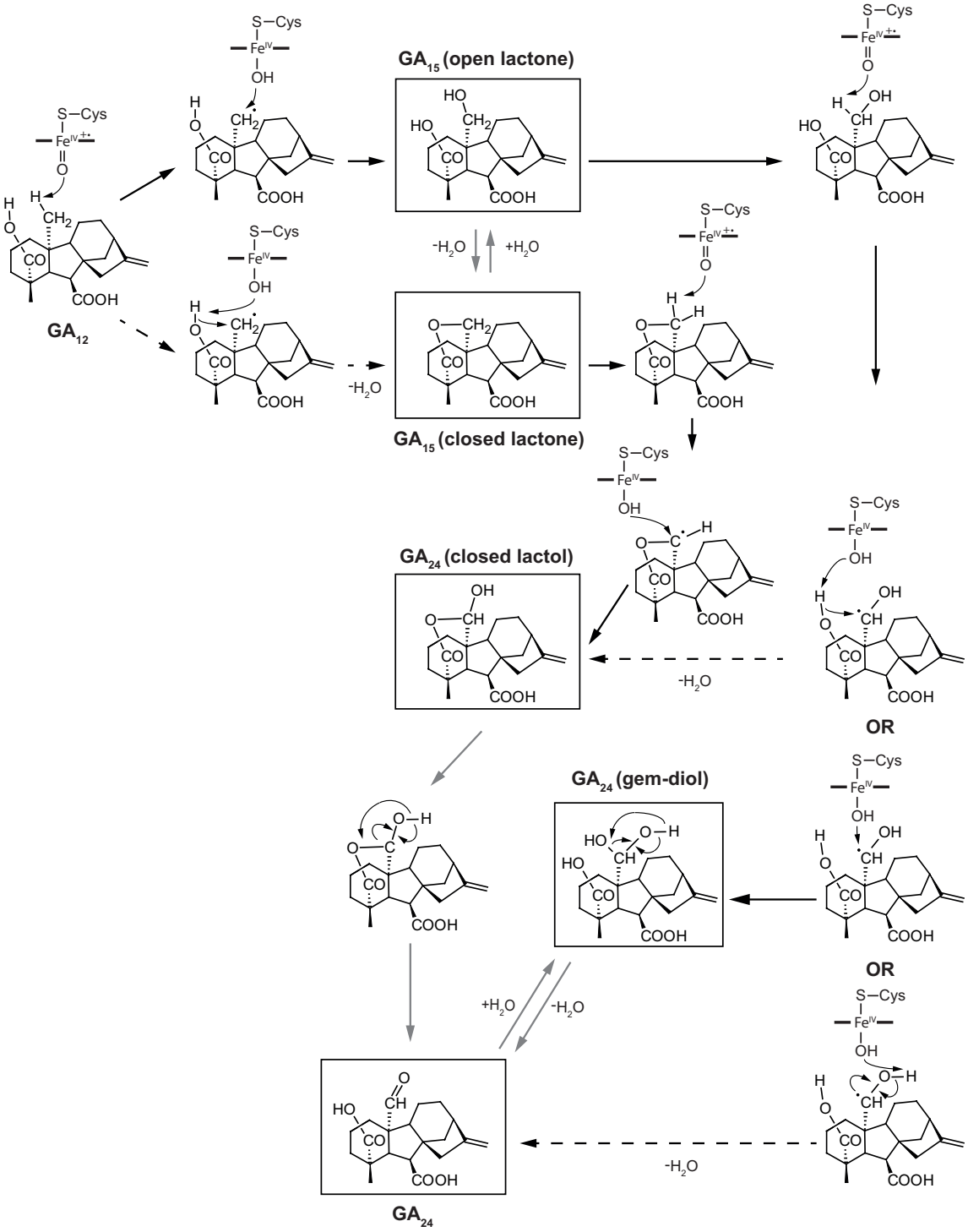
Scheme S1: Position of 18O labels. Schemes depicting the position of the ^{18}O label, from GA_{12}, GA_{15}, GA_{24}, $MeGA_{12}$ and $MeGA_{15}$ into GA_9 or $MeGA_9$.

Scheme S2



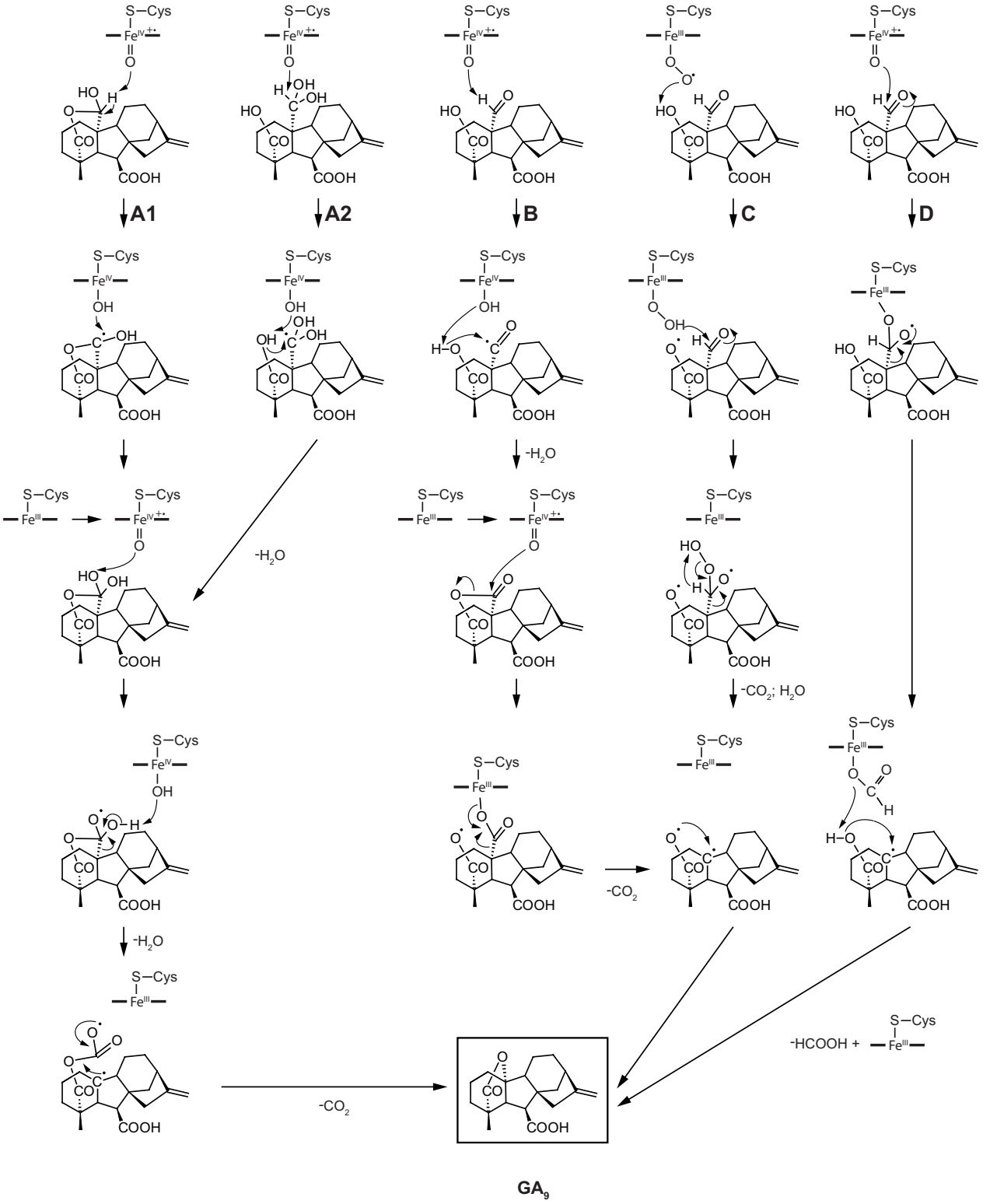
Scheme S2: CYP reaction cycle showing active complexes, use of hydrogen peroxide (H_2O_2) or *tert*-butyl hydroperoxide (*t*BuOOH), and alternative reactivity for Compound II discussed in the text (largely adapted from Krest et al, 2013).

Scheme S3



Scheme S3: Proposed reaction mechansims from GA_{12} to GA_{24}. Potential reactions mechanisms modified after MacMillan (1997) for the transformation of GA_{12} to GA_{24}. Solid arrows in black are enzymatic reactions catalyzed by *Et*CYP112, solid grey arrows are non-enzymatic reactions and dashed black arrows are reactions not catalyzed by *Et*CYP112.

Scheme S4



Scheme S4: Proposed reaction mechansims from GA_{24} to GA_9. Potential reaction mechanisms modified after MacMillan (1997) for the transformation of GA_{24} to GA_9. Pathway A2 was not previously proposed by MacMillan. C-20 is released as CO_2 by pathways A1 A2, B and C, while pathway D releases C-20 as formic acid.