Supporting Information

Materials

Chemical reagents were purchased from Sigma-Aldrich, Ajax Finechem and Chem-Supply. Guaiacol and syringol were gifts from Prof. Kerry Wilkinson, University of Adelaide. Enzymes, biochemical and molecular biology reagents were supplied by Sigma-Aldrich, New England Biolabs and Thermo Fisher Scientific. *S*ubstrate and product stocks were prepared in DMSO. IS (internal standard) stock solutions were prepared in EtOH.

Gene cloning

A codon optimized gene coding for GcoA (based on the NCBI reference sequence WP_027930080; *Amycolatopsis thermoflava*) was purchased as a gBlock from IDT. An *Nde*I restriction site was incorporated at the 5' end (underlined), followed by a double stop codon (italics) and *Kpn*I and *Hind*III restriction sites (underlined).

GcoA Gblock sequence

atttta<u>catATG</u>ACGACGACGGAGCGCCCTGATTTGGCCTGGTTAGATGAAGTTACTATGACACAACTGGAGCGTAATCCAT
ACGAAGTGTACGAACGCTTGCGTGCAGAAGCACCTCTTGCATTCGTACCTGTCTTAGGATCGTACGTAGCGTCGACCGCAGA
GGTTTGTCGCGAGGTCGCAACGTCGCCCGACTTTGAGGCTGTTATCACGCCCGCTGGGGGCCGCACATTCGGCCATCCCGCC
ATTATCGGCGTTAACGGTGACATTCACGCTGACCTTCGCAGCATGGTGGAGCCAGCTCTTCAACCCGCCGAAGTTGATCGTT
GGATTGACGATCTTGTCCGCCCTATCGCTCGCCGTTATCTGGAGCGTTTCGAGAACGACGGGCACGCTGAATTGGTTGCGCA
ATATTGCGAGCCGGTATCTGTGCGCTCCCTTGGCGATCTGCTTGGGCTGCAGGAGGTCGACTCCGACAAGTTACGCGAGTGG
TTCGCAAAGTTAAACCGTAGTTTTACTAACGCTGCGGTTGATGAGAACGGGGAGTTCGCAAACCCGGAGGGATTTGCAGAAG
GGGATCAGGCGAAGGCCGAAATTCGCGCTGTTGTCGACCCTTTAATTGACCGTTGGATTGAGCACCCCGATGATTCAGCGAT
TAGTCACTGGCTGCACGATGGGATGCCTCCGGGCCAAACGCGTGACCGCGAATATATTTATCCCACAATCTATGTCTATCTT
CTTGGCGCGATGCAGGAACCAGGGCATGGAATGGCATCAACTCTGGTCGGGTTATTCTCCCGTCCTGAGCAACTTGAAGAGG
TCGTCGATGATCCAACTTTAATCCCGCGCGCGATCGCAGAGGGGCTGCGTTGGACATCCCCTATCTGGTCTGCAACAGCCCG
TATCTCAACGAAGCCTGTTACAATTGCGGGTGTCGACCTGCCCGCTGGTACACCCGTGATGCTGAGCTATGGAAGCGCCAAC
CATGATACGGGAAAGTATGAAGCCCCCAGCCAGTATGATCTTCATCGTCCTCCTCTTCCCCACTTGGCTTTCGGAGCTGGCA
ATCATGCGTGTGCTGGCATTTACTTTGCAAATCACGTCATGCGCATTGCCTTAGAGGAGTTATTCGAAGCTATTCCGAACTT
GGAACGCGACACCCGTGAAGGGGTAGAGTTTTGGGGCTGGGGCTTTCGCGGACCTACCTCATTACATGTCACGTGGGAGGTG
TAATAG<u>GGTACCAAGCTT</u>ttaatt

GcoA protein sequence

MTTTERPDLAWLDEVTMTQLERNPYEVYERLRAEAPLAFVPVLGSYVASTAEVCREVATSPDFEAVITPAGGRTFGHPAIIG
VNGDIHADLRSMVEPALQPAEVDRWIDDLVRPIARRYLERFENDGHAELVAQYCEPVSVRSLGDLLGLQEVDSDKLREWFAK
LNRSFTNAAVDENGEFANPEGFAEGDQAKAEIRAVVDPLIDRWIEHPDDSAISHWLHDGMPPGQTRDREYIYPTIYVYLLGA
MQEPGHGMASTLVGLFSRPEQLEEVVDDPTLIPRAIAEGLRWTSPIWSATARISTKPVTIAGVDLPAGTPVMLSYGSANHDT
GKYEAPSQYDLHRPPLPHLAFGAGNHACAGIYFANHVMRIALEELFEAIPNLERDTREGVEFWGWGFRGPTSLHVTWEV

The following primers were used to add a 6x His tag (bold) to the C-terminus of the GcoA gene. PCR was performed on the gBlock DNA and the gene was inserted into a pET26 vector using the *Nde*I and *Hind*III restriction sites (underlined). The final sequence was confirmed by DNA sequencing (Australian Genome Research Facility, Adelaide Australia).

FWD: 5' TTAATT<u>catatg</u>acgacgacggagcgccc 3'
REV: 5' TTAATT<u>AAGCTT</u>CTATTA**GTGGTGGTGGTGGTGGTG**cacctcccacgtgacatgtaatg 3'

A codon optimized gene encoding SyoA (based on the NCBI Reference Sequence: WP_037322545.1; *Amycolatopsis thermoflava*) was purchased (Twist Bioscience) cloned in the pET29b plasmid between the *Nde*I and *Xho*I restriction sites of the plasmid. Additional bases incorporated a GGGSGHHHHHH tag (bold) appended to the 3′ end of the gene (C-terminus of the protein), followed by a double stop codon (italics) and *Kpn*I and *Hind*III restriction sites (underlined).

SyoA DNA sequence

ATGACTACTAAGCACACCACGGCCGGCGATACCCAAGAATGGTTAGCGACTGTAACCGTAGAGCAGTTAGAGAATGACCCAT
ACCCTATCTTTGAGCGTTTACGCCGCGAGGCCCCAGTCGCCTGGATCCCGGCCGCCCATGCCTGGGTAGCATCTACGTGGGA
AGCCTGTCGTACGATTGCCGACGATGCAACTAACTTTCGCGGTGGAACTTCACCTATGCACGAGCGCGTACTTGGCACGGAT
CACATCCTGGGAGCCGAGGGTGAGACGCACCAAGACCTTCGCGCAGCCGTAGACCCGCCTCTTAAACCCCGTGCGTTCCGCC
CCTTGCTTGAGGAGCAGGTACGTCCAACGGTCCGTCGTTACCTTGAGGCAATTCGCGGACAGGGCAAGGCAGAACTGATGGC
TGACTACTTTGAGCCGATTTCTGTGCGTTGCGTGGGTGACGTCATTGGGCTTACGGACGTGGACAGTGACACGTTGCGCCGC
TGGTTTCATGCATTAGCGCGTGGAATCGCAAACACGGCAATGGACGCGGAAGGTCGTTTCACTAATCCGGGCGGCTTTGCCC
CTGCGGATGAGGCAGGTGCCGAAATTCGCGAAGTATTAGAGCCGCTCGTAGCCAAGTTATCTGCTGAGCCGGACGGCAGTGC
GCTTAGCCACTACCTTCATCATGGACGCCCTCATGGCGATCCTCGTACACTTGAGCAACTGTTACCGTCCCTCAAGGTGATC
ATTCTCGGCGGGCTGCAGGAACCGGGTCACCAATGTGCAGCCACCTTCCTGGGTCTCACAACTCGTCCTGAACAGTTAAAGC
GCGTGACCGAAGACGCCACGCTCCTGCCACGCGCGCTCACTGAAGGACTCCGCTGGATGAGCCCCGTATTCTCCGCGAGCAG
TCGTTTGCCTTTACGCGAGATTACGATGGGAGAAGCGACGATGCGTCCGGGCCAGACGGTTTGGTTGAGTTATGGGAGTGCA
AACCGCGACGAGGCCGTGTTTGACCGTCCGGATGTATTCGATCTTGATCGTGCCACTCATCCACATCTGGCGTTCGGCACCG
GTCGCCACCTTTGTAGTGGTTCAGCCTATGCCCCACAGGTGGCCCGTATTGCGCTCGAAGAGCTGTTTACAGCCTTTCCCAG
CATTCGTCTTGATCCAGCGCATGAAGTACCTGTGTGGGGCTGGCTGTTCCGCGGGCCTCAACGCCTGGACGTGCTGTGGGAC
GGCGGCGGATCAGGCCATCATCATCATCATCAT*TAATAA*GGTACCAAGCTT

Protein sequence:

MTTKHTTAGDTQEWLATVTVEQLENDPYPIFERLRREAPVAWIPAAHAWVASTWEACRTIADDATNFRGGTSPMHERVLGTD
HILGAEGETHQDLRAAVDPPLKPRAFRPLLEEQVRPTVRRYLEAIRGQGKAELMADYFEPISVRCVGDVIGLTDVDSDTLRR
WFHALARGIANTAMDAEGRFTNPGGFAPADEAGAEIREVLEPLVAKLSAEPDGSALSHYLHHGRPHGDPRTLEQLLPSLKVI
ILGGLQEPGHQCAATFLGLTTRPEQLKRVTEDATLLPRALTEGLRWMSPVFSASSRLPLREITMGEATMRPGQTVWLSYGSA
NRDEAVFDRPDVFDLDRATHPHLAFGTGRHLCSGSAYAPQVARIALEELFTAFPSIRLDPAHEVPVWGWLFRGPQRLDVLWD
GGGSGHHHHHH

Production and purification of GcoA and SyoA

The pET26a vector containing the *gcoA* gene and the pET29 vector containing the *syoA* gene were transformed into *Escherichia coli* BL21 (DE3) competent cells. The cells were then plated on lysogeny broth (LB) agar containing kanamycin (50 μg/ml) and incubated at 37 °C overnight (~16–20 h). A 50 mL LB culture with kanamycin (50 μg/ml) was inoculated and grown overnight (~16–20 h) at 37 °C with shaking (180 rpm). 2 L of the same media were inoculated with 1:100 v/v of overnight culture and incubated, 37 °C with shaking (150 rpm). When the OD_{600} reached 2, 1% v/v ethanol, 0.02% v/v benzyl alcohol and 3 mL/L trace elements (Na_2EDTA (20.1 g), $FeCl_3.6H_2O$ (16.7 g), $CaCl_2.H_2O$ (0.74 g), $CoCl_2.6H_2O$ (0.25 g), $ZnSO_4.7H_2O$ (0.18 g), $MnSO_4.4H_2O$ (0.132 g), $CuSO_4.5H_2O$ (0.10 g)) stock solution were added, and the cultures incubated at 18 °C with shaking (100 rpm).[1] After 30 min, protein expression was induced with addition of 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), and the culture incubated ~20 hours at 18 ˚C with shaking at 100 rpm. Cells were harvested by centrifugation (6200 x*g*, 15 min, 4 ˚C).

The cell pellet was resuspended in 60 mL of ice-cold buffer A (50 mM Tris, 50 mM NaCl, 20 mM imidazole, pH 7.5) and lysed by three rounds of cell disruption. The cell lysate was clarified by centrifugation (40 000 x*g*, 30 min, 4 °C) and protein purified by immobilised nickel affinity

chromatography and anion exchange. The supernatant containing crude cell lysate was applied to a 5 mL HisTrap HP Ni Sepharose column (Cytiva Life Sciences) preequilibrated with buffer 50 mM Tris, 50 mM NaCl, 20 mM Imidazole, pH 7.5. A linear gradient of 20–250 mM imidazole was used to elute the His-tagged proteins at a flow rate of 5 mL/min. The red fractions were pooled and concentrated to ~5 mL by ultrafiltration using a Vivaspin 20 centrifugal concentrator (Sartorius) with a 10 kDa molecular weight cut-off. The sample was buffer exchanged into 50 mM Tris, pH 7.5 by centrifugal ultrafiltration. The protein sample was applied to a 5 mL HiTrap Q FF column (Cytiva Life Sciences) preequilibrated with 50 mM Tris, pH 7.5. The protein was eluted using a linear gradient of 0–100% of 50 mM Tris, pH 7.5, 500 mM KCl at a flow rate of 5 mL/min. The purity was assessed by SDS-PAGE. The red fractions were pooled and concentrated to ~2–4 mL and sterile glycerol was added to a final concentration of 40–50 % v/v. The purified proteins were stored at -20 ˚C and before use, the samples were buffer exchanged into 50 mM Tris, pH 7.5 using a PD-10 desalting column (GE Healthcare).

CO binding assay[2]

The UV-Vis spectrum of ~1–3 µM of GcoA or SyoA (500 µL) in 50 mM Tris, pH 7.5 was recorded from 250 to 700 nm using a Varian Cary 300 UV-Vis Bio Spectrophotometer. A micromolar excess of guaiacol and syringol from a 100 mM stock was added to the solution containing GcoA or SyoA, respectively and the spectrum recorded. A few grains of sodium dithionite were added and the spectrum of the reduced P450 was recorded. To form the P450-CO complex, CO was slowly bubbled through the solution for ~10s and the spectrum was recorded. The concentration and $\varepsilon_{417\ nm}$ of GcoA and SyoA was estimated from the Fe(II)-CO versus Fe(II) difference spectrum using $\varepsilon_{450\text{-}490\ nm}$ = 91 mM^{-1} cm^{-1}.[3]

Spin-state shift analysis

Spin-state shifts induced by substrate binding to the P450 enzymes were measured as previously described with some modification.[1] In brief, 1 µL aliquots of a 100 mM substrate stock solution in DMSO were successively added to 600 µL of ~3 µM of GcoA or SyoA in Tris buffer (50 mM, pH 7.5) and the UV-Vis spectrum recorded using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies) after each addition, until no further shift was observed.

Binding constant analysis

Titrations were performed to determine substrate binding affinity (K_D) according to the previously reported method with modification.[1] Using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies) a baseline was recorded of either 2.5 mL of ~2 µM GcoA or ~3 µM SyoA in potassium phosphate buffer (50 mM, pH 7.4). Aliquots (0.5–5 µL) of 1, 10, or 100 mM substrate stock solutions in DMSO were then added using a 5 µL Hamilton syringe. After each addition of substrate, the difference spectrum was recorded from 300 to 600 nm. Aliquots of substrate were added until no further spectral shift occurred and no more than 10 µL of each stock solution was added to avoid diluting the enzyme. Titrations were performed in triplicate. The peak-to-trough absorbance difference, ΔA (A_{peak} - A_{trough}), was then plotted against substrate concentration. For binding of GcoA to guaiacol the data were fitted to the Morrison (tight-binding) equation (Equation 1).

$$\frac{\Delta A}{\Delta A_{max}} = \frac{([E] + [S] + K_D) - \sqrt{([E] + [S] + K_D)^2 - 4[E][S]}}{2[E]} \quad \textbf{(1)}$$

In this equation, ΔA is the peak-to-trough absorbance difference, ΔA_{max} is the ΔA at saturating substrate concentration, [E] is the P450 concentration and [S] is the concentration of substrate added to the P450. K_D is the dissociation constant of the P450-ligand complex. For all other cases, the data were fitted to the hyperbolic (Michaelis-Menten) equation (Equation 2).

$$\Delta A = \frac{\Delta A_{max} \times [S]}{K_D + [S]} \quad \textbf{(2)}$$

Heme bleaching assay

Using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies) the UV-Vis spectrum of 600 µL of ~2 µM GcoA and SyoA in Tris buffer (50 mM, pH 7.5) was recorded and baselined. 40 mM H_2O_2 was titrated into the solution containing GcoA or SyoA and the UV-Vis spectrum immediately measured every minute until the trough at ~420 nm stopped shifting, indicating complete loss of heme.

***In vitro* H_2O_2-driven turnovers**

In vitro H_2O_2 turnovers were performed at 30 °C and contained 1 µM enzyme, 0.5 mM substrate from a 100 mM stock dissolved in DMSO and 10–20 mM H_2O_2 in Tris buffer (50 mM, pH 7.5) in a total volume of 600 µL. Each reaction was performed in triplicate. Reactions were incubated for 2 mins at 30 ˚C prior to addition of H_2O_2 to initiate the reaction (H_2O_2 was added from a 1 M stock freshly prepared before each experiment from a 30% w/w stock). At time points 0, 60 and 120 mins, 132 µL aliquots of the reaction mixture were removed and quenched with 10 µL of 10 mg mL^{-1} catalase prior to analysis by HPLC.

Control reactions were performed with heat denatured enzymes to determine if background oxidation of the substrates was occurring. Reaction mixtures were heated at 70 °C for 10 mins and cooled to 30 ˚C prior to addition of substrate and H_2O_2. Control reactions were also performed to assess whether the expected products further oxidised when exposed to H_2O_2. Reactions were set up as above, containing catechol or 3-methoxycatechol instead of guaiacol and syringol, respectively.

Kinetic analysis

Reactions were performed at 30 °C and contained a total of 1 µM GcoA or SyoA in Tris buffer (50 mM, pH 7.5) in a total volume of 600 µL. A total of 25–1000 µM guaiacol or 50–2000 µM syringol from a 100, 75, 50, 25, 10, 5 or 2.5 mM stock dissolved in DMSO was added to the enzymes. Reactions were incubated for 2 mins at 30 ˚C prior to addition of a saturating amount of 10–20 mM H_2O_2 to initiate the reaction (H_2O_2 was added from a 200 mM stock freshly prepared before each experiment from a 30% w/w stock). Each reaction was performed in duplicate. After 5 mins, 132 µL aliquots of the reaction mixture were removed and quenched with 10 µL of 10 mg mL^{-1} catalase prior to analysis by HPLC. The concentration of demethylated product was determined by HPLC and used to determine the initial reaction rate. To obtain the K_M and k_{cat} the data were fitted to the Michaelis-Menten equation (Equation 3).

$$\nu = \frac{V_{max} \times [S]}{K_M + [S]} \quad \textbf{(3)}$$

Where Vmax is equal to the product of the catalyst rate constant (kcat) and the concentration of the enzyme, ν is equal to the intial rate of the reaction and [*S*] is the substrate concentration.

Product analysis by HPLC

Analytical High Performance Liquid Chromatography (HPLC) was performed on a Shimadzu LC-20AD equipped with a Phenomenex Kinetex 5u XB-C18 100 Å column (250 mm x 4.6 mm, 5 µM), SIL-20A autosampler, CTO-20A, SPD-20A UV detector and CBM-20Alite communications module. Each sample and standard were injected at a volume of 20 µL. A gradient of 20–95% MeCN in water (with 0.1% TFA) over 30 minutes was used to elute the samples at a rate of 1 mL min^{-1} and the eluate was monitored at 254 nm.

To prepare turnover mixtures for HPLC, 132 µL of the *in vitro* turnover mixture was mixed with 66 µL of MeCN and 2 µL of internal standard (10 mM 9-hydroxyfluorene (9-OHFlu) in EtOH) and centrifuged at 15,800 x*g* for 3 mins to remove particulate matter. Calibration curves were constructed to quantify the product. Solutions of products with concentrations of 20, 50, 100, 200 and 500 µM were prepared for HPLC analysis in the same way as the turnovers. A plot of product peak area/IS peak area vs. product concentration was then constructed. The calibration factor was the slope of this graph and had units of μM^{-1}. Guaiacol eluted at 11.5 min, syringol 11.2 min, catechol 7.2 min and 3-methoxycatechol 7.4 min.

Phylogenetic analysis

Multiple sequence alignments were performed using ClustalX v2.1.[4] A phylogenetic tree was built using command line PhyML v3.3 with default settings.[5] The tree was visualised using ggtree v3.2.1.[6]

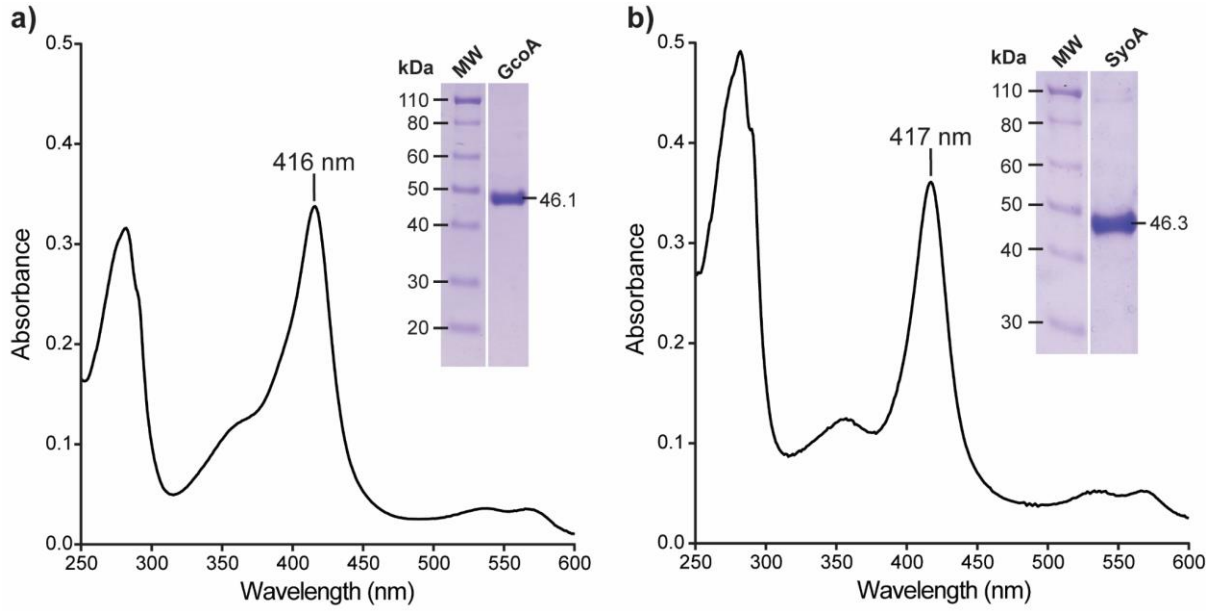

Figure S1. UV-Vis spectrum and SDS-PAGE analysis of purified a) GcoA and b) SyoA. The UV-Vis traces show a characteristic Soret band at ~420 nm indicating successful incorporation of the heme cofactor. SDS-PAGE shows final purification step for the enzymes with the expected size of GcoA (46.1 kDa) and SyoA (46.3 kDa) (inset).

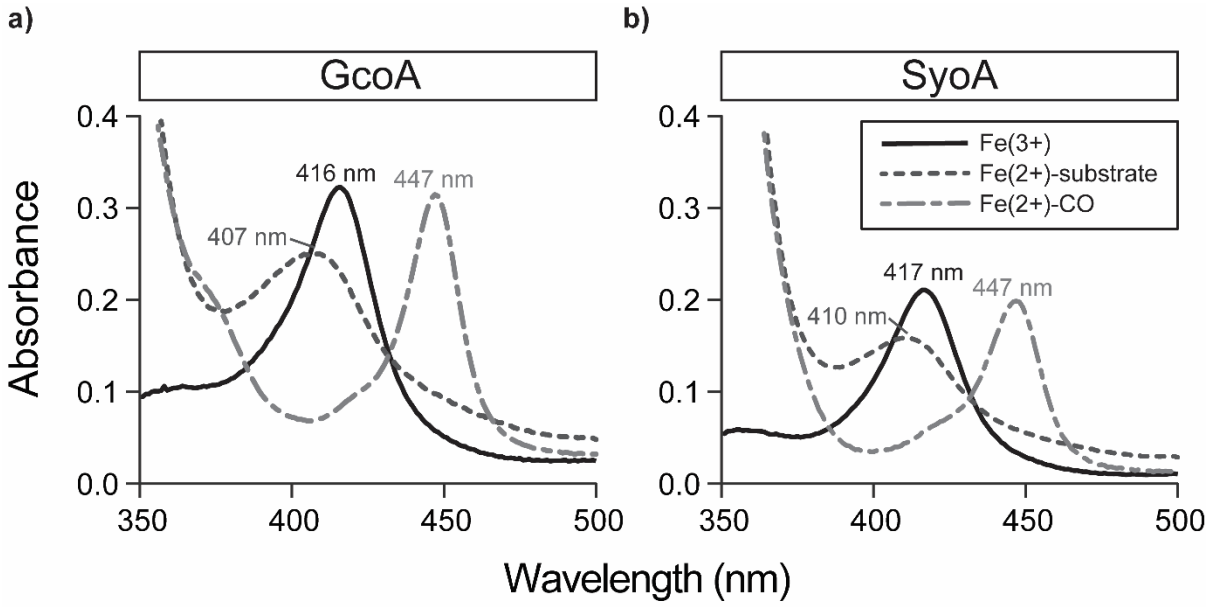

Figure S2. CO binding assay of a) GcoA and b) SyoA. The Soret peak of the oxidised (Fe^{3+}) P450s (black) shifted to the reduced substrate bound (Fe^{2+}-substrate) spectrum (dark grey) after addition of a micromolar excess of sodium dithionite. The spectrum then completely shifted to the reduced CO bound (Fe^{2+}-CO) spectrum (light grey) after CO was bubbled into the solution. The Soret peak failed to shift completely to 447 nm without addition of the preferred substrates (guaiacol for GcoA and syringol for SyoA) prior to addition of dithionite.

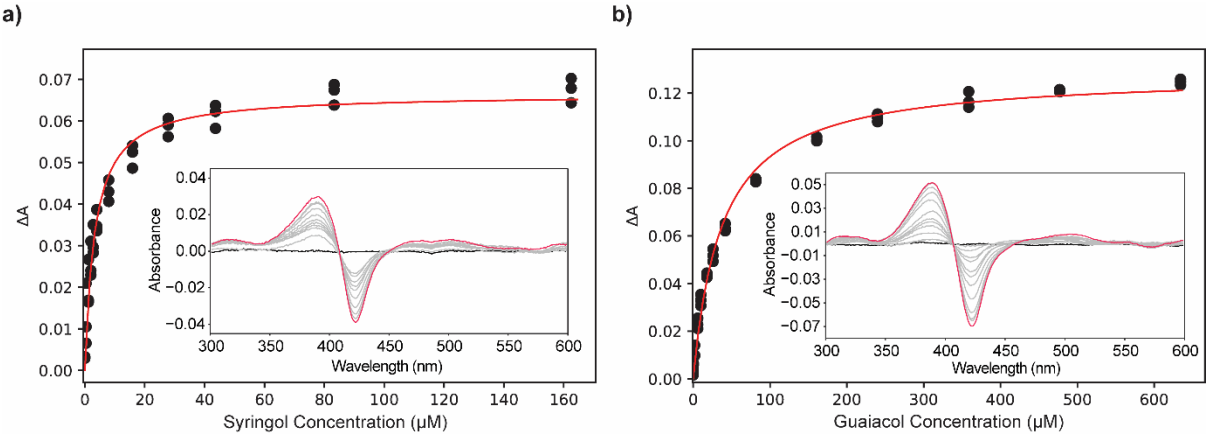

Figure S3. UV-Vis titrations to determine the dissociation constant of a) GcoA with syringol and b) SyoA with guaiacol. The concentration of enzyme used were 1.8 μM GcoA and 3.3 μM SyoA.

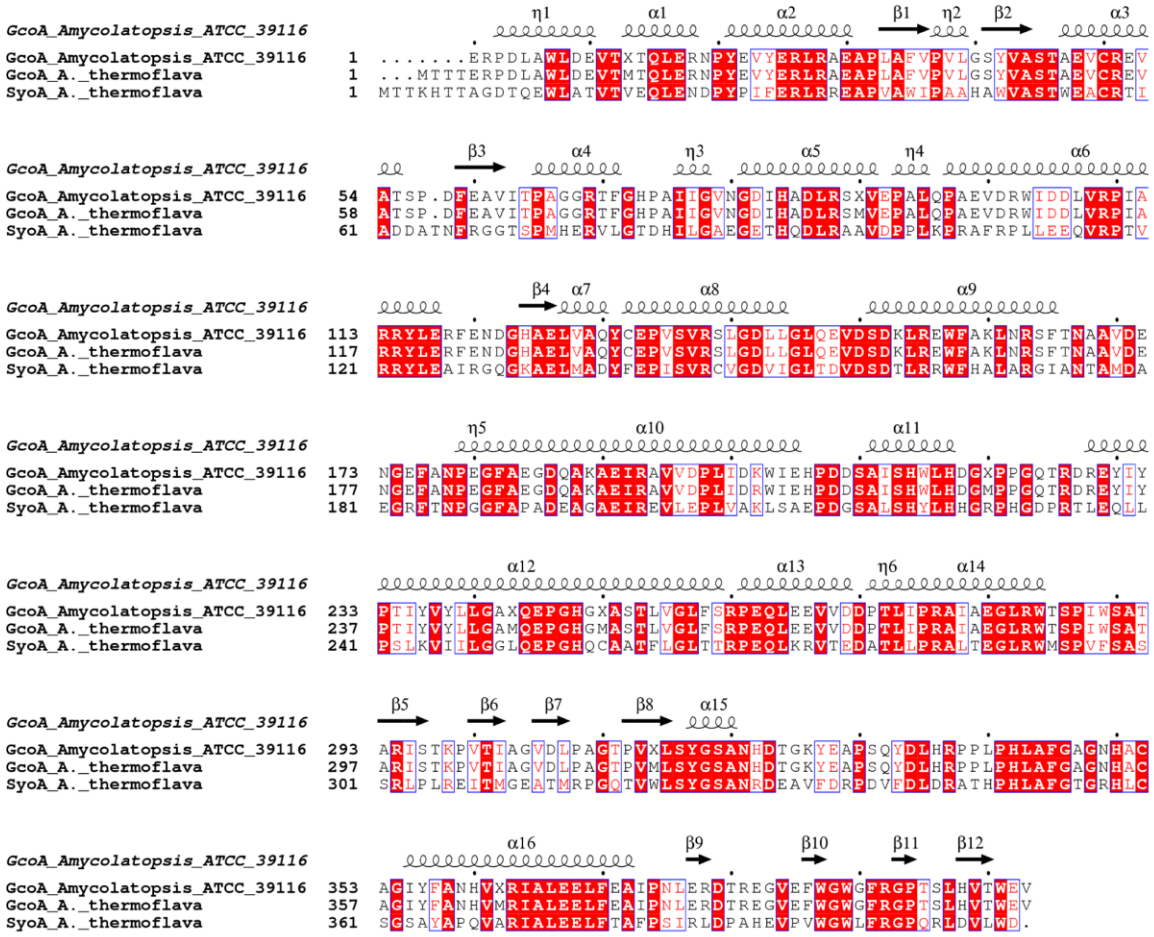

Figure S4. Amino acid sequence alignment of GcoA and SyoA from *A. thermoflava* N1165 and GcoA (PDB: 5ncb) from *Amycolatopsis sp.* ATCC 39116.[7] Selenomethionine residues found in 5ncb are denoted by X.

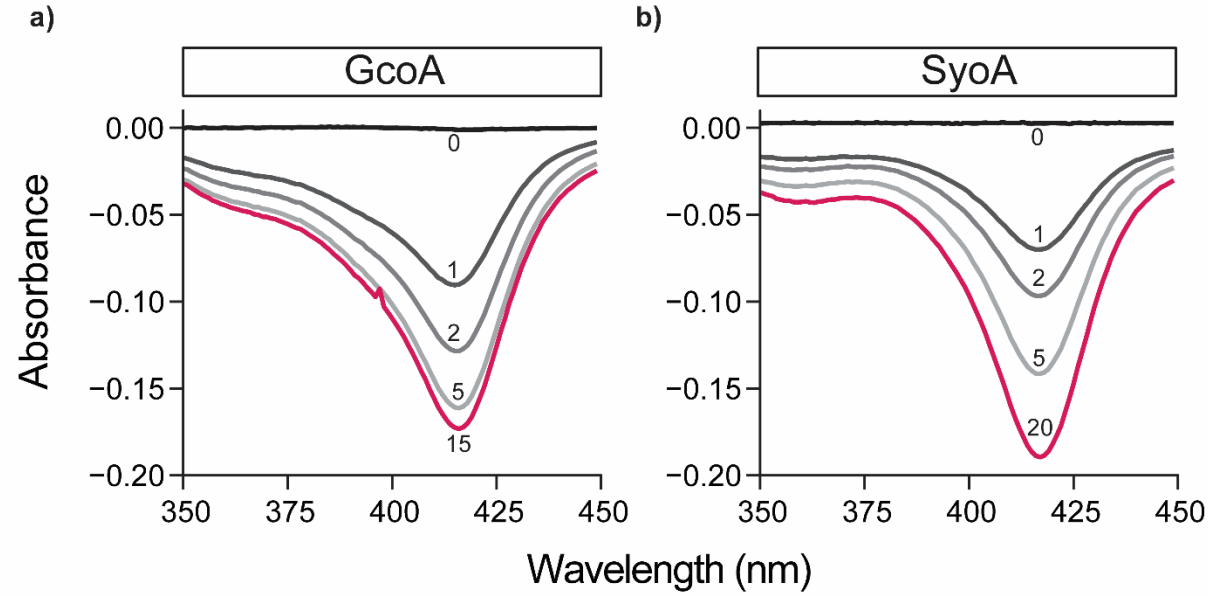

Figure S5. Heme bleaching assay for a) GcoA and b) SyoA. The UV-Vis spectrum of GcoA and SyoA was baselined and monitored post addition of 40 mM hydrogen peroxide until the trough at ~420 nm stopped shifting, indicating complete loss of heme (red trace). Complete loss of heme for GcoA occurred after 15 min and SyoA after 20 min. Numbers indicate time (min) after addition of 40 mM hydrogen peroxide. Assay was performed using 1.6 µM GcoA and 1.8 µM SyoA.

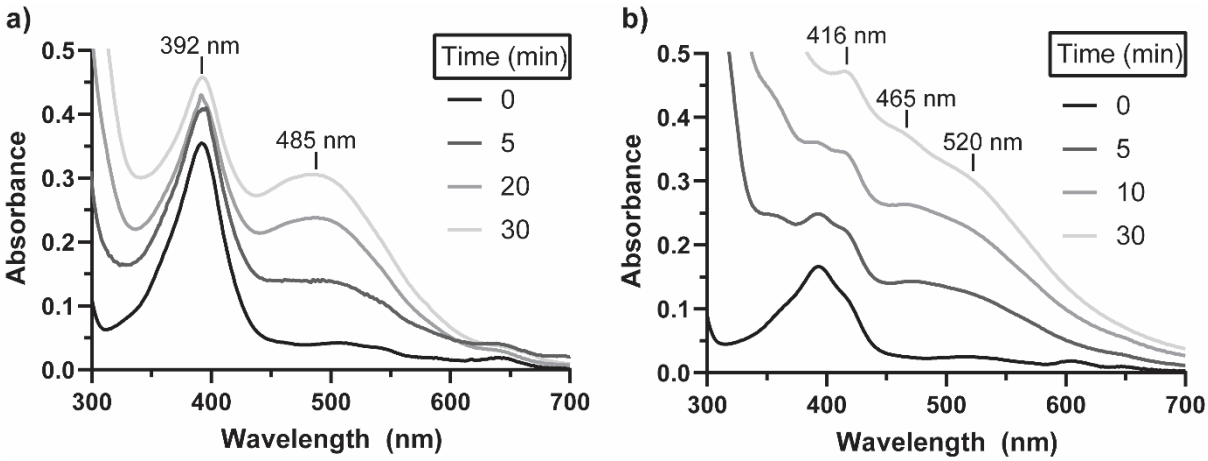

Figure S6. UV-Vis spectrum of the H_2O_2-driven oxidation of a) guaiacol by GcoA and b) syringol by SyoA. Reactions were initiated upon addition of 40 mM H_2O_2 to a cuvette containing either 3.3 µM GcoA or 1.9 µM SyoA and 1 mM guaiacol or syringol, respectively, and the UV-Vis spectrum monitored over 30 min. The colour of the reaction mixtures changed to red over 30 mins as indicated by increasing absorbance at 485 nm for the GcoA guaiacol reaction and 300–600 nm for the SyoA syringol reaction.

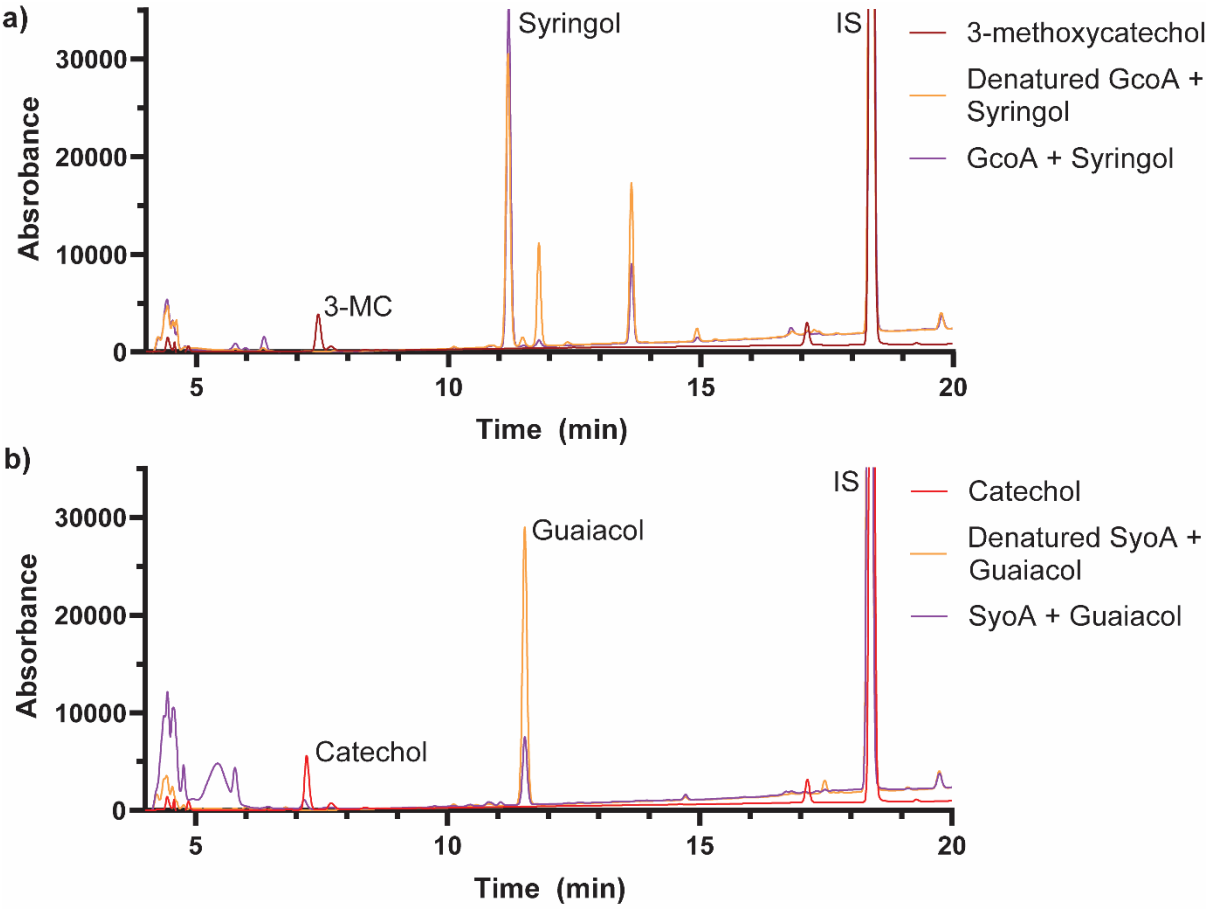

Figure S7. HPLC analysis of the H_2O_2-driven reactions of a) GcoA and syringol and b) SyoA and guaiacol over 120 min. A control reaction with heat denatured P450 is shown in yellow and authentic standards of the expect demethylated products are shown in red. Internal standard (IS) 10 mM 9-hydroxyfluorene eluted 18.4 min. Gradient: 20–95% MeCN in H_2O with 0.1% v/v TFA. Detection wavelength: 254 nm.

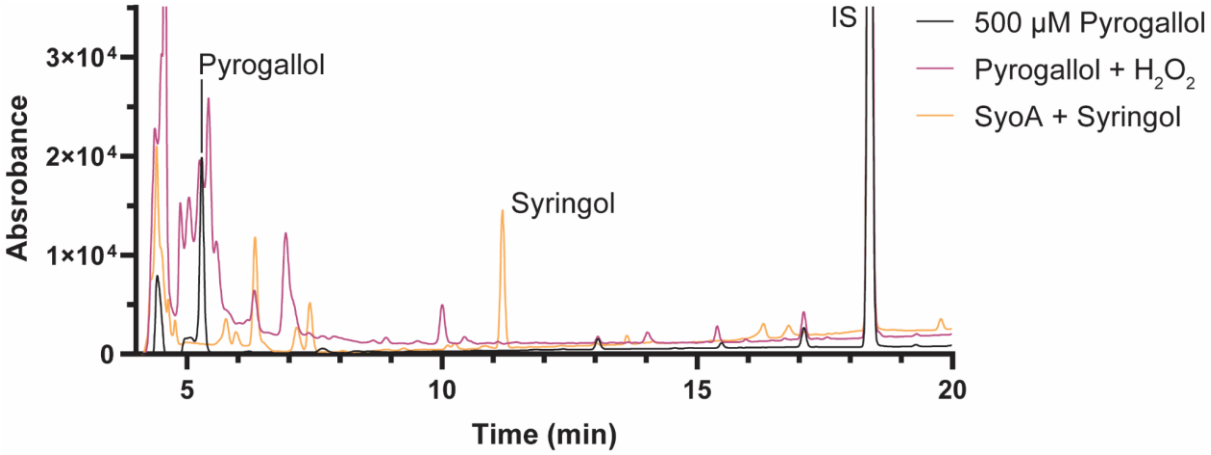

Figure S8. HPLC analysis of pyrogallol oxidation in the presence of 40 mM H_2O_2 compared to the H_2O_2-driven oxidation of syringol by SyoA. An authentic sample of 1,2,3-trihydroxybenzene (pyrogallol) had a retention time of 5.3 min and no peak with the same retention time was observed during the *O*-demethylation of syringol by SyoA. A control reaction using 40 mM H_2O_2 was conducted to compare the oxidation products of pyrogallol to the further oxidation products observed during the reaction with SyoA and syringol. The peaks corresponding to further oxidation of 3-MC did not match those observed during the oxidation of pyrogallol by H_2O_2. These results indicate that SyoA is unlikely to demethylate 3-MC to pyrogallol. Gradient: 20–95% MeCN in H_2O with 0.1% v/v TFA. Detection wavelength: 254 nm.

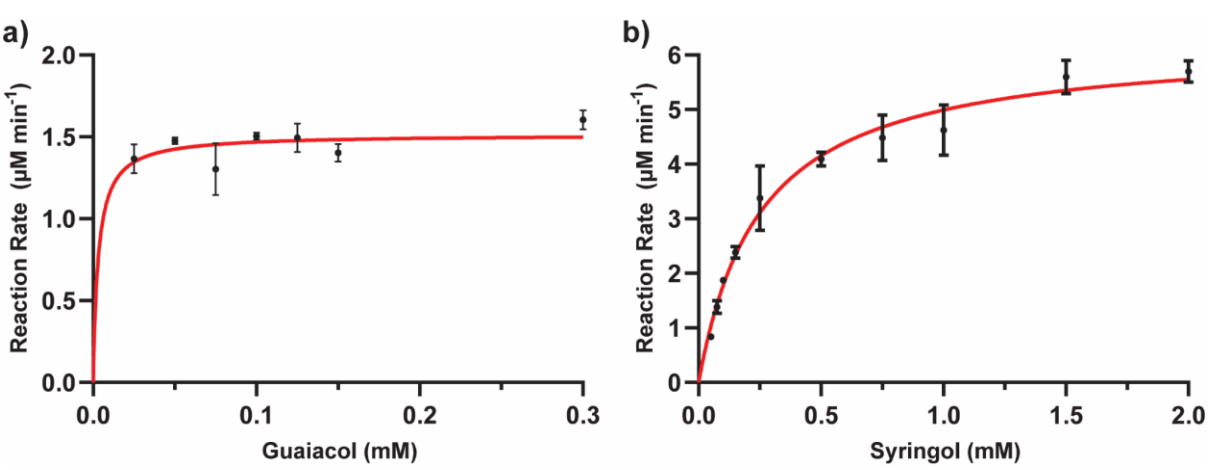

Figure S9. Michaelis-Menten kinetics for a) GcoA and guaiacol and b) SyoA and syringol. The k_{cat} and K_M of syringol oxidation by SyoA were 6.3 ± 0.2 min^{-1} and 253 ± 27 µM, respectively. Error bars represent ± 1 standard deviation from two measurements.

Experiments were conducted to determine the k_{cat} and K_M for the demethylation of guaiacol by GcoA. The k_{cat} was determined to be 1.5 ± 0.05 min^{-1} with the no change in the reaction rate with substrate concentrations > 25 µM. Due to limitations of the methods used, accurate reporting of product formed using less than 25 µM substrate was not feasible. Therefore, the K_M could not be accurately reported. However, the K_M was less than 25 µM.

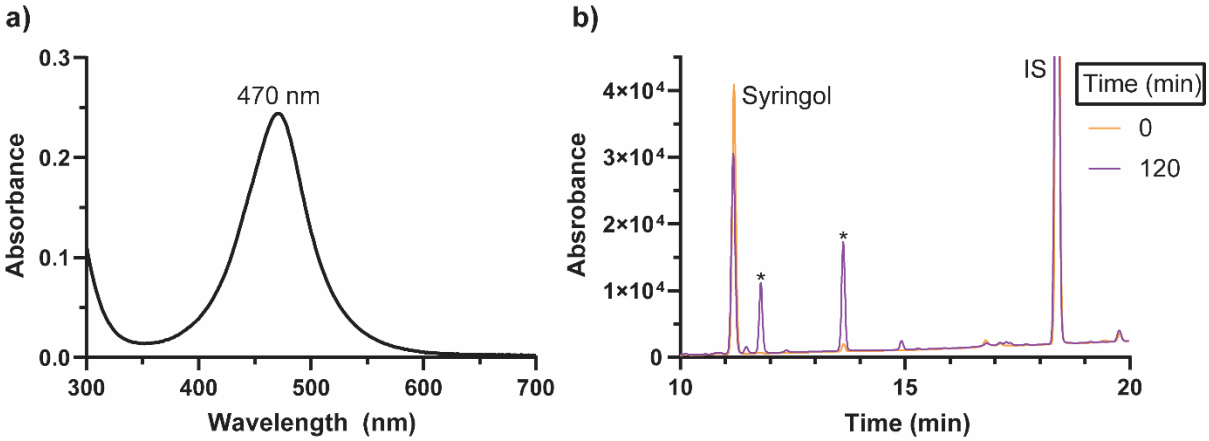

Figure S10. a) UV-Vis spectrum of the oxidation of syringol by 40 mM H_2O_2 after 30 min. b) HPLC analysis of the oxidation of syringol. The oxidation of syringol could occur in the presence of H_2O_2 as indicated by the formation of an absorbance maximum at 470 nm and two products - not observed in the demethylation of syringol to 3-methoxycatechol by SyoA - with retention times of 11.8 and 13.6 min identified by HPLC. Syringol can oxidise to quinones such as coerulignone and 2,6-dimethoxybenzoquinone in the presence of H_2O_2.[8] Only a minor amount of syringol was oxidised and no 3-MC formed in the absence of enzyme, demonstrating the *O*-demethylation of syringol by SyoA occurs as a result of enzyme function. * Denotes major products formed during the oxidation of syringol.

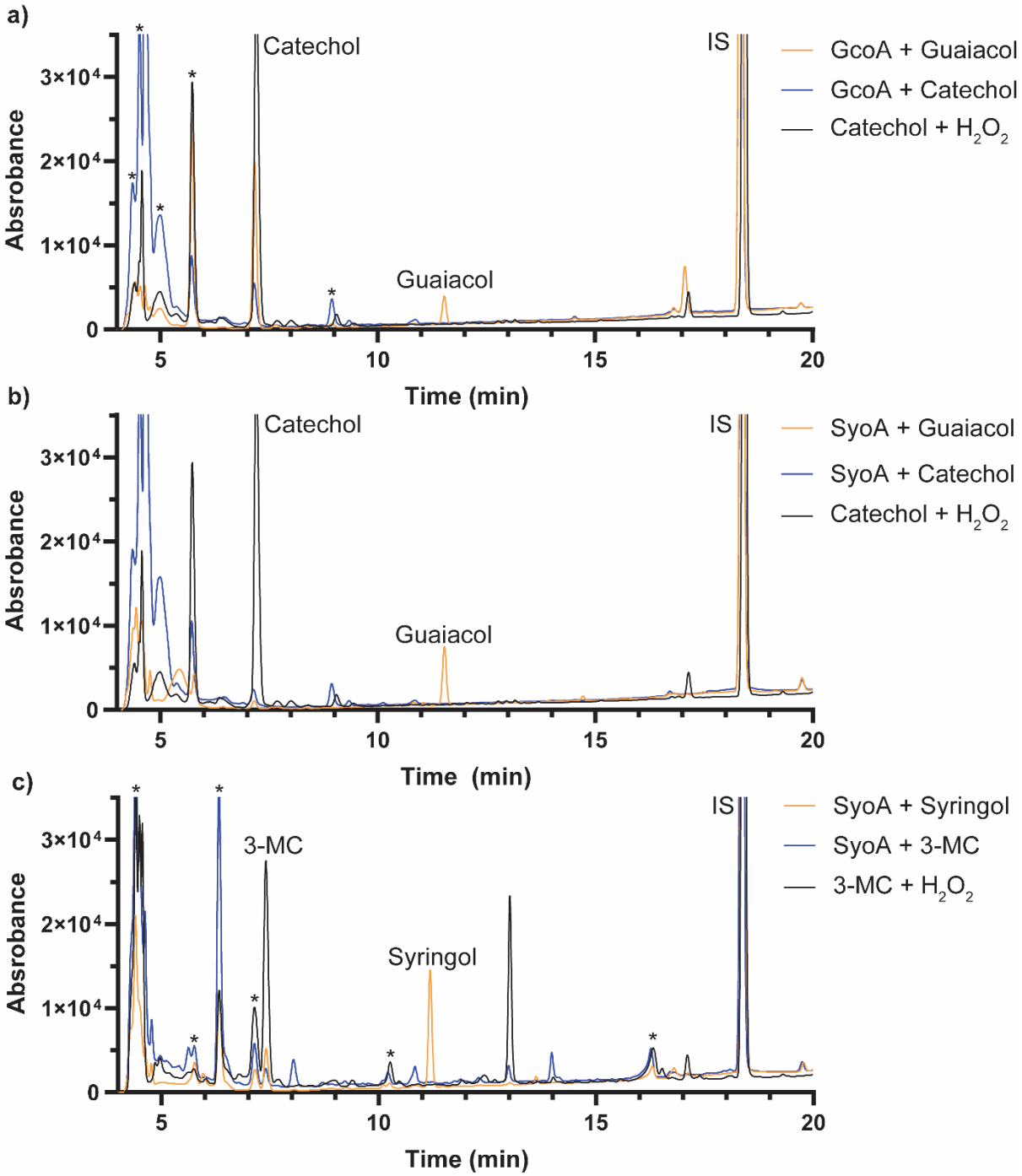

Figure S11. HPLC analysis of a) the oxidation of catechol by H_2O_2 compared to the H_2O_2-driven oxidation of guaiacol by GcoA, b) the oxidation of catechol by H_2O_2 compared to the H_2O_2-driven oxidation of guaiacol by SyoA and c) the oxidation of 3-methoxycatechol by H_2O_2 compared to the H_2O_2-driven oxidation of syringol by SyoA. * Denotes the oxidation products of catechol or 3-methoxycatechol that are also found as further oxidation products in the reactions with guaiacol or syringol, respectively.

References

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