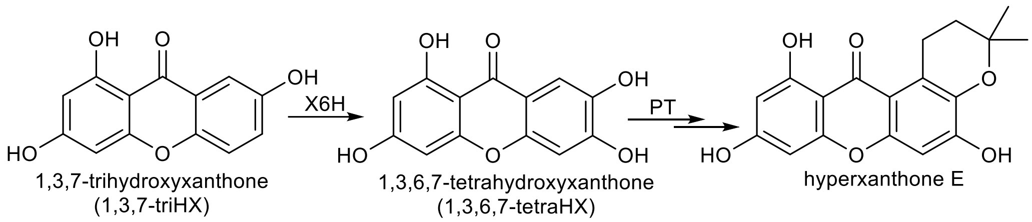
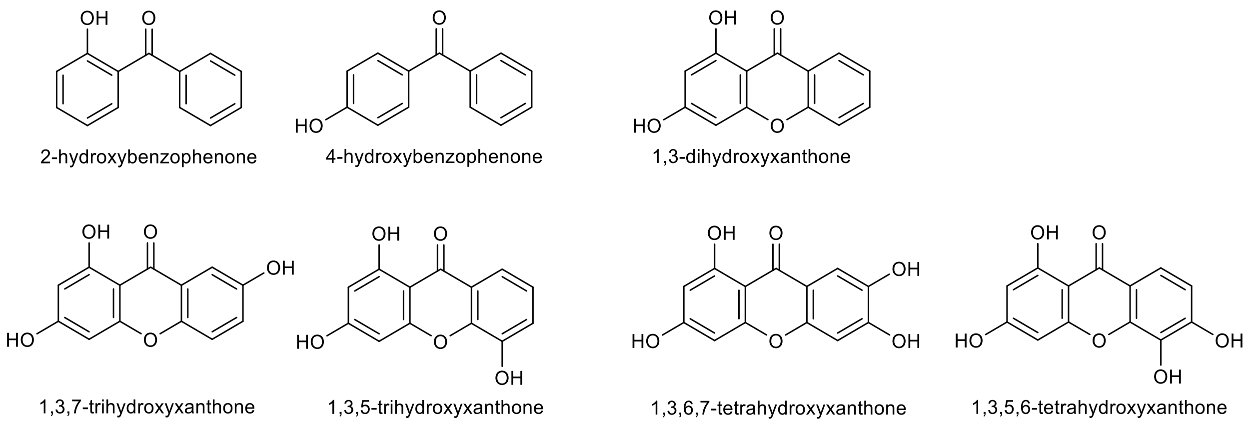
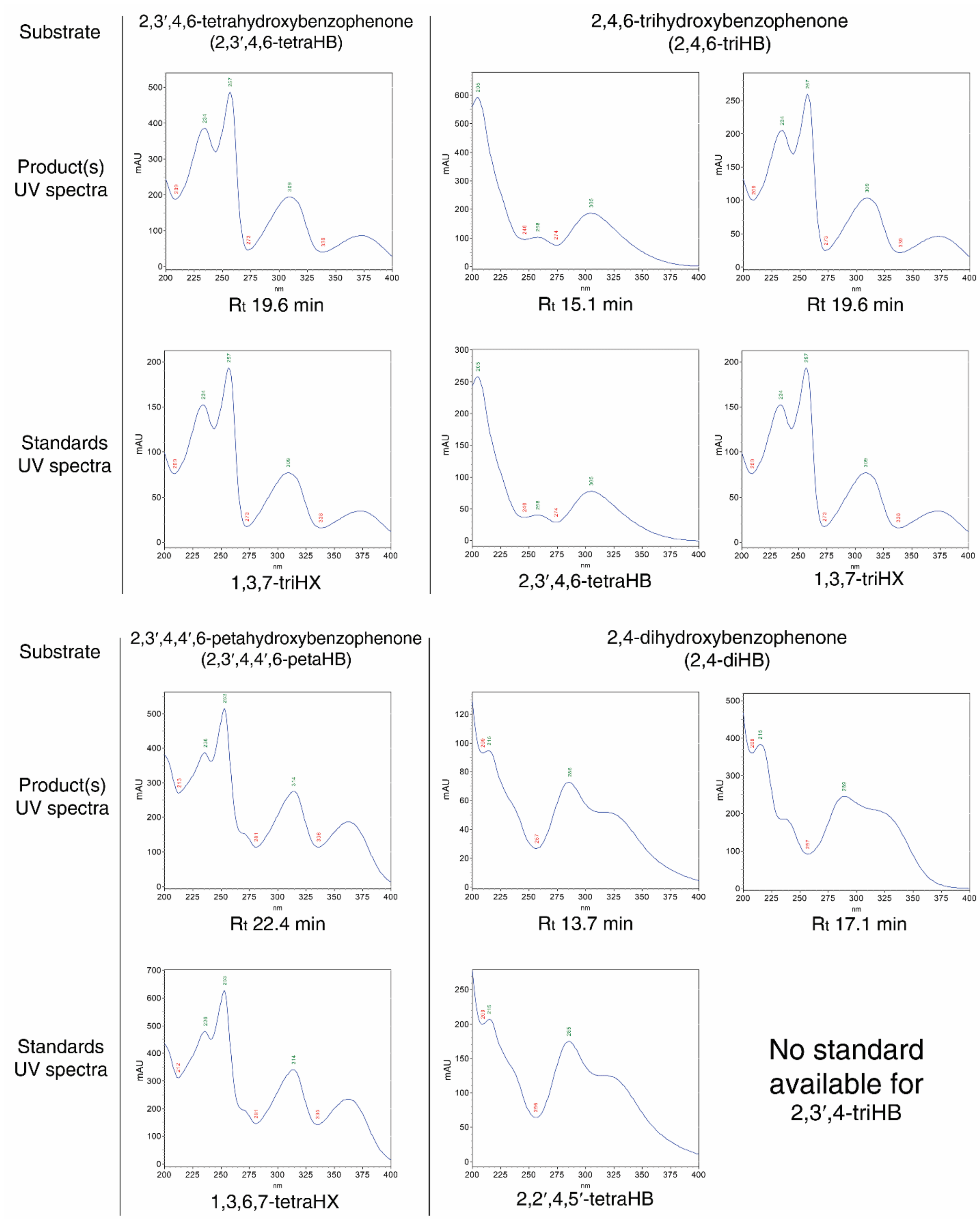


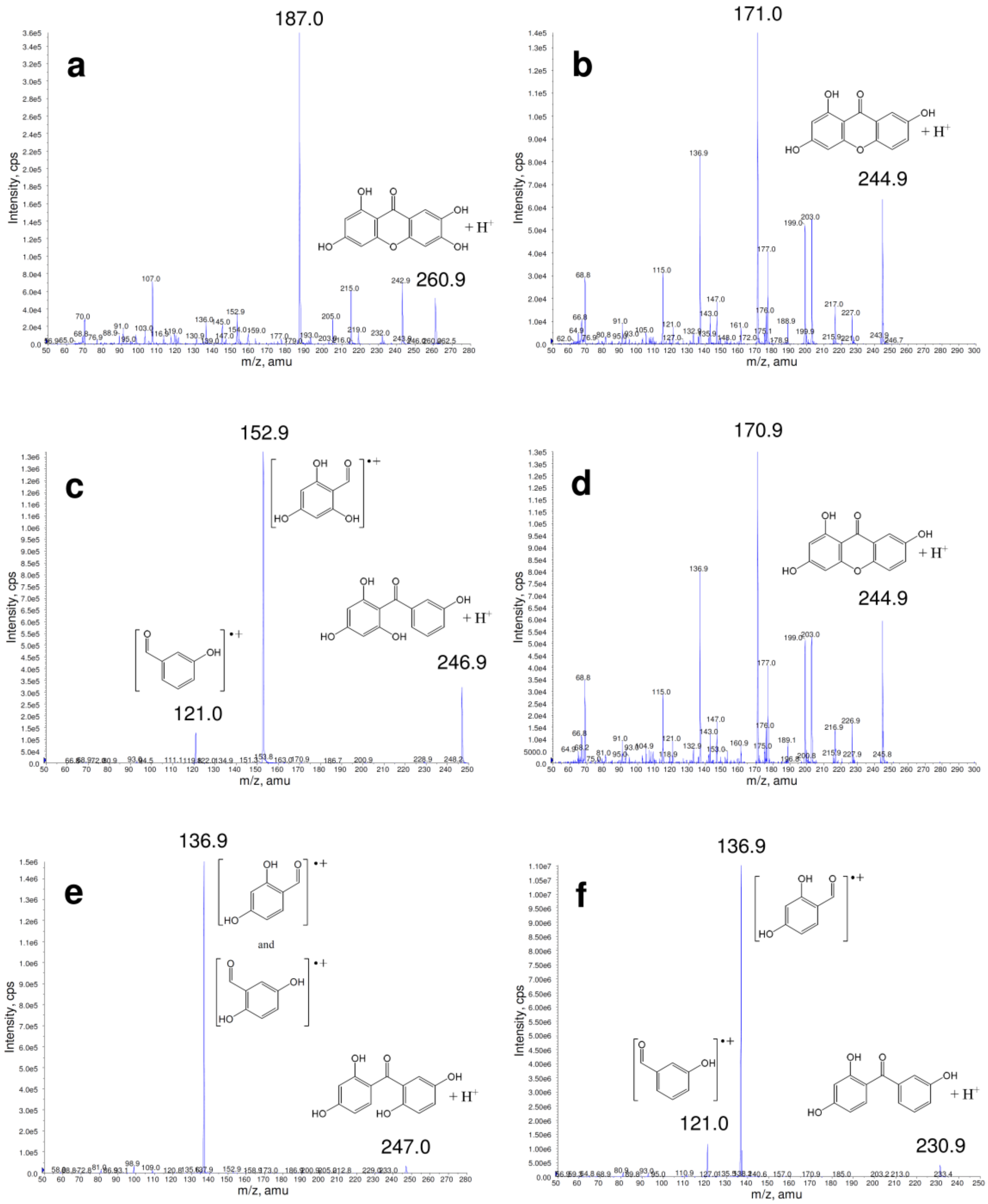
Supplementary Figure 1 | Proposed biosynthesis of hyperxanthone E from 1,3,7-triHX in elicitor-treated *H. calycinum* cell cultures. X6H, xanthone 6-hydroxylase; PT, prenyltransferase.



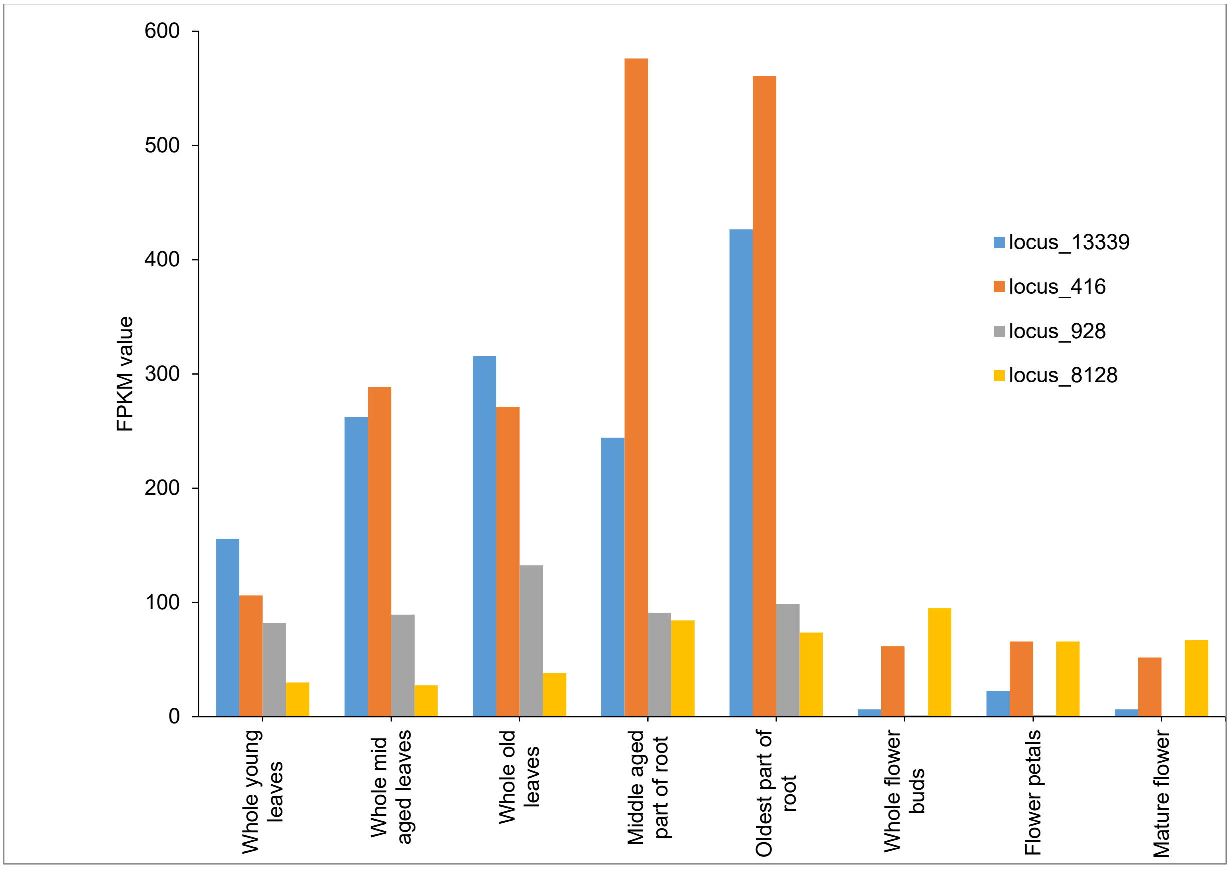
Supplementary Figure 2 | Substrates not accepted by CYP81AA1 and CYP81AA2.



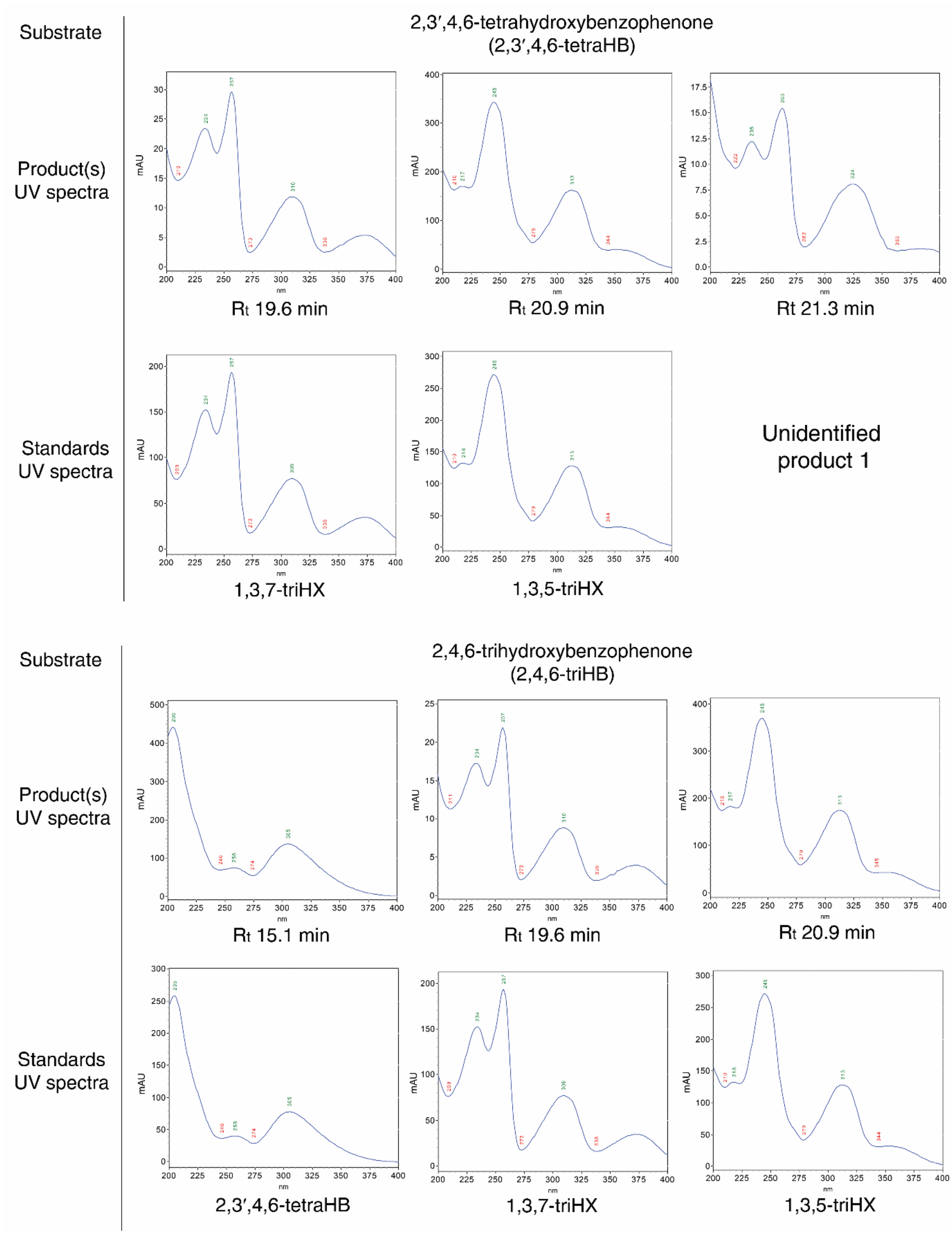
Supplementary Figure 3 | UV spectra of CYP81AA1 products and authentic compounds.



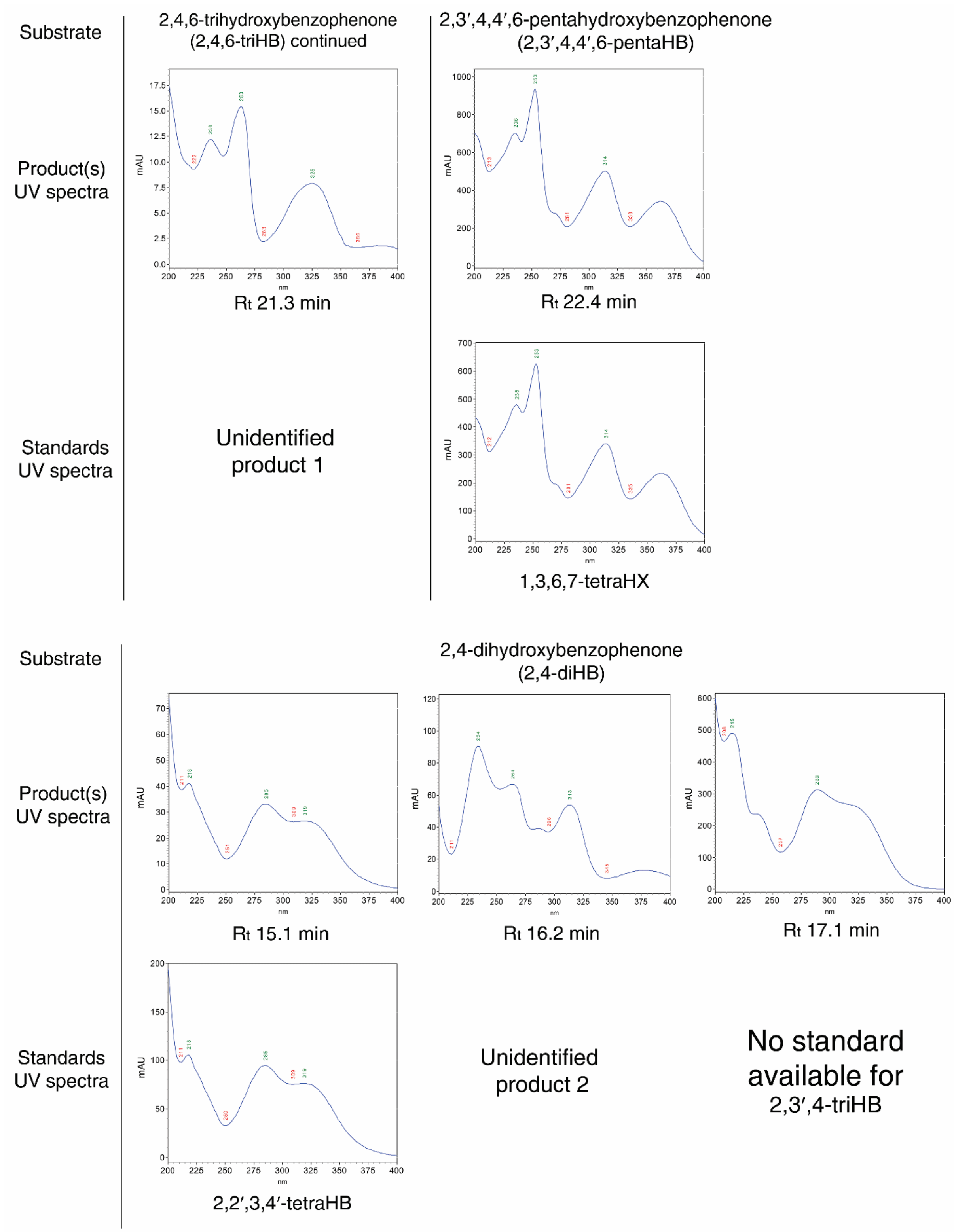
Supplementary Figure 4 | Enhanced product ion (EPI) mass spectra of CYP81AA1 products formed from various substrates. (**a**) 1,3,6,7-tetraHX (R_t = 22.3 min) from 2,3′,4,4′,6-pentaHB. (**b**) 1,3,7-triHX (R_t = 19.6 min) from 2,3′,4,6-tetraHB. (**c**) 2,3′,4,6-tetraHB (R_t = 15.1 min) from 2,4,6-triHB. (**d**) 1,3,7-triHX (R_t = 19.6 min) from 2,4,6-triHB. (**e**) 2,2′,4,5′-tetraHB (R_t = 13.7 min) from 2,4-diHB. (**f**) 2,3′,4-triHB (R_t = 17.1 min) from 2,4-diHB.



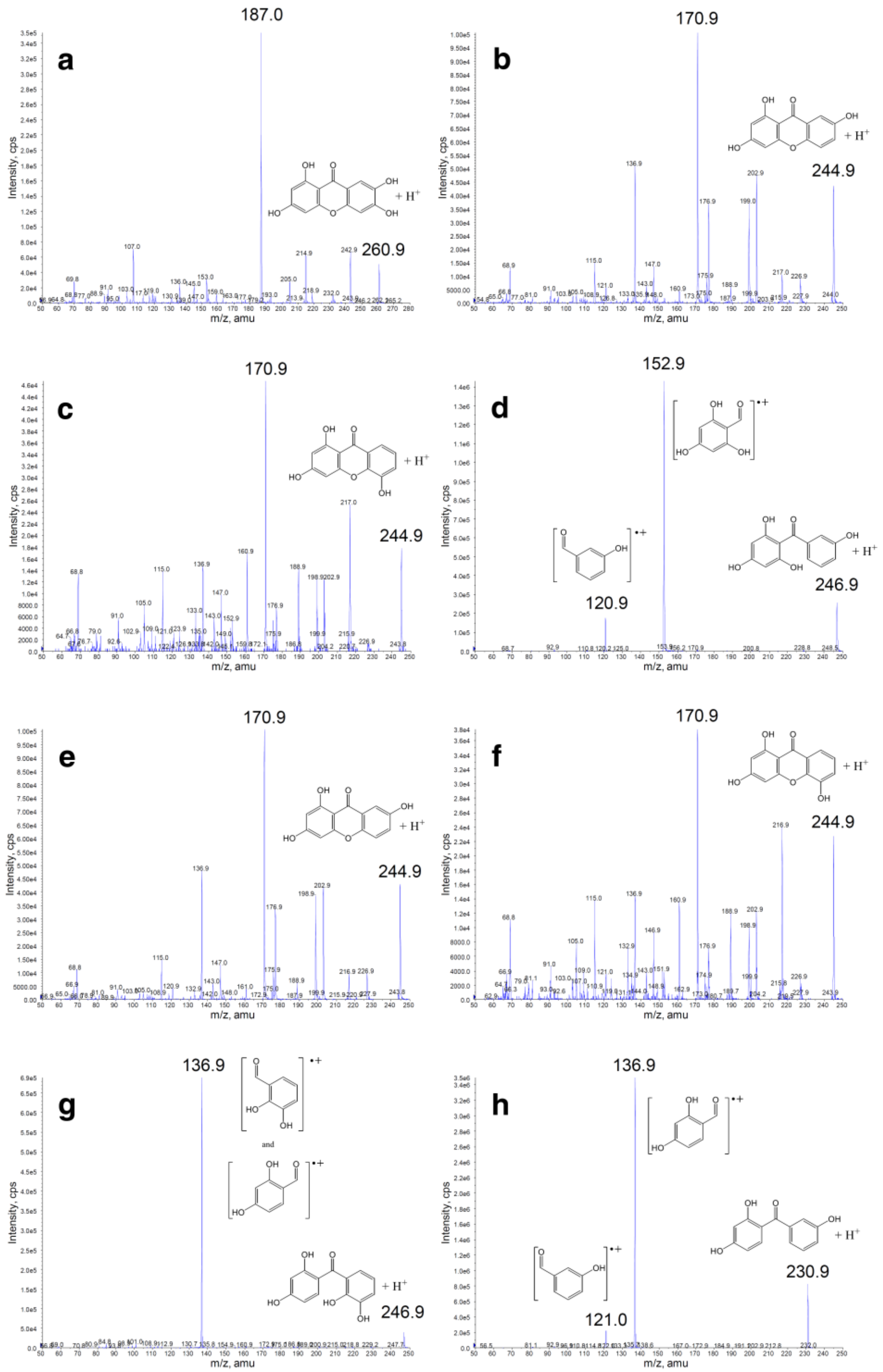
Supplementary Figure 5 | Tissue distribution of transcripts encoded by the loci 13339 (*HpBPS*), 416 (*HpCYP81AA1*), 928 (*HpCYP81AA2*), and 8128 (*HpCYP81AA3*). Data are taken from the MPGR database (http://medicinalplantgenomics.msu.edu). FPKM, fragments per kilobase per million mapped fragments.



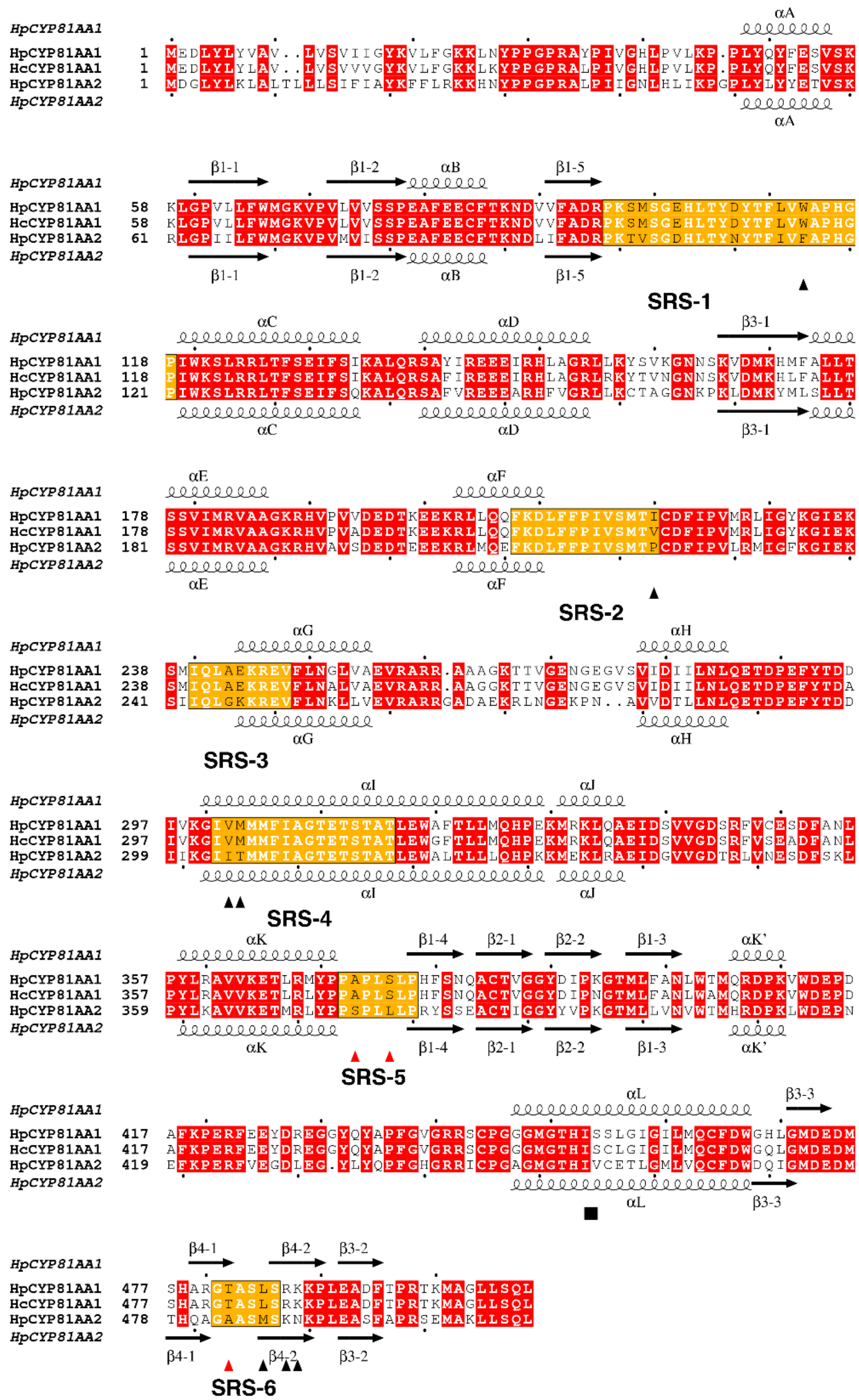
Supplementary Figure 6 | UV spectra of CYP81AA2 products and authentic compounds (I).



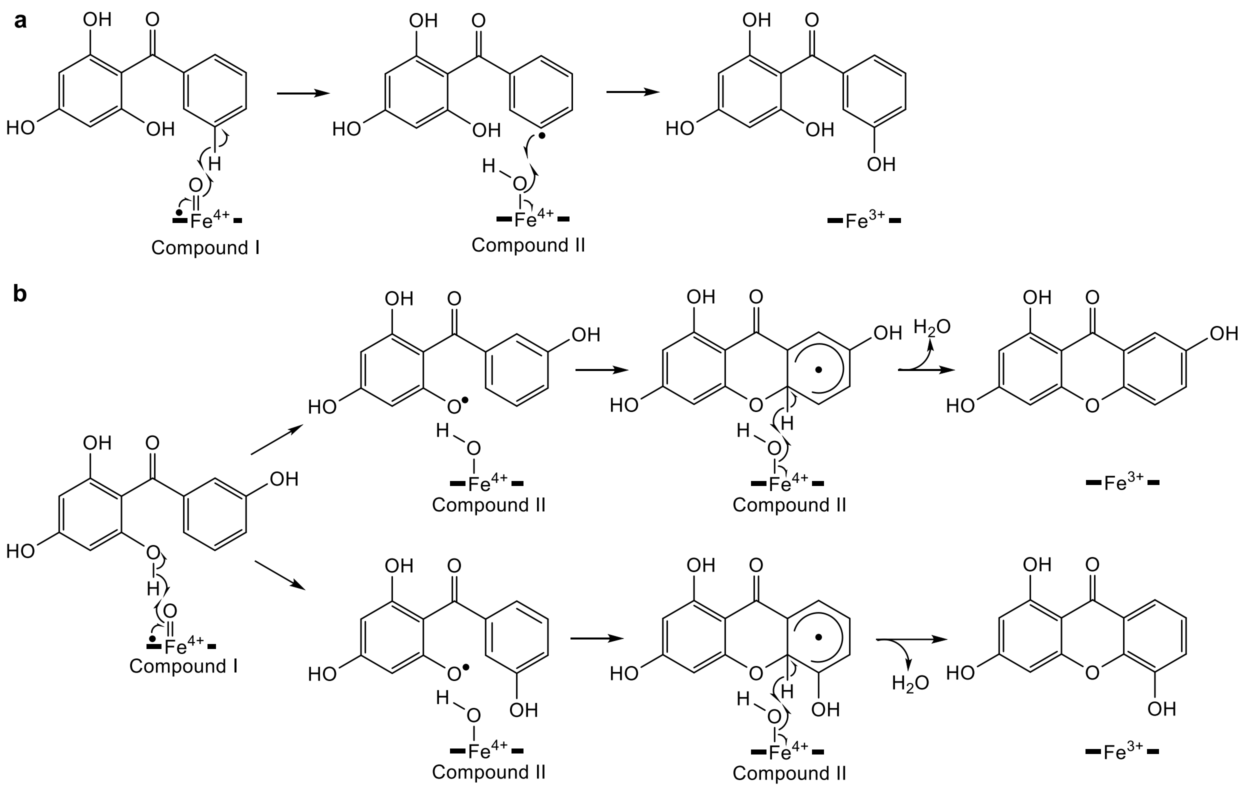
Supplementary Figure 6 | UV spectra of CYP81AA2 products and authentic compounds (II).



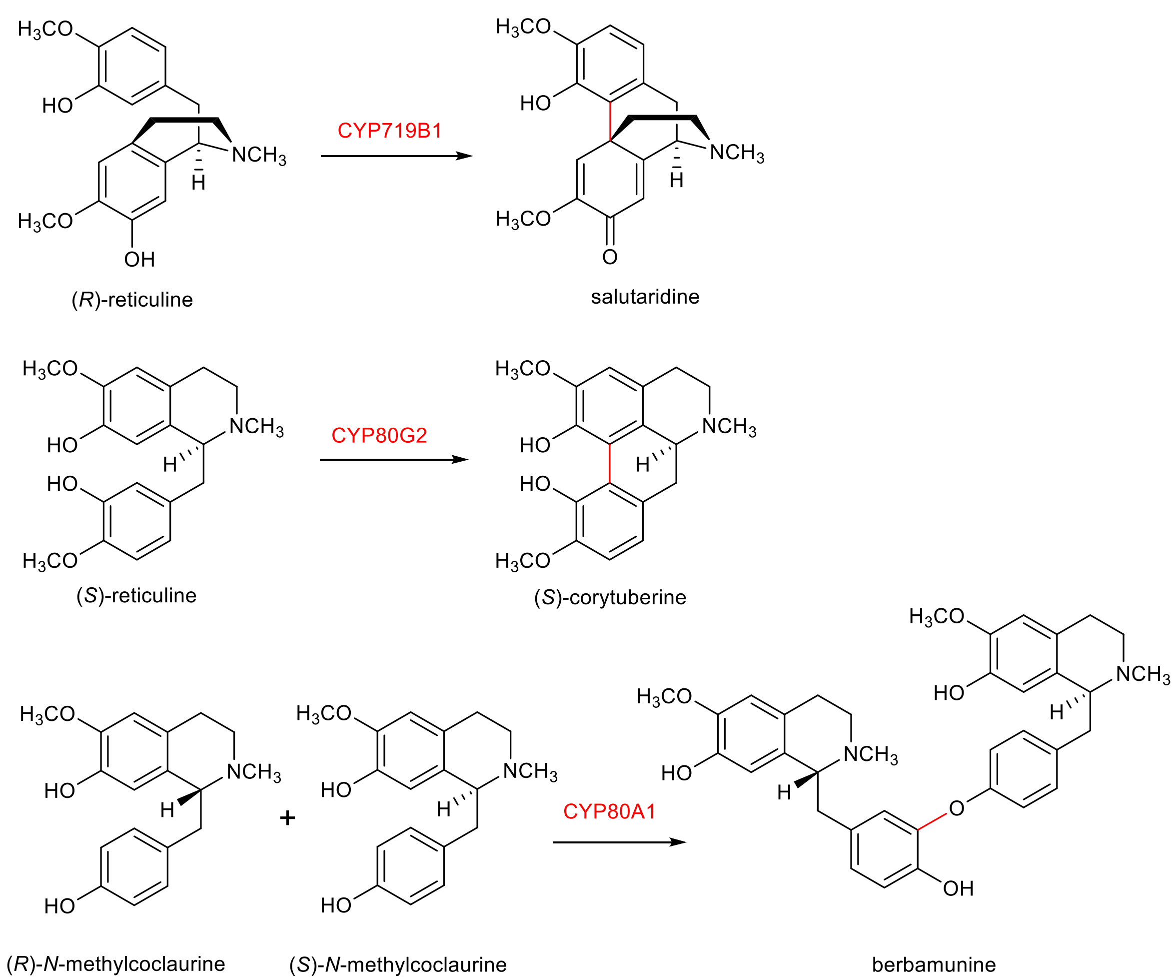
Supplementary Figure 7 | Enhanced product ion (EPI) mass spectra of CYP81AA2 products formed from various substrates. (**a**) 1,3,6,7-tetraHX (R_t = 22.3 min) from 2,3′,4,4′,6-pentaHB. (**b**) 1,3,7-triHX (R_t = 19.6 min) from 2,3′,4,6-tetraHB. (**c**) 1,3,5-triHX (R_t = 20.9 min) from 2,3′,4,6-tetraHB. (**d**) 2,3′,4,6-tetraHB (R_t = 15.1 min) from 2,4,6-triHB. (**e**) 1,3,7-triHX (R_t = 19.6 min) from 2,4,6-triHB. (**f**) 1,3,5-triHX (R_t = 20.9 min) from 2,4,6-triHB. (**g**) 2,2′,3,4′-tetraHB (R_t = 15.1 min) from 2,4-diHB. (**h**) 2,3′,4-triHB (R_t = 17.1 min) from 2,4-diHB.



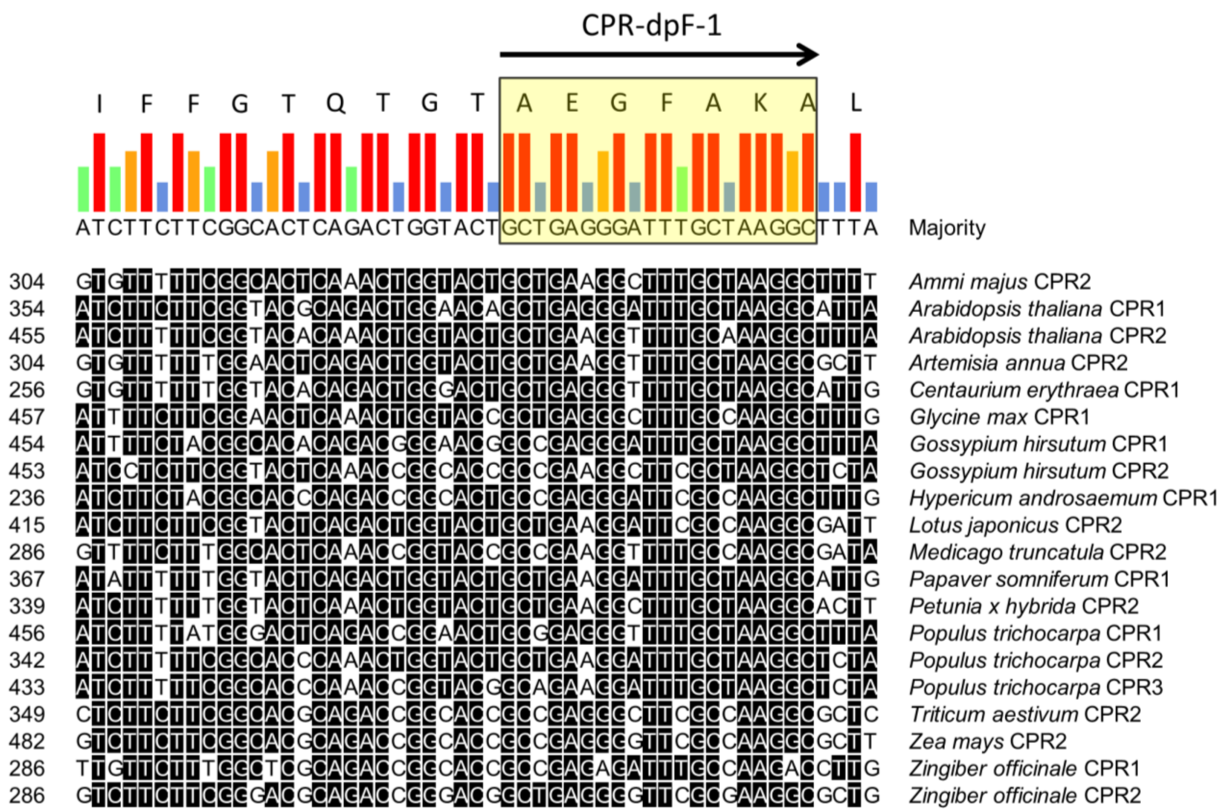
Supplementary Figure 8 | Multiple sequence alignment of HpCYP81AA1, HcCYP81AA1 and HpCYP81AA2. The conserved secondary structures of HpCYP81AA1 and HpCYP81AA2 are indicated on the top and the bottom, respectively. SRSs 1-6 are highlighted in orange. The black triangles point to positions that were mutated and the red triangles refer to the deduced regioselectivity-determining residues in HpCYP81AA2. The black square refers to the position of the C-terminal exchange.



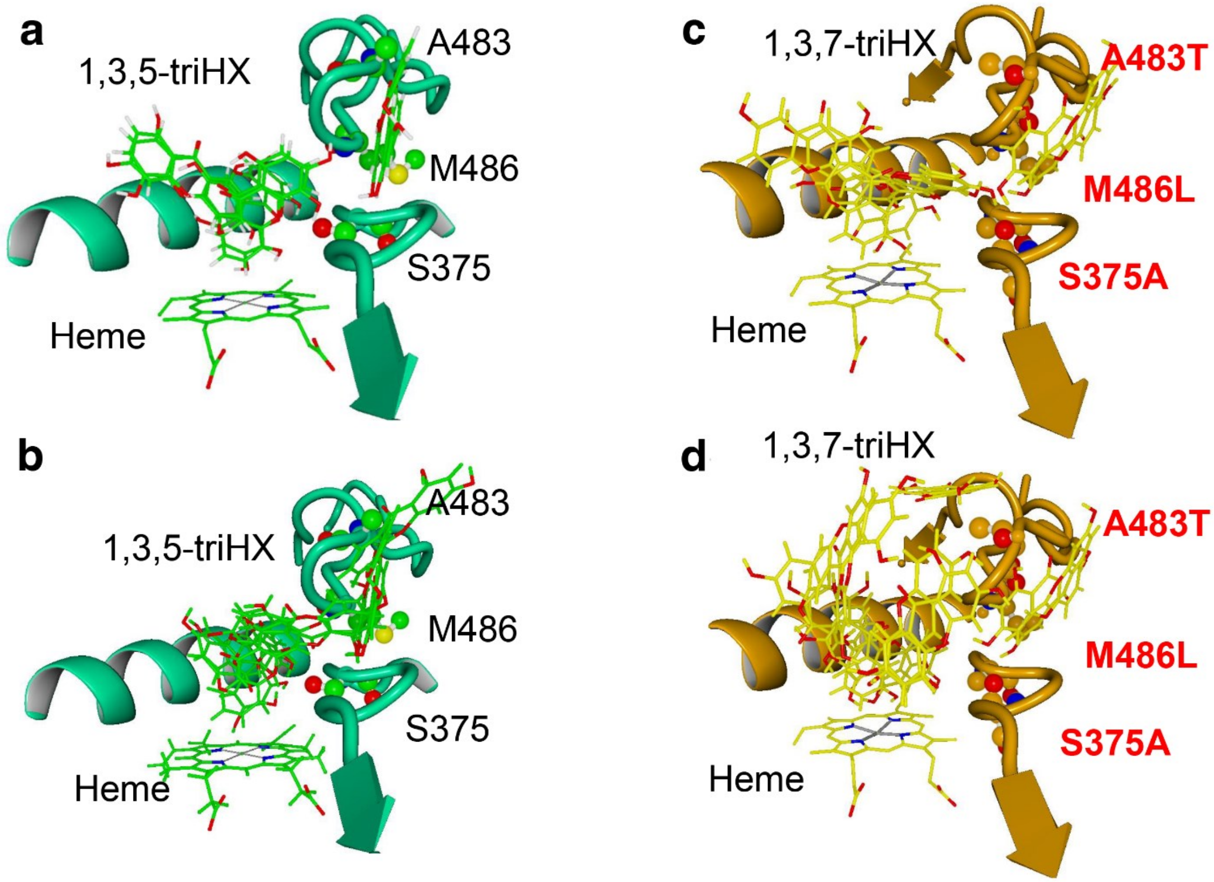
Supplementary Figure 9 | Proposed reaction processes catalyzed by CYP81AA1 and CYP81AA2. (**a**) Hydroxylation reaction at the 3′-position of 2,4,6-triHB catalyzed by both enzymes. (**b**) Regioselective C–O phenol coupling reactions occurring either *para* or *ortho* to the introduced 3′-hydroxy group catalyzed by CYP81AA1 and CYP81AA2, respectively.



Supplementary Figure 10 | Phenol coupling reactions catalyzed by plant CYPs in isoquinoline alkaloids biosyntheses.



Supplementary Figure 11 | Multiple sequence alignment of the FMN binding domains of 20 plant CPRs for designing the CPR-dpF-1 primer.



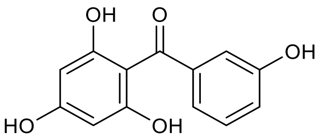
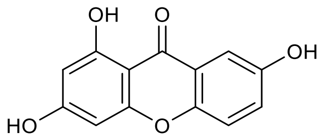
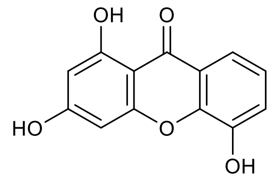
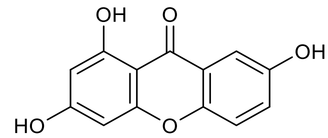
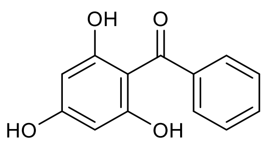
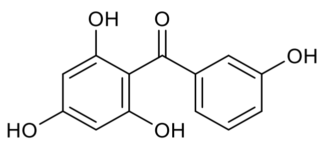
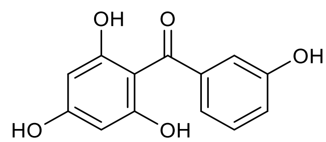
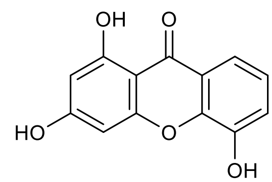
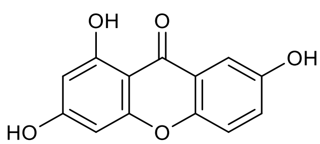
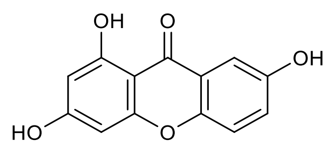
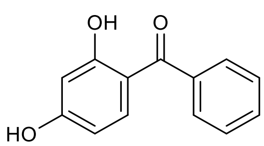
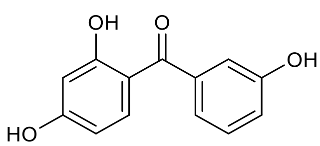
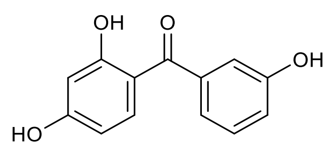
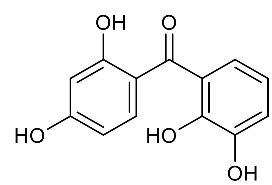
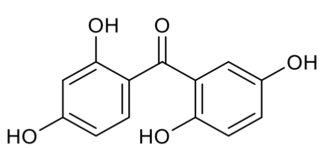
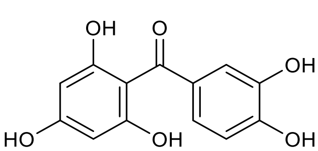
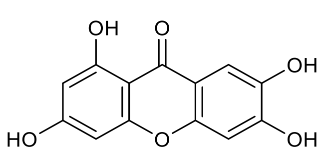
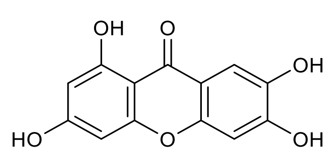
Supplementary Figure 12 | Automated product docking using AUTODOCK VINA. The active sites of wild-type CYP81AA2 (lime) with bound 1,3,5-triHX (green sticks) and the sextuple mutant (mut6) of CYP81AA2 (orange) with bound 1,3,7-triHX (yellow sticks) are illustrated. (**a**) CYP81AA2 1,3,5-triHX docking with rigid receptor, overlay of 7 clusters representing 25 docking results. (**b**) CYP81AA2 1,3,5-triHX docking with flexible receptor sidechains, overlay of 9 clusters representing 100 docking results. (**c**) CYP81AA2(mut6) 1,3,7-triHX docking with rigid receptor, overlay of 8 clusters representing 25 docking results. (**d**) CYP81AA2(mut6) 1,3,7-triHX docking with flexible receptor sidechains, overlay of 12 clusters representing 100 docking results.

Supplementary Table 1 | CYP contigs identified in a *H. calycinum* SSH library.

Contig number	Number of copies	Closest NCBI match	Closest MPGR[a] match (% identity)
21	22	CYP81C	hpa_locus_416 (95.0)
41	7	CYP81C	hpa_locus_416 (91.6)
50	11	CYP81C	hpa_locus_416 (97.2)
56	2	CYP71A	hpa_locus_18081 (98.2)
59	33	CYP81C	hpa_locus_416 (97.3)
62	36	CYP72A	hpa_locus_60 (78.2)
86	3	CYP81D	hpa_locus_12223 (70.8)
103	2	CYP81C	hpa_locus_416 (97.4)
110	3	CYP81C	hpa_locus_416 (97.2)
158	2	CYP706	hpa_locus_19031 (81.9)
181	3	CYP706	hpa_locus_19031 (82.2)
199	3	CYP81D	hpa_locus_13843 (89.7)
219	5	CYP81C	hpa_locus_416 (92.0)

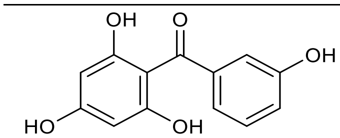
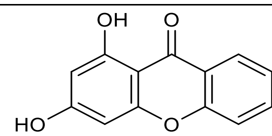
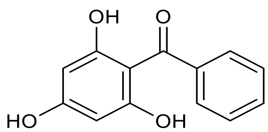
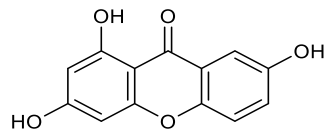
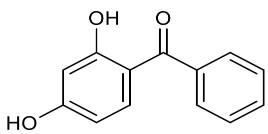
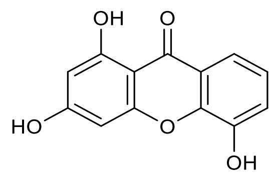
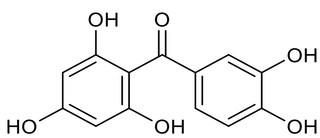
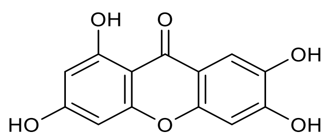
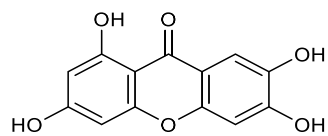
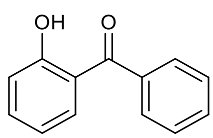
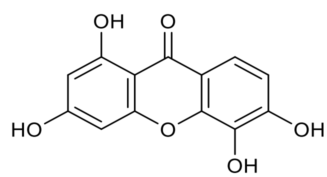
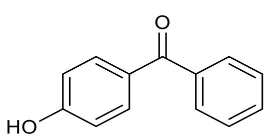
[a] Medicinal Plant Genomics Resource (http://medicinalplantgenomics.msu.edu)

Supplementary Table 2 | Product profiles of CYP81AA1 and CYP81AA2[a].

Substrate	Product(s)		
	CYP81AA1	CYP81AA2	
 2,3′,4,6-tetraHB	 1,3,7-triHX	 1,3,5-triHX	 1,3,7-triHX (minor) + unidentified product 1 (minor)
 2,4,6-triHB	 2,3′,4,6-tetraHB	 2,3′,4,6-tetraHB	 1,3,5-triHX + unidentified product 1 (minor)
	 1,3,7-triHX	 1,3,7-triHX (minor)	
 2,4-diHB	 2,3′,4-triHB	 2,3′,4-triHB + unidentified product 2	 2,2′,3,4′-tetraHB
	 2,2′,4,5′-tetraHB		
 2,3′,4,4′,6-pentaHB	 1,3,6,7-tetraHX	 1,3,6,7-tetraHX	

[a] Non-accepted substrates are depicted in Supplementary Fig. 1

Supplementary Table 3 | Product profile of CYP81AA3.

Substrate	Product	Substrate	Product
 2,3′,4,6-tetraHB	n.d.	 1,3-diHX	n.d.
 2,4,6-triHB	n.d.	 1,3,7-triHX	n.d.
 2,4-diHB	n.d.	 1,3,5-triHX	n.d.
 2,3′,4,4′,6-pentaHB	 1,3,6,7-tetraHX	 1,3,6,7-tetraHX	n.d.
 2-HB	n.d.	 1,3,5,6-tetraHX	n.d.
 4-HB	n.d.		

n.d. = not detected

Supplementary Table 4 | Contact residues located within a 4 Å radius of the bound inhibitor and their standard positions and SRS numbers. Divergent residues are highlighted in red.

CYP81AA1		CYP81AA2		Standard numbering position	SRS number
Residue	Position	Residue	Position		
PHE	110	PHE	113	85	1
PHE	212	PHE	215	181	2
ILE	215	ILE	218	184	2
VAL	216	VAL	219	185	2
ILE	307	ILE	309	263	4
ALA	308	ALA	310	264	4
GLU	311	GLU	313	267	4
THR	312	THR	314	268	4
ALA	315	ALA	317	271	4
TYR	370	TYR	372	325	5
PRO	372	PRO	374	327	5
ALA	373	SER	375	328	5
LEU	377	LEU	379	331	5
THR	482	MET	486	437/439	6
ALA	483	SER	487	437.3/440	6
SER	484	LYS	488	438/441	6
LEU	485	ASN	489	439/441.1	6

Supplementary Table 5 | Product profiles of CYP81AA1 mutants.

Enzyme mutant	1,3,7-triHX (% of total product)	1,3,5-triHX
Wild-type CYP81AA1	100	0
CYP81AA1-AA2	100	0
CYP81AA1 A373S	94.9 ± 1.1	5.1 ± 1.1
CYP81AA1-AA2 A373S	93.2 ± 0.2	6.8 ± 0.2
CYP81AA1 A373S/S376L	95.0 ± 0.4	5.0 ± 0.4
CYP81AA1-AA2 A373S/S376L	96.0 ± 0.8	4.0 ± 0.8
CYP81AA1 A373S/S376L/T482A	95.5 ± 0.3	4.5 ± 0.3
CYP81AA1 A373S/S376L/T482A/L485M/R487K/K488N	93.5 ± 0.6	6.5 ± 0.6
CYP81AA1 A373S/S376L/T482V	94.6 ± 0.5	5.4 ± 0.5
CYP81AA1 A373S/S376L/T482F	95.9 ± 0.3	4.1 ± 0.3
CYP81AA1 W113F/A373S/S376L/T482A	95.2 ± 0.1	4.8 ± 0.1
CYP81AA1 I220P/A373S/S376L/T482A	99.4 ± 0.1	0.6 ± 0.1
CYP81AA1 A373S/S376L/T482A/L485M/R487K/K488N/V302I/M303T	No activity detected	
CYP81AA1 W113F/I220P/A373S/S376L/T482A/L485M/R487K/K488N/V302I/M303T	No activity detected	

Supplementary Table 6 | Accession numbers of the plant CPR sequences used to design CPR-dpF-1.

Name	**Accession No.**	**Name**	**Accession No.**
Ammi majus CPR2	AY532374.1	*Medicago truncatula* CPR2	XM_003610061.1
Arabidopsis thaliana CPR1	NM_118585.3	*Papaver somniferum* CPR1	U67185.1
Arabidopsis thaliana CPR2	NM_179141.2	*Petunia* x *hybrida* CPR2	DQ099545.1
Artemisia annua CPR2	DQ984181.1	*Populus trichocarpa* CPR1	XM_002307300.2
Centaurium erythraea CPR1	AY596976.1	*Populus trichocarpa* CPR2	AF302497.1
Glycine max CPR1	NM_001249813.1	*Populus trichocarpa* CPR3	AF302498.1
Gossypium hirsutum CPR1	FJ719368.1	*Triticum aestivum* CPR2	AJ303373.1
Gossypium hirsutum CPR2	FJ719369.1	*Zea mays* CPR2	EU956822.1
Hypericum androsaemum CPR1	AY520902.1	*Zingiber officinale* CPR1	AB566408.1
Lotus japonicus CPR2	AB433810.1	*Zingiber officinale* CPR2	AB566409.1

Supplementary Table 7 | List of primers used for core fragment amplification, RACE, qRT-PCR and cloning.

Primer name	Sequence 5′→3′
Core fragments amplification	
Contig59-F1	AAGGTCCCCGTCCTCGTTGTC
Contig21-R1	CGTATTCCTCGAACCTCTC
CPR-dpF-1	GCNGARGGNTTYGCHAAGGC
CPR-R1	CTTGGACAATGGTGTGGAGAGTTC
3′ and 5′ RACE	
CYP81-3RACE1	TCCTAATGCAACACCCAGAG
CYP81-3RACE2	CTACCTAAGGGCCGTCGTGA
CYP81-5RACE1	CCGCTTCTCCTCCTTGGTGTCC
CYP81-5RACE2	CGTCGTTCTTGGTGAAGCACTCCTC
CPR-3RACE1	GCTCCATTCAGGGGTTTCC
CPR-3RACE2	GGTTAGCCCTGAAAGAATCC
CPR-5RACE1	ATCCATTTCGGGCCACAATGCCTCTC
CPR-5RACE2	TGCCTGTTCCCTAAGCCAAACACTGC
51544-3RACE1	GACGCCATGCGACTTCATCC
51544-3RACE2	TCCTTAACGGCTTACTTGCCAATA
qRT-PCR	
Hc81AA1-qF	TCTCGATGACCGTATGTGACTT
Hc81AA1-qR	CGTTAAGGAAGACCTCCCTCT
HcCPR2-qF	TGACTATGCTGCGGATGATGAA
HcCPR2-qR	GCCAAGAAGAGGACAACCAAATC
HcBPS-qF	AAGGAAAGAAGAGGGCTAGTGT
HcBPS-qR	ATGTGCTCGCTGTTAGTGTTC
Actin-qF	CGGCAGTGGTTGTGAACAT
Actin-qR	TCTCGCTGGTCGTGATCTG
Histone-H2A-qF	AACATCTACTCTTTGGACGACTTG
Histone-H2A-qR	AATTGCTGGAGGTGGAGTTATTC
Cloning*	
HcCPR2-*Bam*HI-F	GTAAT<u>GGATCC</u>GATGGAACCGACGGGGAGC
HcCPR2-*Hin*dIII-R	GTCTG<u>AAGCTT</u>TCACCATACGTCGCGAAGGTAC
Hc81AA1-*Eco*RI-F	ATT<u>GAATTC</u>GGATGGAGGACTTGTACTTGTACC
Hc81AA1-*Pac*I-R	ACG<u>TTAATTAA</u>TTAGAGCTGGGAAAGGAGACC
Hp81AA1-*Spe*I-F	TATG<u>ACTAGT</u>AATGGAGGACTTGTATCTGTACG
Hp81AA1-*Pac*I-R	ACG<u>TTAATTAA</u>TTAGAGCTGGGAGAGGAGG
Hp81AA2-*Eco*RI-F	ATT<u>GAATTC</u>ATGGACGGTTTATACTTAAAACTAGCC
Hp81AA2-*Pac*I-R	ACG<u>TTAATTAA</u>CTAGAGTTGGGAAAGGAGCTTG
Hp81AA2-*Spe*I-F	ATT<u>ACTAGT</u>AATGGACGGTTTATACTTAAAACTAGC
Hp81AA3-*Spe*I-F	TATG<u>ACTAGT</u>AATGGAATTGTATTTGTATCTAGCCG
Hp81AA3-*Pac*I-R	ACG<u>TTAATTAA</u>CTAAGAGAGTAGAGAACTGAGG

* Introduced restriction site sequences are underlined

Supplementary Table 8 | Primers used for C-terminal exchanges and site-directed mutagenesis.

Primer name	Sequence 5′→3′
Overlapping primers used for C-terminal exchanges	
AA1-AA2-ex	CGGAGGGATGGGGACCCACATTGTCTGCGAGACATTGGGAATG
AA2-AA1-ex	CCGGAATGGGAACCCATATCTCGTCCCTGGGGATTGGAATTCTTA
Primers used for site-directed mutagenesis (mutated positions are highlighted in red)	
AA1-A373S-F	CCGCCGTCCCCACTCTCCCTCCCCCACTTCTCCAACCAAG
AA1-A373S-R	GTGGGGACGGCGGGTACATCCTGAGCGTCTCCTTCACGAC
AA1-ASSL-F	CGCCGTCCCCACTCTTGCTCCCCCACTTCTCCAACCAAG
AA1-T482V-F	AGCCACGCGCGTGGTGTCGCGAGTCTGTCAAGGAAGAAG
AA1-T482F-F	AGCCACGCGCGTGGTTTCGCGAGTCTGTCAAGGAAGAAG
AA1-T482uni-R	ACGCGCGTGGCTCATGTCCTCGTCCATGCCGAGGTGGC
AA1-T482A-F	CGTGGTGCCGCGAGTCTGTCAAGGAAGAAGCCATTGGAG
AA1-T482A-R	TCGCGGCACCACGCGCGTGGCTCATGTCCTCGTCCATGC
AA1-TALMRKKN-F	CGTGGTGCCGCGAGTATGTCAAAGAACAAGCCATTGGAG
AA1-VIMT-F	GAATTATTACGATGATGTTTATTGCCGGGACCGAGACG
AA1-VIMT-R	TCATCGTAATAATTCCCTTGACGATGTCATCCGTG
AA1-W113F-F	TCGTGTTCGCCCCGCACGGCCCCATCTGGAAGAGCCTC
AA1-W113F-R	GGGGCGAACACGAGGAAGGTGTAGTCGTAGGTGAGGTGC
AA1-I220P-F	ATGACCCCATGCGACTTCATCCCGGTGATGAGGCTGATC
AA1-I220P-R	TCGCATGGGGTCATGGAGACGATAGGAAAGAAGAGATCC
AA2-A483T-F	GCCGGTACGGCTAGTATGTCCAAGAACAAGCCATTGG
AA2-A483T-R	TAGCCGTACCGGCTTGGTGGGTCATGTCCTCGTCCATAC
AA2-ATMLKRNK-F	GCCGGTACGGCTAGTCTGTCCAGGAAGAAGCCATTGG
AA2-L378S-F	CGCTCTCGCTACCTCGTTATTCGAGCGAGGCTTGCAC
AA2-L378S-R	GGTAGCGAGAGCGGGGACGGAGGATACAGCCTCATCGTC
AA2-S375A-F	CCTCCGGCCCCGCTCTTGCTACCTCGTTATTCGAGCGAG
AA2-S375A-R	GCGGGGCCGGAGGATACAGCCTCATCGTCTCCTTCACG
AA2-SALS-F	CCTCCGGCCCCGCTCTCGCTACCTCGTTATTCGAGCGAG

Supplementary Table 9 | Templates and primers used to generate mutations.

Template	Primers	Mutant
Mutants of CYP81AA1		
Wild-type CYP81AA1	AA1-A373S-F AA1-A373S-R	A373S
AA1-AA2	AA1-A373S-F AA1-A373S-R	AA1-AA2 A373S
A373S	AA1-AS-SL-F AA1-A373S-R	A373S/S376L
AA1-AA2 A373S	AA1-AS-SL-F AA1-A373S-R	AA1-AA2 A373S/S376L
A373S/S376L	AA1-T482A-F AA1-T482A-R	A373S/S376L/T482A
A373S/S376L	AA1-TALMRKKN-F AA1-T482A-R	A373S/S376L/T482A/L485M/R487K/ K488N
A373S/S376L	AA1-T482V-F AA1-T482uni-R	A373S/S376L/T482V
A373S/S376L	AA1-T482F-F AA1-T482uni-R	A373S/S376L/T482F
A373S/S376L/T482A	AA1-W113F-F AA1-W113F-R	W113F/A373S/S376L/T482A
CYP81AA1 A373S/S376L/T482A	AA1-I220P-F AA1-I220P-R	I220P/A373S/S376L/T482A
A373S/S376L/T482A/L485M/R487 K/K488N	AA1-VIMT-F AA1-VIMT-R	A373S/S376L/T482A/L485M/R487K/ K488N/V302I/M303T
A373S/S376L/T482A/L485M/R487 K/K488N/V302I/M303T	AA1-W113F-F AA1-W113F-R	W113F/A373S/S376L/T482A/L485M/ R487K/K488N/V302I/M303T
W113F/A373S/S376L/T482A/L485 M/R487K/K488N/V302I/M303T	AA1-I220P-F AA1-I220P-R	W113F/I220P/A373S/S376L/T482A/ L485M/R487K/K488N/V302I/M303T
Mutants of CYP81AA2		
Wild-type CYP81AA2	AA2-S375A-F AA2-S375A-R	S375A
Wild-type CYP81AA2	AA2-L378S-F AA2-L378S-R	L378S
Wild-type CYP81AA2	AA2-A483T-F AA2-A483T-R	A483T
S375A	AA2-SALS-F AA2-S375A-F	S375A/L378S
S375A	AA2-A483T-F AA2-A483T-R	S375A/A483T
L378S	AA2-A483T-F AA2-A483T-R	L378S/A483T
S375A/L378S	AA2-A483T-F AA2-A483T-R	S375A/L378S/A483T
S375A/L378S	AA2-ATMLKRNK-F AA2-A483T-R	S375A/L378S/A483T/M486L/K488R/ N489K

Supplementary Methods

Synthesis of standards. 1,3,7-Trihydroxyxanthone, 1,3,5-trihydroxyxanthone, and 1,3,5,6-tetrahydroxyxanthone were synthesized by the Grover, Shah and Shah method[1,2]. 2,2′,3,4′-Tetrahydroxybenzophenone and 2,2′,4,5′-tetrahydroxybenzophenone were prepared in the laboratory of Prof. Dr. Qidong You, Pharmaceutical University, Nanjing, China[3]. 2,3′,4,6-Tetrahydroxybenzophenone and 1,3-dihydroxyxanthone were from our laboratory collection[4]. 1,3,6,7-Tetrahydroxyxanthone was isolated from *H. perforatum in vitro* roots as previously reported[5]. All other chemicals were commercially available.

Supplementary References

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