New Phytologist

***New Phytologist* Supporting Information Figs S1–S6 and Tables S1 & S2**

Article title: Multifunctional oxidosqualene cyclases and cytochrome P450 involved in the biosynthesis of apple fruit triterpenic acids

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The following Supporting Information is available for this article:

Fig. S1 Chromatograms of typical LC-APCI-MS analysis of the products of MdOSC3.

Fig. S2 Expression analysis of triterpene biosynthetic genes (MdOSC1, MdOSC3, MdOSC4, MdOSC5) by real-time qPCR on RNA extracted from apple skin tissues.

Fig. S3 Mass spectra of lupeol, germanicol, β-amyrin, and α-amyrin.

Fig. S4 Chromatographic trace of extract of *Nicotiana benthamiana* leaf transiently transformed with MdOSC4 and the same sample spiked with taraxerol.

Fig. S5 Alignment of P450 predicted amino acid sequences from apple (CYP716A175) and other species.

Fig. S6 Mass spectra comparison (MS1, MS2, and MS3) between betulinic acid, ursolic acid, oleanolic acid, and a putative morolic acid.

Table S1 Primer sequences and properties of triterpene-related and housekeeping genes in this study.

Table S2 Summary of the skin triterpene composition of 20 apple cultivars

Table S1 Primer sequences and properties of triterpene-related and housekeeping genes used to (a) isolate oxidosqualene cyclases and (b) used on the panel of 20 apple cultivars

(a) Primers used to isolate Oxidosqualene Cyclases

Primer name	Sequence
attB1 F_OSC4_MR	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTGGAAGCTTAAGGTCG
attB2 R_OSC4_MR	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATCACGCTTTGGAAGG
attB1 F_OSC5_RG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTGGAAGCTTAAGGTCG
attB2 R_OSC5_RG	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGACATCAACAAATGGAACCC

(b) Primers used to measure gene expression by PCR

Genes	Accession Number	Primer Sequences (5'-3')	Size (bp)	T_m (°C)	E	References
eF-1α	AJ223969.1	F: ACTGTTCCTGTTGGACGTGTTG R: GAGTTGGAAGCAACGTACCC	208	82.5	1.861	Giorno *et al*. (2012)

IMPA-9	CN909679	F : TCGTGAACTCAGGCGCTTACTG	205	83.5	1.953	Giorno *et al*. (2012)
		R : AAGCAACGGTAAAGCGGGCAAC				
MdGAPDH	EB146750	F : TGAGGGCAAGCTGAAGGGTATCTT	185	82.5	1.874	Malladi & Hirst (2010)
		R: TCAAGTCAACCACACGGGTACTGT				
MdACTIN	EB127077	F: ACCATCTGCAACTCATCCGAACCT	185	82	1.919	Malladi & Hirst (2010)
		R: ACAATGCTAGGGAACACGGCTCTT				
MdOSC1	FJ032006.1	F : TTGTACTACTAATCCAGTGATCAAGATGTGG	238	84	1.857	Brendolise *et al*. (2011)
		R : CTCTCTTAGTATCTGAAAACGCCATAGGAG				
MdOSC3	FJ032008.1	F : GCAATCGTGATCAAAGAAGATGTGGAGG	232	84	1.882	Brendolise *et al*. (2011)
		R : TTCTCTTAAAATCTGAAAACGCCATAGG				
MdOSC4	KT383435	F : CTCTCGAAGTAACAATGAAGCACA	124	79	1.864	

		R : TAATCACCATTTGGGTCTTCAG			
MdOSC5	KT383436	F : TATGCATCCAGCAAAAATGTTT	152	77.5	1.851
		R : TCCAATTAATTTCGCCATAAGGT			
CYP716A175	EB148173	F : AGGCACGTTCCTCGCTTC	70	77.5	1.974
		R : CAAACCCTAAGAGGAGGGTCA			

Table S2 Summary of the skin triterpene composition of 20 apple cultivars separated into three russeting groups: russeted (four cultivars), semi-russeted (nine cultivars), and waxy (seven cultivars)

		Triterpene Acids (nmol g^{-1} DW)			Triterpene-caffeates (nmol g^{-1} DW)		
	Total Triterpenes	Ursolic acid	Oleanolic acid	Betulinic acid	Oleanolic acid -3-trans-caffeate	Betulinic acid -3-trans-caffeate	Betulinic acid -3-cis-caffeate
Russeted							
Average	6899^{a}	1569^{a}	800^{a}	1628^{b}	416^{b}	2115^{c}	371^{c}
Min–Max	4971–8075	670–2802	500–1270	1094–2095	248–511	1287–3722	266–586
Semi-russeted							
Average	5567^{a}	1981^{ab}	1515^{b}	1028^{ab}	205^{b}	693^{b}	145^{b}
Min–Max	4228–7636	1464–2504	991–1974	527–1712	72–406	291–1312	68–257
Waxy							
Average	5864^{a}	2839^{b}	2045^{b}	687^{a}	90^{a}	159^{a}	45^{a}
Min	3562–7379	1680–3816	1203–2540	324–834	23–175	82–252	25–73

Data are expressed in nmol g^{-1} DW. Data were obtained after HPLC-DAD analysis as described in the Materials and Methods section. Averages with no letter in common within a column are significantly different ($P < 0.05$; One-way ANOVA, Tukey's pairwise results).

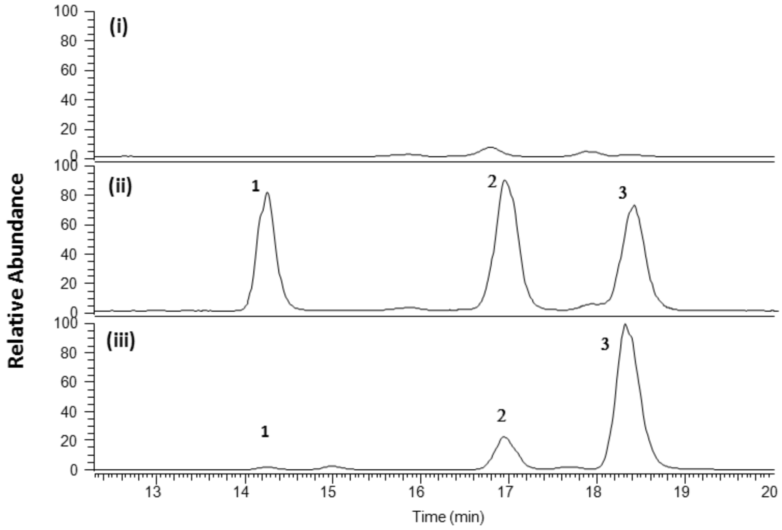

Fig. S1 Chromatograms of typical LC-APCI-MS analysis of the products of MdOSC3 (iii) after transient expression in *Nicotiana benthamiana*, following the methodology described in the Materials and Methods section. p19 was used as a negative control (i) and mixed with authentic standards at 20 µg ml^{-1} (1, lupeol; 2, β-amyrin; 3, α-amyrin) (ii). Compounds were identified and quantified on the basis of their mass spectral data (Fig. S3). Chromatograms are presented as selected ion plots of the m/z 409.8 [MH-H2O]+ ion.

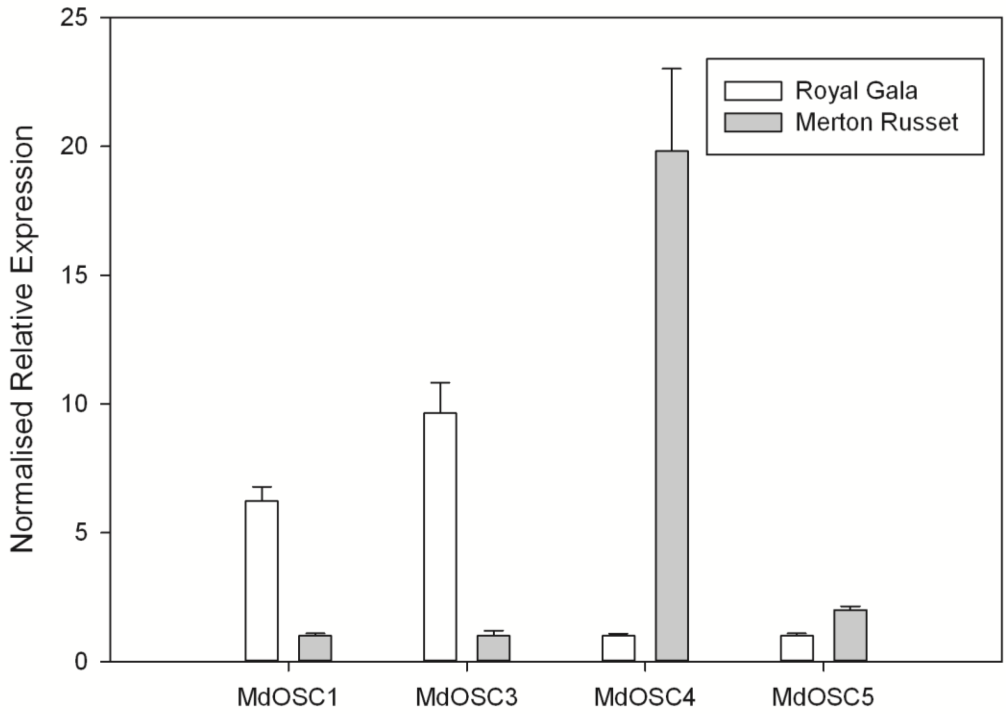

Fig. S2 Expression analysis of triterpene biosynthetic genes (MdOSC1, MdOSC3, MdOSC4, MdOSC5) by real-time qPCR on RNA extracted from apple skin tissues. Error bars show SE (n = 3).

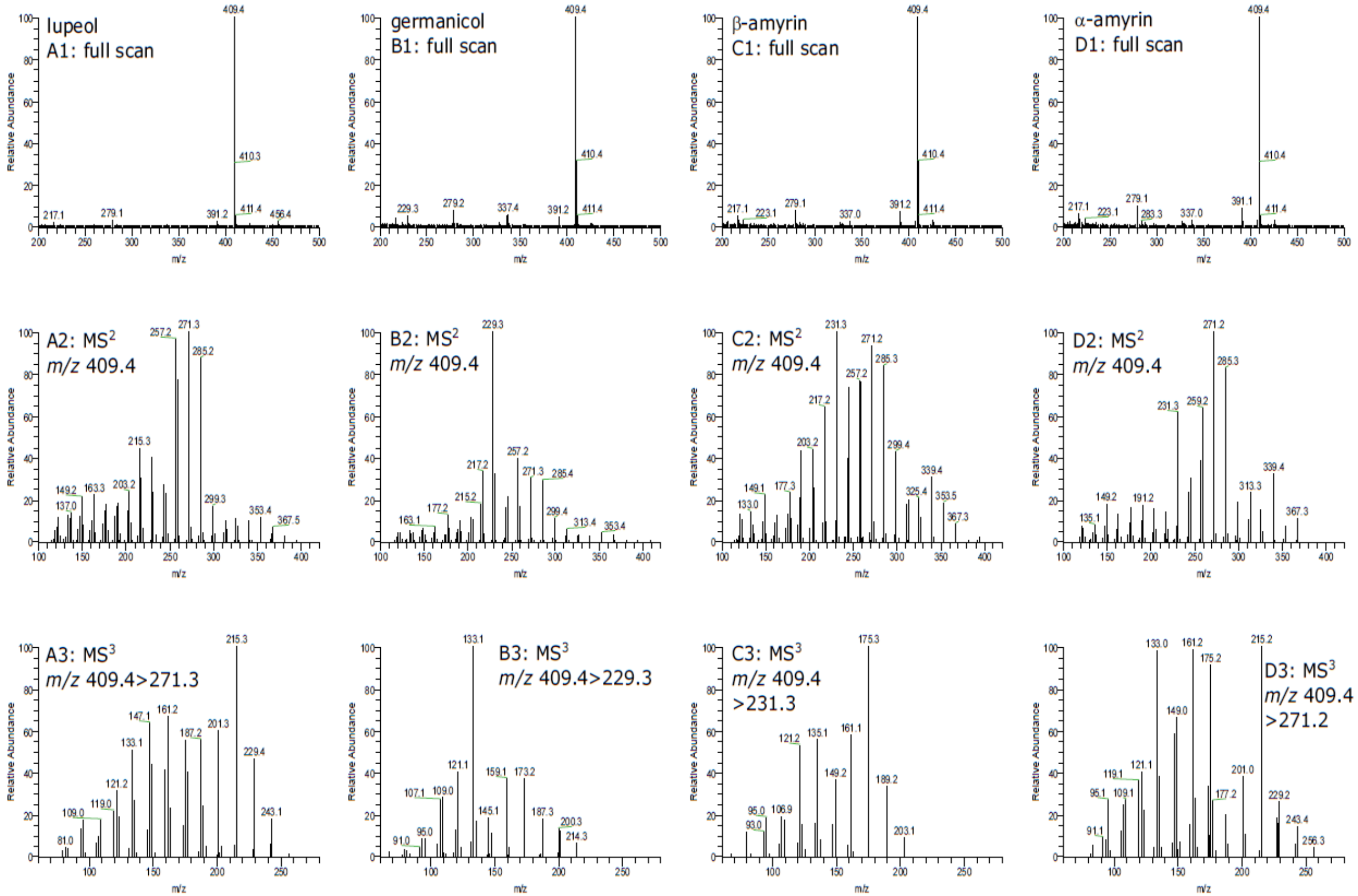

Fig. S3 Mass spectra comparison (MS1, MS2, and MS3) between lupeol, germanicol, β-amyrin, and α-amyrin.

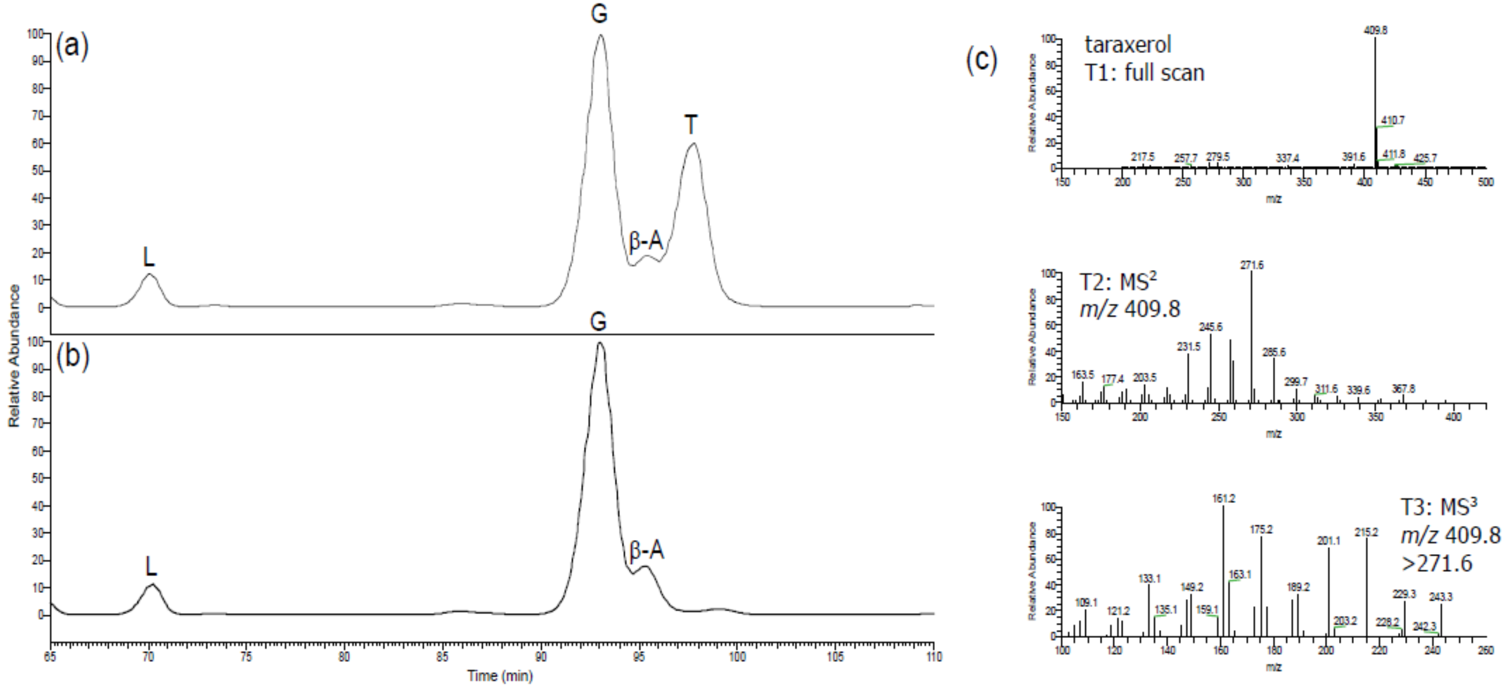

Fig. S4 Chromatographic trace of extract of *Nicotiana benthamiana* leaf transiently transformed with MdOSC4 (b) and the same sample spiked with taraxerol (T) (a) showing no taraxerol in the leaf sample. Mass spectra of the taraxerol peak (MS1, MS2, and MS3) (c). Transient expression of MdOSC4 (b) led to the formation of germanicol (G), β-amyrin (β-A), and lupeol (L). The presence of taraxerol (T) could therefore be excluded.

Fig. S5 Alignment of P450 predicted amino acid sequences from apple (CYP716A175) and other species. The prefix is the cytochrome P450 designation, and the suffix identifies the species as follows: At, *Arabidopsis thaliana*; Aa, *Artemisia annua*; Mt, *Medicago truncatula*; Vv, *Vitis vinifera*; Cp, *Carica papay*; Pt, *Populus trichocarpa*; St, *Solanum tuberosum*; Nt, *Nicotiana tobaccum*. GenBank (GB) numbers (or Arabidopsis identifiers) in the order listed in the alignment are: AT5G36140 (CYP716A2-At), AT5G36110 (CYP716A1–At), XP_002264643 (CYP716A20-Vv), XP_002280969.1 (CYP716A19-Vv), XP_002309057.1 (CYP716A8-Pt), ABC94483.1 (CYP716A14-Aa), XP_002324668.2 (CYP716A3-Pt), XP_002325241 (CYP716A6-Pt), XP_006338129 (CYP716A13-St), no GB identifier (CYP716A36-Nt), CBN88268.1 (CYP716A12-Mt), BAJ84106 (CYP716A15-Vv), BAJ84106 (CYP716A17-Vv), no GB identifier (CYP716A16-Cp). The text/background colour code refers to the degree of similarity between the different sequences: red/yellow, identical; dark blue/turquoise, conservative; black/green, block of similar; black/white, nonsimilar.

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 CYP716A1-At   ------MYMAIMIILFLSS-ILLSLLLLLRKHLSHFSYPNLPPGNTGLPL
CYP716A20-Vv   -MELLLLSFLLLMALSLS--FWIRFFVHKLEKSSG---INLPPGKMGFPF
CYP716A19-Vv   -MELSLLHILPWATLFTT--LSLSFLIYKLMIISHGTPRNLPSGNTGLPY
 CYP716A8-Pt   -MELPFISLLPYGILFIISAVSLSYLINKHKYYLS-SLNNLPPGNTGLPL
CYP716A14-Aa   MVDLILIYSIFLSLIVVLVPLSLYFVFYKSKPVVD---RKLPPGQTGWPV
 CYP716A3-Pt   --------------------------------------------------
 CYP716A6-Pt   ----MQNFFLNLLLAVFFITFFISIIVYKHRSKFK--HPNLPPGSSGLPY
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CYP716A12-Mt   --MEPNFYLSLLLLFVSFISLSLFFIFYKQKSP-----LNLPPGKMGYPI
CYP716A15-Vv   ---MEVFFLSLLLIFVLSVSIGLHLLFYKHRSHFT--GPNLPPGKIGWPM
CYP716A17-Vv   ---MEVFFLSLLLIFVLSVSIGLHLLFYKHRSHFT--GPNLPPGKIGWPM
CYP716A16-Cp   ---MEHLYLTLLLAFVSFVSLSLFIIFYKHRSPYA--YPNLPPGKIGLPF
  CYP716A175   ---MEHFYLTLLLGFVSFITLSLSVLFYRHRAQFV—GTNLPPGKVGYPV

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CYP716A20-Vv   IGESLEFLRMGRKGTPERFIQDRMAKYSTQ---IFKTCLLGEPTAVVCGA
CYP716A19-Vv   IGESIQFLSNGRKGHPEKFISERMLKFSSK---VFKTSLFGETAAVFCGS
 CYP716A8-Pt   IGESLEFLTTGQKGQPEKFILDRMAKFSSK---VFKTSLFCEPTAVFCGA
CYP716A14-Aa   IGETLEFLTNGWKGHPEKFIFDRMARFSPH---VFKTSLMLEDAAVFCGS
 CYP716A3-Pt   ----------------------------------------------MLNGV
 CYP716A6-Pt   IGETLELLLTGCKGHPQKFFLDRKAKYASE---VFKTNLFCQPAAVLCGA
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CYP716A36-Nt   IGESFEFLSTGWKGHPEKFVFDRMTKYSSS---VFKTHLLGEKAAVFCGP
CYP716A12-Mt   IGESLEFLSTGWKGHPEKFIFDRMRKYSSE---LFKTSIVGESTVVCCGA
CYP716A15-Vv   VGESLEFLSTGWKGHPEKFIFDRISKYSSE---VFKTSLLGEPAAVFAGA
CYP716A17-Vv   VGESLEFLSTGWKGHPEKFIFDRISKYSSE---VFKTSLLGEPAAVFAGA
CYP716A16-Cp   VGESLEFLSTGWKGHPEKFIFDRIARYSSV---IFKTNLLGEPAAVFCGA
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  CYP716A19-Vv   AGNKFLFSNENKLVTAWWPSSVNKIFPSSLQTSSQ-EESKKMRKLLPGFL
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  CYP716A14-Aa   AGNKFLFSNENKLVKAWWPASVEKILPSAKETTNQ-------RKMLSRSF
   CYP716A3-Pt   AGNKFLFSNENRLVETWWPEFVNKIFPSAVEKSPK-EEAKRMRRLFPPFL
   CYP716A6-Pt   AGNRFLFSNKNKVLKAWYPDFVCKIFPSSVQRPLI-EQVDRLRTLLPELL
  CYP716A13-St   SGNKFLFSNENKLVQAWWPNSVNKVFPSSTQTSSK-EEAIKMRKMLPNFF
  CYP716A36-Nt   TCNKFLFSNENKLVQAWWPDSVNKVFPSSTQTSSK-EEAIKMRKMLPNFF
  CYP716A12-Mt   ASNKFLFSNENKLVTAWWPDSVNKIFPTTSLDSNLKEESIKMRKLLPQFF
  CYP716A15-Vv   AGNKFLFSNENKLVHAWWPSSVDKVFPSSTQTSSK-EEAKKMRKLLPQFF
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  CYP716A20-Vv   KPEALQKYVGIMDSIAKWHLDNHWDLNETVTVFPLAKQYTFMVACRLFLS
  CYP716A19-Vv   KPEALQRYISIMDVIAQRHFESSWNNKEEVTVFPLAKMFTFWLACRLFLS
   CYP716A8-Pt   KPESLQRYISVMDVIAQRHLASDWEGKQEVSVFPLAKTYTFWLACRLFLS
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  CYP716A13-St   KPEALQRYVGIMDHITQRHFASGWENKEQVVVFPLTKRYTFWLACRLFLS
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  CYP716A12-Mt   KPEALQRYVGVMDVIAQRHFVTHWDNKNEITVYPLAKRYTFLLACRLFMS
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     CYP716A175  KPEALQRYIGIMDTVARRHFAEGWENKKEVEVFPLAKNYTFWLAARLFVS

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   CYP716A1-At   MEDPARVRQLEEQFNTVAVGIFSIPIDLPGTRFNRAIKASRLLRKEVSAI
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   CYP716A3-Pt   IEDPSHIARFSHPFNHITSGIFTIPIAFPGTPFNRAIKATKLIRIELLAI
   CYP716A6-Pt   IEDPDHIAKLASPFNLVVSGIFSIPIDLPGTPLSRAIKASTIIRTELFAI
  CYP716A13-St   VEDPNHVAKFADPFDVLASGLISIPIDLPGTPFNRAIKASNFIRKELVRI
  CYP716A36-Nt   VEDPNHVAKFADPFNVLASGLISIPIDLPGTPFNRAIKASNLIRKELLLI
  CYP716A12-Mt   VEDENHVAKFSDPFQLIAAGIISLPIDLPGTPFNKAIKASNFIRKELIKI
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  CYP716A17-Vv   IEDPAHVAKFEKPFHVLASGLITVPIDLPGTPFHRAIKASNFIRKELRAI
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   CYP716A8-Pt   IKQRKIDLAENKASPT-QDILSHMLTTADDNGQCMKKIDIADKILGLLVG
  CYP716A14-Aa   IKQRKIDLADGKASPT-QDILSQMLCDEESQN--IAEADTADVIIGLLIG
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  CYP716A17-Vv   IKQRKIDLAEGKASQN-QDILSHMLLATDEDGCHMNEMEIADKILGLLIG
  CYP716A16-Cp   IKQRKIDLGNGSASPT-QDILSHMLLTSDESGQFMSELDIADKILGLLIG
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CYP716A20-Vv   KMKYSWNVANEVMRLAPPLQGSFREAITDFTYAGFSIPKGWKFLYWSTNA
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CYP716A8-Pt    RMRYSWNVACEVMRVAPPLQGAFREAMTEFNYAGFTIPKGWK-LYWSANT
CYP716A14-Aa   KMKYSWNVACEVLRLAPPLQGSFREAMTDFVYNGYSIPKGWK-LYWSALS
CYP716A3-Pt    KMKYSWNVACETLRLSPPFIGNFKEAIKDFTFNGFAIPKGWKASHFLTLY
CYP716A6-Pt    NMTYSWNVIREVLRLCPTFP-NVREAIHDFDFNGFSIPKGWKAKYFP---
CYP716A13-St   KMKYSWNVACEVLRLAPPLQGAFREALSDFIFNGFSIPKGWK-IYWSANS
CYP716A36-Nt   KMKYSWNVACEVLRLASPLQGAFREAINDFIFNGFYIPKGWK-LYWSANS
CYP716A12-Mt   KMKYSWNVACEVMRLSPPLQGGFREAITDFMFNGFSIPKGWK-LYWSANS
CYP716A15-Vv   NMRYSWNVACEVMRLAPPLQGAFREAITDFVFNGFSIPKGWK-LYWSANS
CYP716A17-Vv   KMRYSWNVACEVMRLAPPLQGAFREAITDFVFNGFSIPKGWK-LYWSANS
CYP716A16-Cp   KMKYSWNVACEVLRLAPPLQGAFRQALYDFIFDGFSIPKGWK-LYWSANS
CYP716A175     KMKYSWNVAQEVLRLAPPLQGAFREALSDFVFNGFTIPKGWK-LYWSANS

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CYP716A2-At    THK-----NPEYFPEPEKFEPSRFEGSGPKPYTYVPFGGGSRICPGREYA
CYP716A1-At    THM-----NPDYFPEPERFEPNRFEGSGPKPYTYVPFGGGPRMCPGKEYA
CYP716A20-Vv   THK-----NPDYFPDPEKFDPSRFEGNGPIPYTYVPFGGGPRMCPGKEYA
CYP716A19-Vv   THR-----NPEFFPKPEKFDPSRFDGKGPAPYTYVPFGGGPRMCPGKEYA
CYP716A8-Pt    THK-----NPECFPEPENFDPSRFEGNGPAPYTFVPFGGGPRMCPGKEYA
CYP716A14-Aa   THK-----NPEVFTEPQKLDPSRFDGKGPAPYTFVPFGGGPHMCPGREYA
CYP716A3-Pt    WSASSTHKNPEYFSEPEKFDPSRFEGKGPAPYTFIPFGGGPRMCPGNEYA
CYP716A6-Pt    ---------------EPERFDPSRFEGTGPAPYTFVPFGGGPMMCPGQGFA
CYP716A13-St   THK-----SGEFFPDPEKFDPSRFEGSGPAPYTFVPFGGGPRMCPGKEYA
CYP716A36-Nt   THK-----NPEYFPEPQKFDPSRFEGSGPAPYTFVPFGGGPRMCPGKEYA
CYP716A12-Mt   THK-----NAECFPMPEKFDPTRFEGNGPAPYTFVPFGGGPRMCPGKEYA
CYP716A15-Vv   THK-----SPECFPQPENFDPTRFEGNGPAPYTFVPFGGGPRMCPGKEYA
CYP716A17-Vv   THK-----SPECFPQPENFDPTRFEGDGPAPYTFVPFGGGPRMCPGKEYA
CYP716A16-Cp   THK-----DPECFPEPEKFDPTRFEGNGPAPYTFVPFGGGPRMCPGKEYA
CYP716A175     THK-----NAAYFPEPFKFDPTRFEGNGPAPYTFVPFGGGPRMCPGKEYA

               451                                               502
CYP716A2-At    RLEILIFMHNLVKRFKWEKVFPKENKLVADPAPIPAKGLPI-----------
CYP716A1-At    RLEILIFMHNLVNRFKWEKVFPNENKIVVDPLPIPDKGLPI-----------
CYP716A20-Vv   RLEILVFIHNVVRRFSWYKLHPNE-DVIVDPMPMPAKGLPIRLRHH------
CYP716A19-Vv   RLEVLVFMHNLVRRFKWEKLLPDE-KIIVDPMPIPAKGLPIRLHHHQP----
CYP716A8-Pt    RLEILVFLHNLVKKFRWEKLLPKE-RIIVDPMPIPSKGLPIRLHPHEAA---
CYP716A14-Aa   RLEILVFMHHLVIKYKWEKVIPNE-QIIVNPMPKLAKGLPLRLYPRYA----
CYP716A3-Pt    RLEILVFMHNLVKRFKFERLVLDE-KIVFDPTPKPERGLPVRLLPHKA----
CYP716A6-Pt    RLEMLIFMHNLVKRFKFDKFVAEE-KIMFSPMPIPEKGVPIRLFPHRP----
CYP716A13-St   RLEILVFMHHLVKRFKFEKIIPDE-KIIVNPMPIPAKGLPVRLYPHHHKP--
CYP716A36-Nt   RLEILVFMHHLVKRFKWEKVIPNE-KIVVDPMPIPAKGLPVRLYPHKA----
CYP716A12-Mt   RLEILVFMHNLVKRFKWEKVIPDE-KIIVDPFPIPAKDLPIRLYPHKA----
CYP716A15-Vv   RLEILVFMHNVVKRFKWDKLLPDE-KIIVDPMPMPAKGLPVRLHPHKP----
CYP716A17-Vv   RLEILVFMHNVVKRFKWDKLLPDE-KIIVDPMPMPAKGLPVRLHPRKP----
CYP716A16-Cp   RLEILVFMHNVIKRFKWEKVLPDE-KIIVDPMPVPAKGLPVRLYPHAHAL--
CYP716A175     RLEILVFMHNLVKRFKWEKVLPDE-QIVVDPLPMPAKGLPVRLFPHPKTATT
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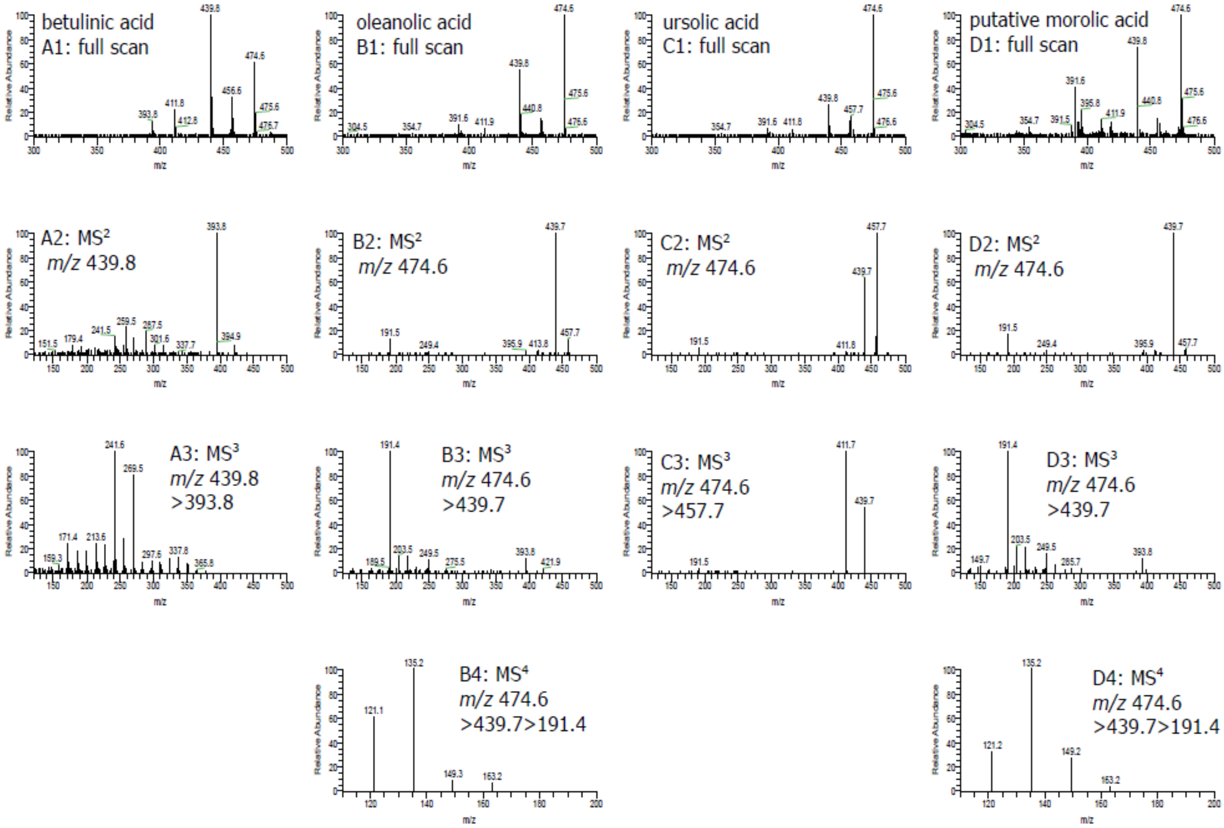

Fig. S6 Mass spectra comparison (MS1, MS2, and MS3) between betulinic acid, ursolic acid, oleanolic acid, and a putative morolic acid.

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