

<Electronic Supporting Information>

Supplementary Figure S1. Amino acid alignment of various oxidosqualenes

		*	20	*	40	*	60	*	80	*	100	
EtAS	:	MWKLKIAE-----	GG-NDEYLYSTN	-NYVGRQTWVFD	PQ-PPT	PQELAQVQQA	RNLNFYNNRY	HVKPSSDLLW	RQFLREK	-NFKQTIPQ	AKINEGE	: 87
PNY1	:	MWKLKIAE-----	GNKNDPYLYSTN	-NFVGRQTWEF	DPDYVAS	PGELEEVQV	RRQFWDNRY	QVKPSGDLL	WRMQFLREK	-NFRQTIPQ	VKVGDD	: 89
BPY	:	MWRLKIAE-----	GG-SDPYIYSTN	-NFVGRQTWEF	DPQ-AGS	PQERAEVBE	ARRNFYDNRY	QVKPSGDLL	WRMQFLREK	-NFKQTIPQ	VKVEDGE	: 87
PSY	:	MWRLKIAE-----	GG-NDPYLFSTN	-NFVGRQTWEY	DPE-AGS	EEERAQVBE	ARRNFYNNR	FEVKPCGDLL	WRQVLRN	-NFKQTIGG	VKIEDDE	: 87
AtLUP1	:	MWKLKIGK-----	GNGEDPHLFSSN	-NFVGRQTWKF	DHK-AGS	PEERAABE	ARRGFLDN	RFRVKGCS	DLLWRMQ	FLREK-KFE	QGIPLKAT	: 88
TRW	:	MWKLKIAE-----	GG-DDEWLT	TTN-NHVGRQH	WQFDPD	-AGTEEERAE	IEBKIRLN	NFKLNRFQ	FKQSADLL	MRTQLR	KEN-PINKI	: 87
OEW	:	MWKLKIAE-----	G--TGPWL	TTN-NHIGRQH	WQFDPD	-AGTPDERVE	VBRLREEF	KKNRERT	KQSADLL	MRLMQL	VKEN-QRVQI	: 86
BPW	:	MWKLKIAE-----	GG-PG--LV	SGN-DFIGRQH	WQFDPD	-AGTPQERAE	VBKVREEF	TKNRFQ	MKSADLL	MRLMQL	VKEN-PCQPI	: 85
AtCAS1	:	MWKLKIAE-----	GGSPWL	RTTN-NHVGRQ	FWQFDPN	-LGTPEDLAA	VBARKS	FSDNRFV	QKHSADLL	MRLQFS	REN-LISPV	: 86
HsLAS	:	MTEGTC	LRRRGGPYKTE	-PATDLGR	WRRLN-CERGR	QTWTYLQ	-DERAGRE	QTGLEAY	ALGLD	TKNY-----	FKDLP	: 68
ScLAS	:	MTEF-----	YSDTIGL	PKTDPRL	WRRLRT	DELGRES	WEYL----	TPQQA	-----	NDPPST	FQTQWLLQ	: 75
SHC	:	M-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	: 2
AtLUP2	:	MWKLKIGE-----	GNGEDPYLFSSN	-NFVGRQTWEF	DPK-AGT	PEERAABE	DARNYLDNR	PRVKGCS	DLLWRMQ	FLKEA-KFE	QVI PPVK	: 88
OsOSC6	:	MWRLKIAAESGG	-----	GS	GSSPLLHTGN	-GFLGRAV	WEFDPD	-AGTPEERA	EVARLR	RDRD	TRHREFQ	: 92
		Mw lki		n	gr w d		r	nr	k	dll r q		
		*	120	*	140	*	160	*	180	*	200	
EtAS	:	D--ITYEK	ATTALRR	AVHFFS	ALQASD	-GHWPA	ENAGELF	FLPPLVM	CCLYIT	GHDDT	VFPAPHR	: 183
PNY1	:	A--VTYEA	ATTTLRR	AVHFFS	ALQASD	-GHWPA	ENSGELF	FLPPLVM	CVYIT	GHDDT	VFPAPHR	: 185
BPY	:	E--ITYEK	STAAALRR	AVHFFS	ALQASD	-GHWPA	ENAGELF	FLPPLVM	CMYIT	GHDDT	VFPAPHR	: 183
PSY	:	E--ITYEK	TTTTLRR	GTHHLA	TLOTSD	-GHWPA	QIAGELF	FMPPLV	FCVYIT	GHDDT	VFPAPHR	: 183
AtLUP1	:	E--ITYET	TNTALRR	GVRYET	ALQASD	-GHWPG	EITGELF	FLPPLIF	CLYIT	GHDDT	VFPAPHR	: 184
TRW	:	E--VTND	AVTTTLK	RAISFY	STIQAH	D-GHWPA	ESAGELF	FLPPLV	IALLY	VTGAM	NDILTPA	: 183
OEW	:	G--ITEE	AVITTLR	RAISFY	STIQAH	D-GHWPA	ESAGELF	FLPPLV	LALYV	TGAIN	VLSREH	: 182
BPW	:	V--ITEE	AVITTLR	RSLSFY	SSIQAHD	-GHWPG	ESAGELF	FLQPFV	MALYIT	GDINT	IFSPA	: 181
AtCAS1	:	D--VTEE	MOVETTL	KRGLDF	YSTIQAH	D-GHWPG	DYGGEM	FLPLGL	ITLIT	SGALNT	VLSEQH	: 182
HsLAS	:	KAHTA	FEFEGAL	----	NGMTFY	VGLQAED	-GHW	TGDYGG	PLFLPL	GLLIT	CHVA--	: 159
ScLAS	:	-----	ACHNGA	SFEKLL	QEPD	SGIFPC	QYKGEM	FMTIGY	VAVNYI	AG---	IEIPE	: 161
SHC	:	EQLVE	APAYART	LDR	AVEYLL	SCQ-KDE	GYW	GPLLSN	VMTMAE	YVLL	CHILDR	: 96
AtLUP2	:	G--ITYK	NATDALRR	AVSFY	SALQSSD	-GHWPA	EITGELF	FLPPLV	FCFYIT	GHDDT	VFPAPHR	: 184
OsOSC6	:	N--VTEE	TILSSLR	RALNQ	STLQAH	D-GHWPG	DYSGILF	IMPLIL	FMSH	VTGTL	DVLSLEH	: 188
		t	l r	Q D Gns	p 16	6tg	h	eb kyby	nqne	DGgw	g h eg st 6f 3 l Y	

Figure 10: Multiple sequence alignment of the PNY1 protein (283 amino acids) with its orthologs from Arabidopsis thaliana (AtLUP1, 288 aa), Arabidopsis lyrata (AtLAS1, 280 aa), Arabidopsis lyrata (AtLAS2, 255 aa), Arabidopsis lyrata (AtLAS3, 257 aa), Arabidopsis lyrata (AtLAS4, 189 aa), Arabidopsis lyrata (AtLAS5, 283 aa), Arabidopsis lyrata (AtLAS6, 285 aa), Arabidopsis lyrata (AtLAS7, 379 aa), Arabidopsis lyrata (AtLAS8, 380 aa), Arabidopsis lyrata (AtLAS9, 379 aa), Arabidopsis lyrata (AtLAS10, 379 aa), Arabidopsis lyrata (AtLAS11, 377 aa), Arabidopsis lyrata (AtLAS12, 377 aa), Arabidopsis lyrata (AtLAS13, 376 aa), Arabidopsis lyrata (AtLAS14, 376 aa), Arabidopsis lyrata (AtLAS15, 346 aa), Arabidopsis lyrata (AtLAS16, 349 aa), Arabidopsis lyrata (AtLAS17, 271 aa), Arabidopsis lyrata (AtLAS18, 380 aa), Arabidopsis lyrata (AtLAS19, 381 aa). The alignment shows conserved regions across the sequences, with positions 220, 240, 260, 280, and 300 highlighted. The PNY1 Y261 mutation is indicated by a green box.

F474

		*	420	*	440	*	460	*	480	*	!
EtAS	:	CM	-AEDPNGVPEKKHLARI	PDYMWVAEDGMKMQSF	-GSQQWDTGFAIQALLASNDTE	--EIGQVLKKGHDEIKKSQVKENP	-SGDFKSMHRHISKGSWIF	:	474		
PNY1	:	CM	-VEDPNGDYFRKHLARI	PDYIWWAEDGMKMQSF	-GSQEWDTGFSIQALLSDLTHT	--EIGPTLMKGHDEIKKSQVKDNP	-SGDFKSMYRHISKGSWIF	:	475		
BPY	:	CM	-VEDPNGDYFKKHLARI	PDYIWWAEDGIKMQSF	-GSQEWDTGFAIQALLASNDTD	--EIGPTLARGHDEIKKSQVKDNP	-SGDFESMHRHISKGSWIF	:	474		
PSY	:	CM	-VEDPNGDAFKKHLARV	PDYLMWISEDGMTMQSF	-GSQEWDTGFAVQALLATNDIE	--EIKPALAKGHDEIKKSQVTENP	-SGDFKSMHRHISKGSWIF	:	474		
AtLUP1	:	CM	-VENPNGDYFKKHLARI	PDYMWVAEDGMKMQSF	-GCQLWDTGFAIQALLASNDLP	--ETDDALKRGHNYIKASQVREN	-SGDFRSMYRHISKGSWIF	:	472		
TRW	:	TM	-VEDPNGDAYKRHLARI	PDYFWVAEDGMKMQSF	-GCMWDAAFAIQAFSSNDTE	--EYGPTLKKAHEFVKASQVRDNP	-PGDFSKMYRHTSKGSWIF	:	474		
OEW	:	CM	-VEDPNSEAYKRHLARI	PDYFWVAEDGLKMQSF	-GCMWDAAFAIQAILSSNDLAE	--EYGPTLMKAHNEVKASQVQENP	-SGDFNEMYRHTSKGSWIF	:	472		
BPW	:	CM	-AEDPNGEAYKLHLGRIP	PDNYWVAEDGLKIQSF	-GCMWDAGFAIQAILSCNINE	--EYWPTLRKAHEFVKASQVPENP	-SGDFKAMYRHINKGSWIF	:	471		
AtCAS1	:	CM	-VEDPNSEAEKLHLPRIH	DFLWLAEDGMKMQGYNGS	QLWDTGFAIQAILATNDIVE	--EYGPVLEKAHSEVKNSQVLEDC	-PGDLNYWYRHISKGSWIF	:	472		
HsLAS	:	RM	YVDGPASTAEQEHVSRI	PDYLMWGLDGMKMQGTNGS	QIWDTAFAIQALLEAGGHRPE	FFSSCLQKAHEFLRLSQVPDNP	-P-DYQKYRQMRKGGEF	:	444		
ScLAS	:	TL	IEEGVDSEAEQRLQYRF	KDALFHGPGQMTIMGTNGV	QTDCAFAIQYFFVAGLAERPE	EFYNTIVSAYKELCHAQFDTE	CVPGS----YRDKRKGAMCE	:	445		
SHC	:	LK	ILDMTQHPAEIKGWEGL	ELYGVELDYGGWVFQASIS	PVWDTGLAVLALRAAGLPADHD	---RLVKAGEWLLDRQIT---	VPGDWAVKRPNLKPGGEAF	:	365		
AtLUP2	:	CM	-IENPNGDHEKKHLARI	PDYMWVAEDGLKMQSF	-GSQLWDTVFAIQALLACDLS	SD--ETDDVLRKGHSFIKKSQVREN	-SGDFKSMYRHISKGSWIL	:	475		
OsOSC6	:	CM	-IEDPNSDAEKRHLPR	IYDFLWLAEDGMKAQVYD	GCQWETAETVQAICSTGLVD	--EFSTTLEKAYGELKNSQVLHDL	PNG--KSFYRHRSGSWIL	:	476		
			w e p 5 h r d	edG k q	g q Wd fa6qa	l e 6 h 56 sq	gd rh kG 5 f				

D⁴⁸⁶CTAE

		*	520	*	540	*	560	*	580	*	600
EtAS	:	SD	QDHGMQVSDCTAEGLKCCLL	FSMMPPEIVGEKMDAQHLY	NAVNILISLQS----	KNGGLAWE	PAGAQQWLEMLNPTEFE	ADIVIEHEHYVICTASAI	:	569	
PNY1	:	SD	QDHGMQVSDCTAEGLKCCLL	FSTMPPEIVGKKIKPERLY	DSNVNLLSLQR----	KNGGLSAWE	PAGAEWLELLNPTEFE	ADIVIEHEHYVICTSSAI	:	570	
BPY	:	SD	QDHGMQVSDCTAEGLKCCLL	FSIMPPPEIVGEKMEPEQLY	DSNVNLLSLQS----	KNGGLAWE	PAGAEWLELLNSTEFE	ADIVIEHEHYICTASAM	:	569	
PSY	:	SD	QDHGMQVSDCTAEGLKCCLL	LSLLPPPEIVGEKMEPERL	FDNVNLLSLQS----	KNGGLAWE	PAGAEWLELLNPTEFE	ADIVVEHEHYVICTGSAI	:	569	
AtLUP1	:	SD	RDHGMQVSDCTAEALKCCLL	LSMMMSADIVGQKIDDEQLY	DSNVNLLSLQS----	GNGGVNAWE	PSRAYKWLELLNPTEFM	ANTMVEREFVICTSSVI	:	567	
TRW	:	SI	QDHGMQVSDCTAEGLKVSLL	YSQMNP-KLVGEKVETEHL	YDAVNVILSLQS----	ENGGFPAWE	PQRAYAWLEKFNPTFE	FEDVLIEREHYVICTSSAI	:	569	
OEW	:	SM	QDHGMQVSDCTAEGLKAALL	FSQMPPPELVGAETETGHLY	DAVNVILTSLQS----	ASGGFPAWE	PQKAYRWLEKLNPTFE	FEDVLIERDYVICTSSAV	:	567	
BPW	:	SM	QDHGMQVSDCTAEGLKVAIL	FSQMPPDLVGEKIEKERLY	DAVNVILSLQS----	SNGGFPAWE	PQRAYGWLEKFNPTFE	FEDTLIEREHYVICTSPAV	:	566	
AtCAS1	:	ST	ADHGMPISDCTAEGLKAALL	LSKVPKAIVGEPIDAKRLYE	AVNVIIISLQN----	ADGGLATYEL	TRSYPMLELINPAETEG	DIVIDYPYVICTASAI	:	567	
HsLAS	:	ST	LDGGMIVSDCTAEALKAVLL	LQEKCP-HVT-EHIPRERLC	DAVAVLLNMRN----	PDGGFATYET	KRGHLELLNPSEVEGD	IMIDYTYVICTSAMM	:	538	
ScLAS	:	ST	KTQGYTVADCTAEAIKAI	IMVKNSPVFSEVHHMIS	SERLFEIGIDVLLNLQNI	GSFEYGSFATY	EKIKAPLAMETLNPAE	VEGDMIVEYPYVICTSSV	:	545	
SHC	:	QF	DNVYYPDVDDTA-----	VVWALNTLRLPDERRRR	DAMTKGFRWIVGMQS----	SNGGWGAYD	VDNNTSDLPNHI-PFCDE	GEVT-DPPSEIVTAHVL	:	453	
AtLUP2	:	SD	RDHGMQVSDCTAEALKCCML	LSMMPAEVVGQKIDPEQLY	DSNVNLLSLQG----	EKGGLTAW	EPVRAQEWLELLNPTE	DEETCVMAEREHYVICTSAVI	:	570	
OsOSC6	:	ST	ADNGMSVPE	DCGETLQALLGLSKISP	-KLVGDPKKEKSLYDAVD	CLLSFSN----	KDGTFSSECTRTASWTE	ILNPSESERNIVVDYPH	VICTSSAI	571	
			s g5 sDCtae k 6 s	vg 6 66 q	Gg 5e	w e np e f	EtAS 564C	ect	6		

D485C486TAE involved in EtAs β -amyrin synthase

		*	
EtAS	: -----	:	-
PNY1	: -----	:	-
BPY	: QVVNCIGQSLYKKYK	:	779
PSY	: -----	:	-
AtLUP1	: -----FIVN---	:	757
TRW	: -----QNI--	:	758
OEW	: -----LHAQT	:	758
BPW	: -----LEA--	:	755
AtCAS1	: -----LLQQGE	:	759
HsLAS	: -----	:	-
ScLAS	: -----	:	-
SHC	: -----	:	-
AtLUP2	: -----FATHQDL	:	763
OsOSC6	: -----VASKD	:	760

The triterpene cyclases amino acids sequences were aligned using Clustal W, as implemented in the (CLC Sequence Viewer (CLC bio), and the figure was made by GenDoc (<http://www.nrbsc.org/gfx/genedoc/>). EtAS: *Euphorbia tirucalli* β -amyrin synthase (AB206469),¹ PNY1: *Panax ginseng* β -amyrin synthase (AB009030),² BPY: *Betula platyphylla* β -amyrin synthase (AB055512),³ PSY: *Pisum sativum* β -amyrin synthase (AB034802),⁴ AtLUP1: *Arabidopsis thaliana* multifunctional triterpene cyclase (At1g78970),⁵ TRW: *Taraxacum officinale* lupeol synthase (AB025345),⁶ OEW: *Olea europaea* lupeol synthase (AB025343),⁶ BPW: *Betula platyphylla* lupeol synthase (AB055511),³ AtCAS1: *Arabidopsis thaliana* cycloartenol synthase (At2g07050),⁷ HsLAS: *Homo sapiens* lanosterol synthase (P48449),^{8,9} ScLAS: *Saccharomyces cerevisiae* lanosterol synthase (P38604),¹⁰ SHC: *Alicyclobacillus acidocaldarius* squalene-hopene cyclase,^{11,12} AtLUP2: *Arabidopsis thaliana* multifunctional triterpene cyclase (At1g78960),¹³ OsOSC6: *Oryza sativa* Achilleol B synthase (AK070534)¹⁴

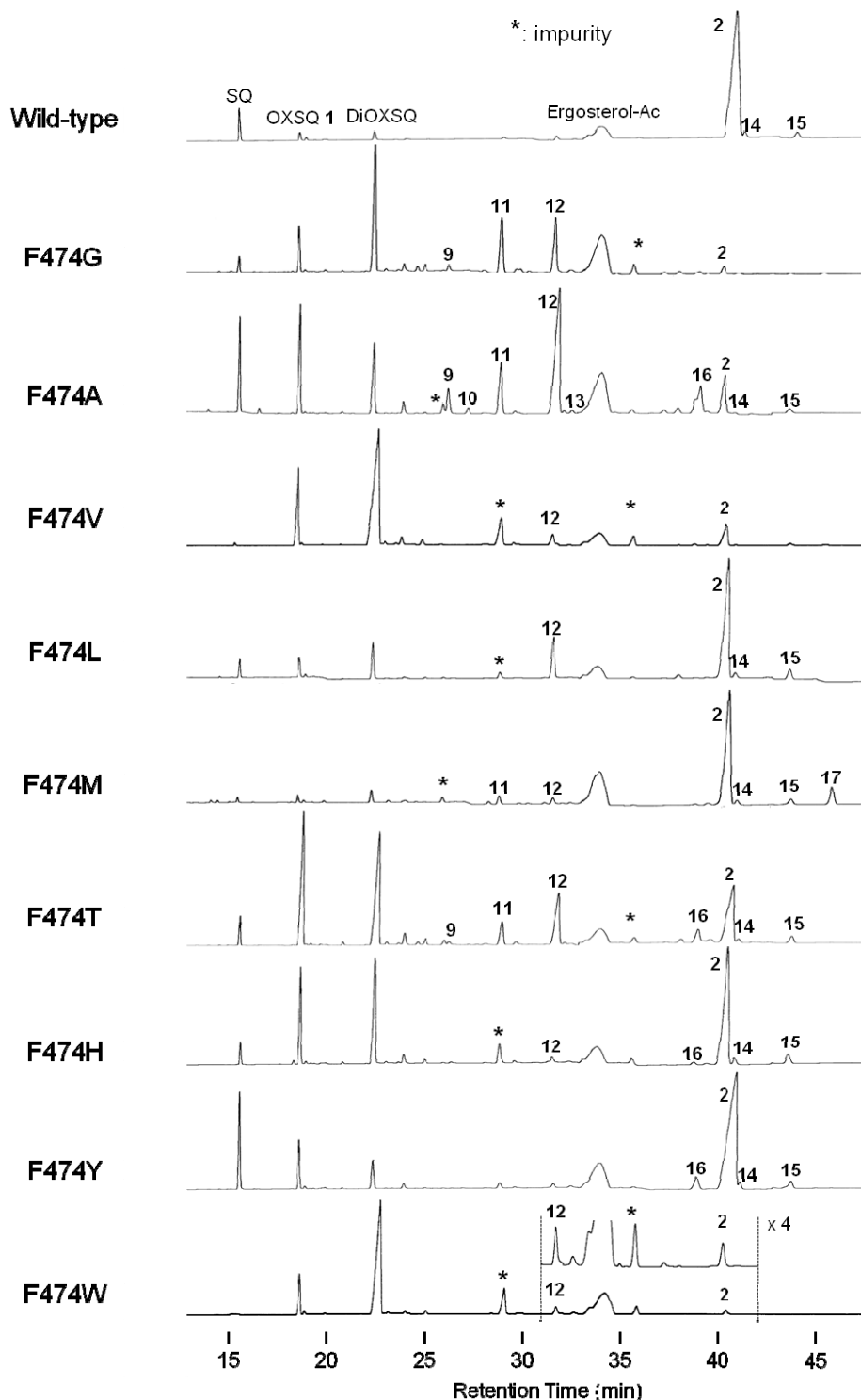
The underlined lines colored with yellow: QW motifs, with the consensus sequence [K/R][G/A]X2-3[F/Y/W][L/I/V]X3QX2-5-GXW. The functions of D⁴⁸⁶CTAE motif, C564, and F728 have been analyzed by us: R. Ito, Y. Masukawa and T. Hoshino, *FEBS J.*, **2013**;280:1267-1280; R. Ito, I Hashimoto, Y. Masukawa, T. Hoshino, *Chem. Eur. J.*, **2013**, 19, 17150-17158.

References

- 1) M. Kajikawa, K. T. Yamato, H. Fukuzawa, Y. Sakai, H. Uchida, and Kanji Ohyama, *Phytochemistry*, **66**(15), 1759-1766 (2005).
- 2) T. Kushiro, M. Shibuya, and Y. Ebizuka, *Eur. J. Biochem.*, **256**(1), 238-244 (1998).
- 3) H. Zhang, M. Shibuya, S. Yokota, and Y. Ebizuka, *Biol. Pharm. Bull.*, **26**(5), 642-650 (2003).

- 4) M. Morita, M. Shibuya, T. Kushiro, K. Masuda, and Y. Ebizuka, *Eur. J. Biochem.*, **267**(12), 3453-3460 (2000).
- 5) J.B. R. Herrera, B. Bartel, W. K. Wilson, S. P. T. Matsuda, *Phytochemistry*, **49**(7), 1905-1911 (1998).
- 6) M. Shibuya, H. Zhang, A. Endo, K. Shishikura, T. Kushiro, and Y. Ebizuka, *Eur. J. Biochem.*, **266**(1), 302-307 (1999).
- 7) E. J. Corey, S. P. T. Matsuda, and B. Bartel, *Proc. Natl. Acad. Sci.*, **90**, 11628-11632 (1993).
- 8) C. H. Baker, S. P. T. Matsuda, D. R. Liu, and E. J. Corey, *Biochem. Biophys. Res. Commun.*, **213**(1), 154-160 (1995).
- 9) C. K. Sung, M. Shibuya, U. Sankawa, and Y. Ebizuka, *Biol. Phar. Bull.*, **18**(10), 1459-1461 (1995).
- 10) E. J. Corey, S. P. T. Matsuda, and B. Bartel, *Proc. Natl. Acad. Sci.*, **91**(6), 2211-2215 (1994).
- 11) D. Ochs, C. Kaletta, K.-D. Entian, A. Beck-Sickinger, and K. Poralla, *J. Bacteriol.*, **174**(1), 298-302 (1992).
- 12) T. Sato, Y. Kanai, and T. Hoshino, *Biosci. Biotechnol. Biochem.*, **62**(2), 407-411 (1998).
- 13) T. Husselstein-Muller, H. Schaller, and P. Benveniste, *Plant Mol. Biol.*, **45**(1), 75-92 (2001).
- 14) R. Ito, K. Mori, I. Hashimoto, C. Nakano, T. Sato, and T. Hoshino, *Org. Lett.*, **13**(10), 2678-2681 (2011).

Supplementary Figure S2. GC profiles of the hexane-extract obtained from the wild-type and the site-directed mutants. Each of the strain was cultured in a 100 mL medium as described in the Experimental section. The cultured cells were collected by centrifugation. To the collected pellets, were added 15% KOH/MeOH and heated at 80°C. The lipophilic materials were extracted with hexane and the hexane extract was evaporated under reduced pressure. The residues were acetylated with Ac₂O/py. One ml of hexane was added and 1.0 µl of the solution was injected. GC column: J&W, DB-1, capillary (Length 30 m, I.D.0.32 mm, Film Thickness 0.25 µm), Injection temp. 300°C; Column temp.:190-250°C (10°C/min), 250-270°C (0.35°C/min).

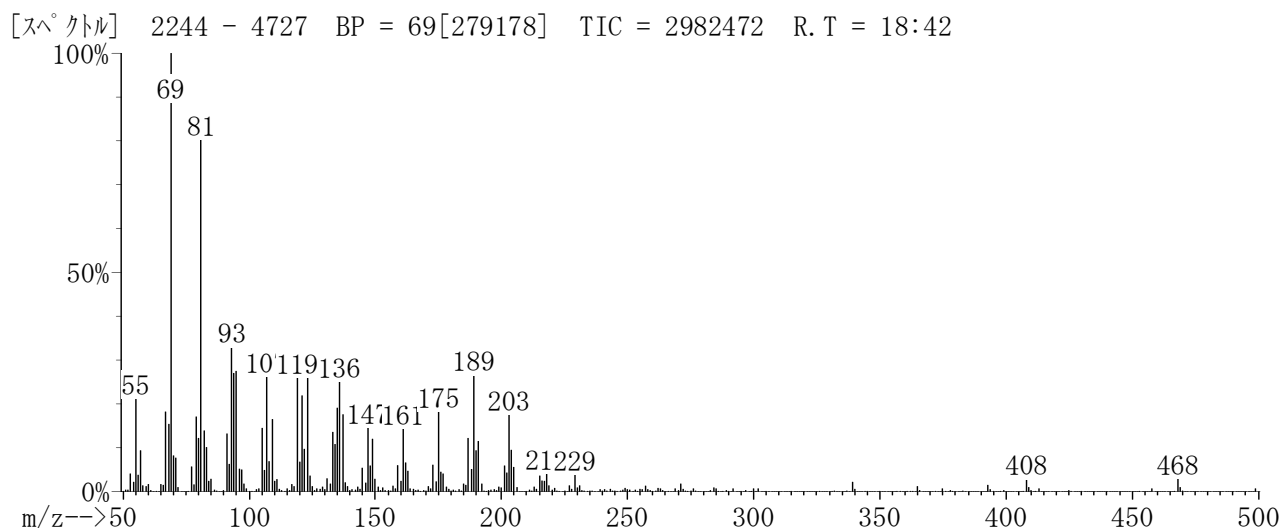


Supplementary Figure S3.

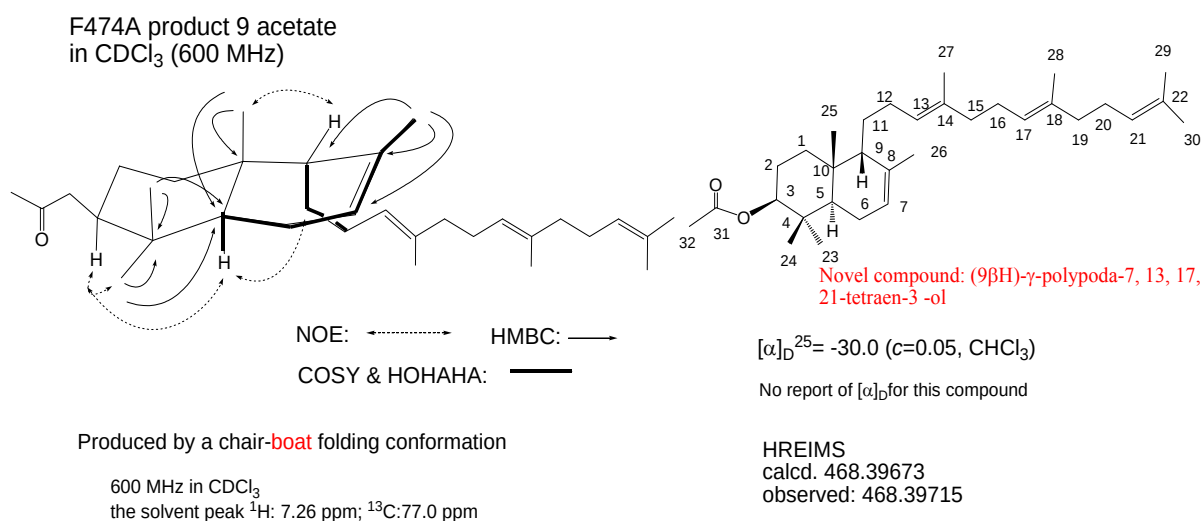
Spectroscopic Data of Triterpene products accumulated in the Mutagenesis Experiments.

Fig. S3. 1. Product 9 acetate produced by F474A mutant

3.1.1 EIMS



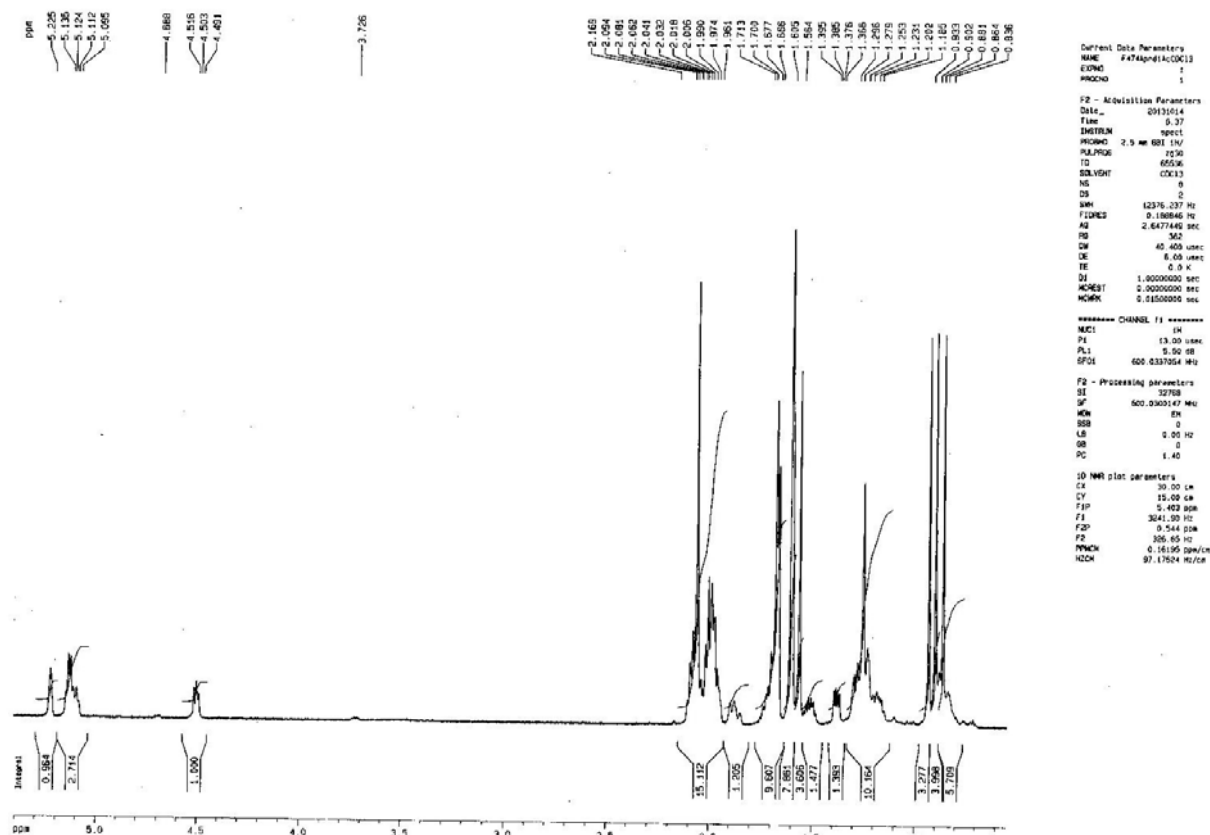
3.1.2. NMR data analyses, $[\alpha]_D^{25}$, and HREIMS



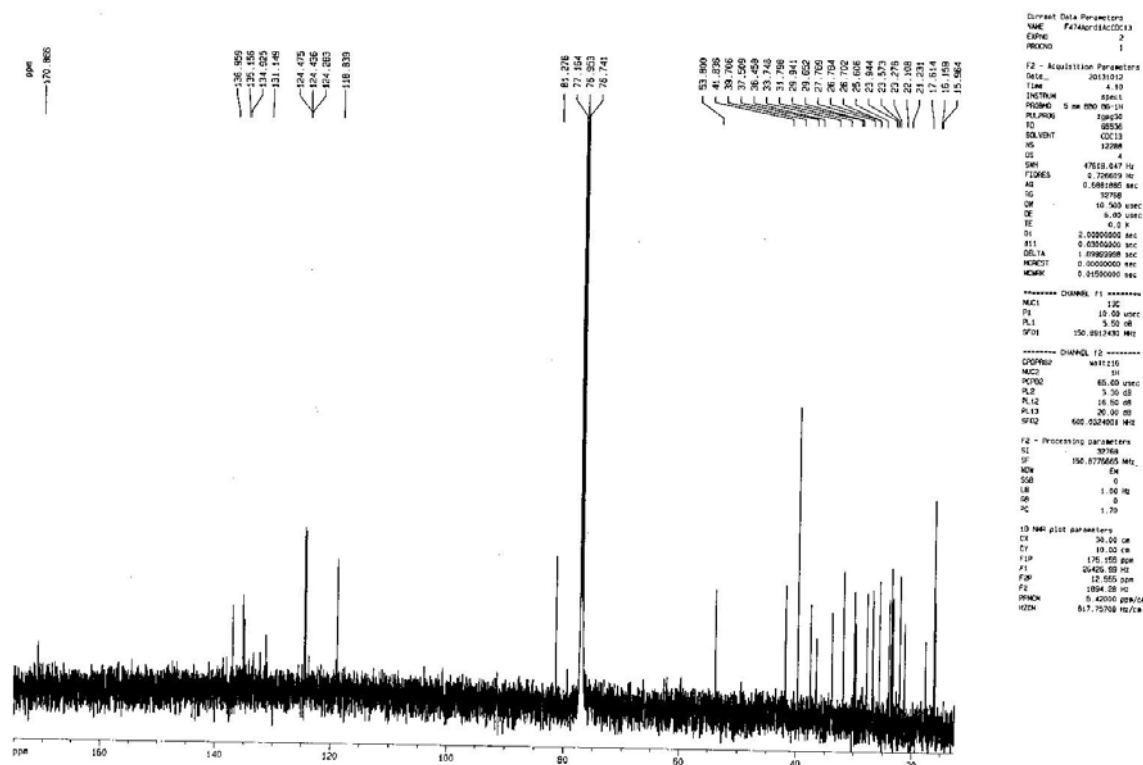
NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	1.28 (m); 1.71 (m)	33.75 (t)	9	1.23 (m)	53.80 (d)	17	5.12 (m) ^e	124.4 (d) ^f	25	0.902 (3H, s)	22.11 (q)
2	1.70 (2H, m)	23.94 (t)	10	—	36.46 (s)	18	—	134.9 (s) ^g	26	1.666 (3H, bs)	23.28 (q)
3	4.50 (dd, 8.4, 6.6 Hz)	81.28 (d)	11	1.18 (m); 1.51 (m)	31.77 (t)	19	1.98 (2H, m)	39.71 (t) ^a	27	1.606 (3H, s) ^c	16.16 (q) ^d
4	—	37.51 (s)	12	2.02 (2H, m)	29.94 (t)	20	2.07 (2H, m)	26.78 (t) ^b	28	1.606 (3H, s) ^c	15.96 (q) ^d
5	1.38 (dd, J=11.4, 5.4 Hz)	41.84 (d)	13	5.12 (m) ^e	124.5 (d) ^f	21	5.12 (m) ^e	124.3 (d)	29	1.599 (3H)	17.61 (q)
6	1.86 (1H, m); 1.96 (1H, m)	23.57 (t)	14	—	135.2 (s) ^g	22	—	131.1 (s)	30	1.678 (3H, s)	25.61 (q)
7	5.22 (1H, br s)	118.8 (d)	15	1.98 (2H, m)	39.71 (t) ^a	23	0.865 (3H, s)	27.77 (q) ^d	31	—	170.8 (s)
8	—	136.9 (s)	16	2.07 (2H, m)	26.78 (t) ^b	24	0.934 (3H, s)	15.96 (q) ^d	32	2.063 (3H, s)	21.23 (q)

The assignments of the symbols a-f are indistinguishable between the same symbols, due to the very close chemical shifts.

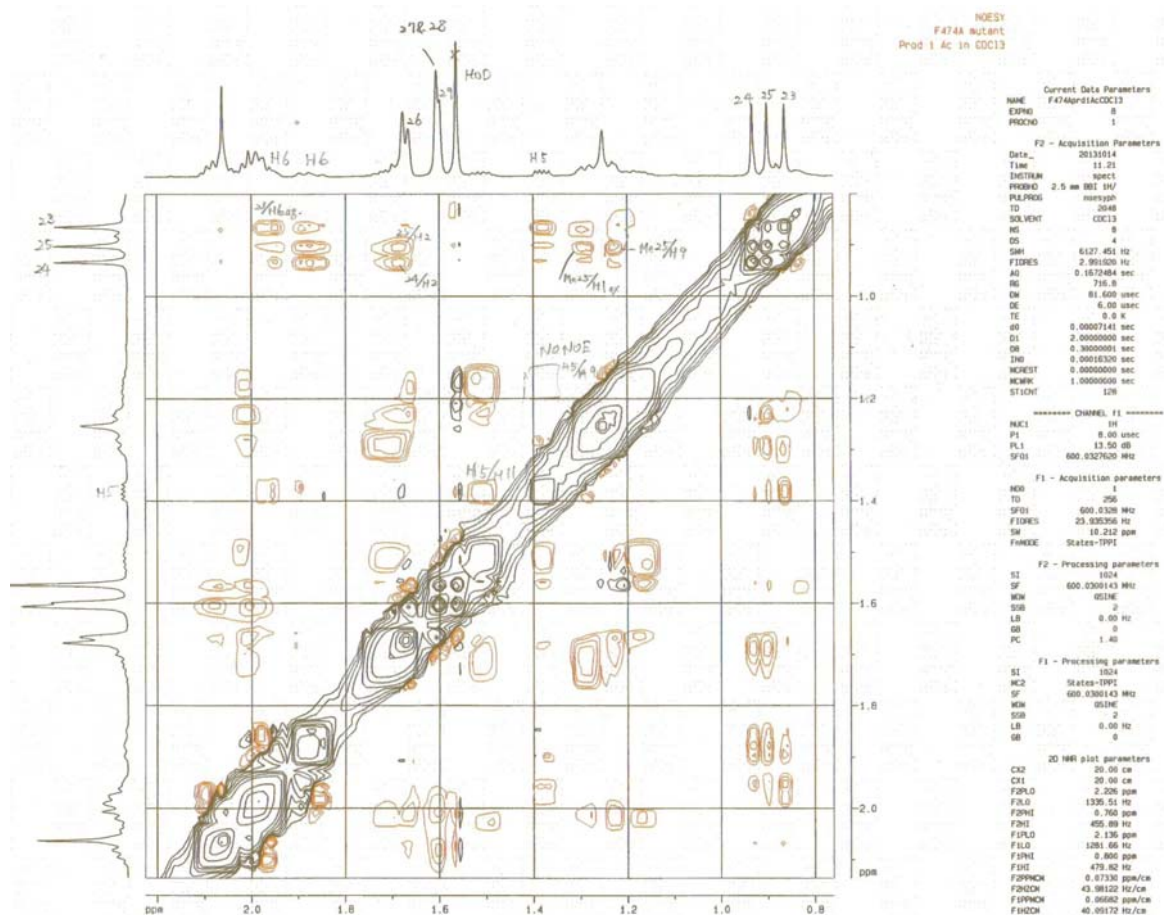
¹H-NMR in CDCl₃(600 MHz)



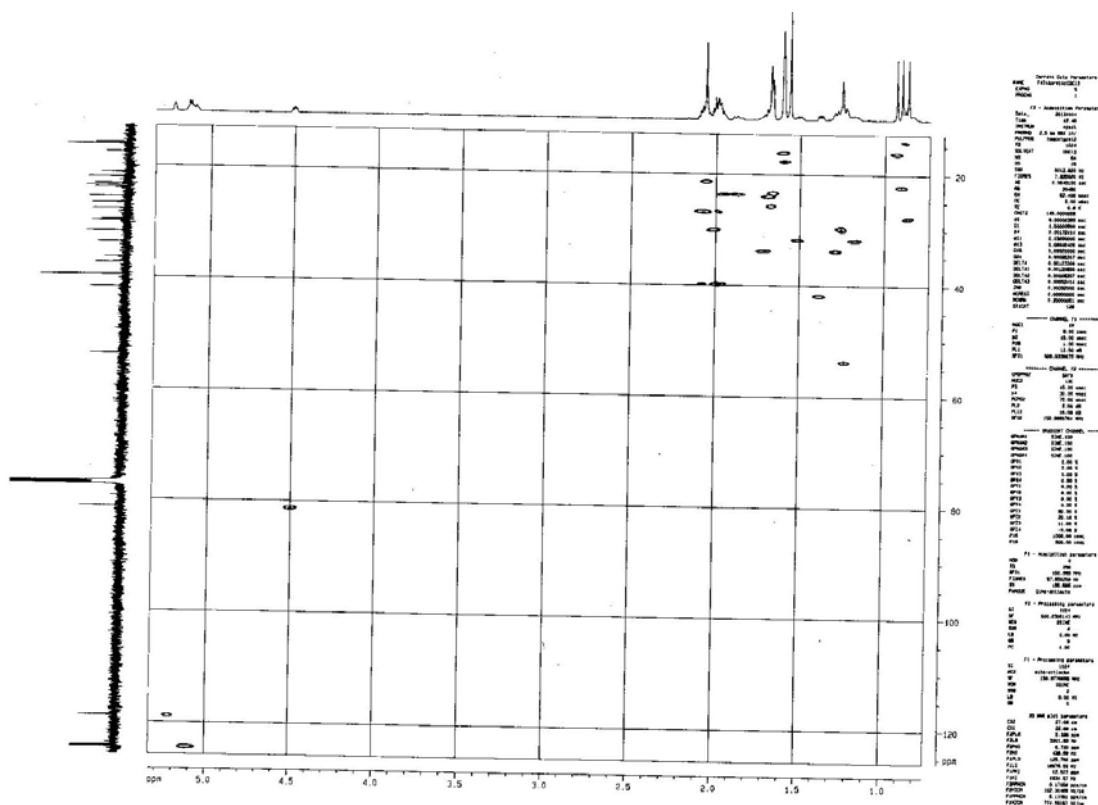
3.1.4 ¹³C NMR in CDCl₃(150 MHz)



3.1.7 NOESY in CDCl₃



3.1.8 HSQC in CDCl₃



3.1.9. HMBC in CDCl₃

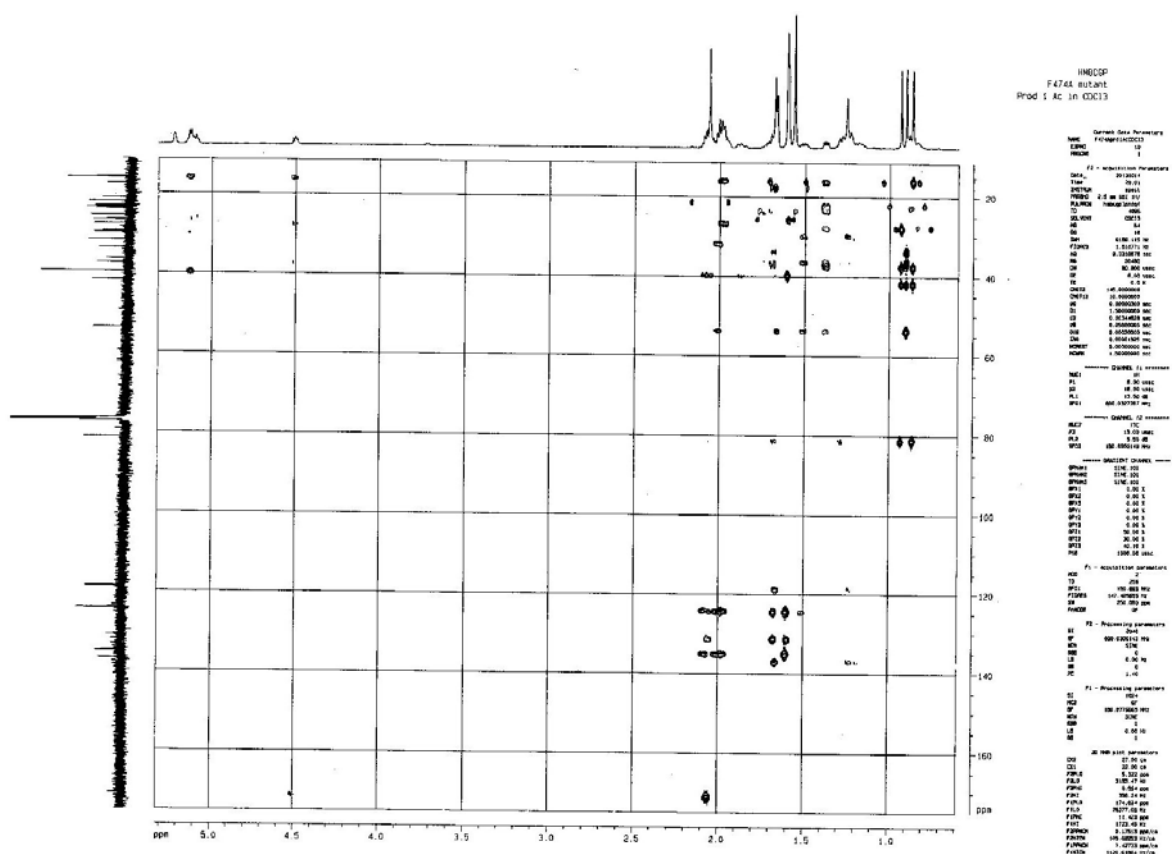
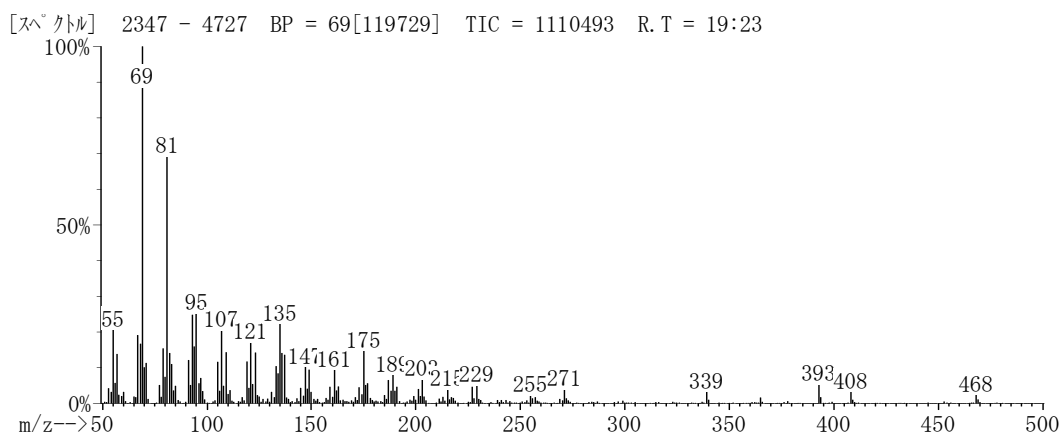


Figure S3.2. Product 10 acetate produced by F474A mutant

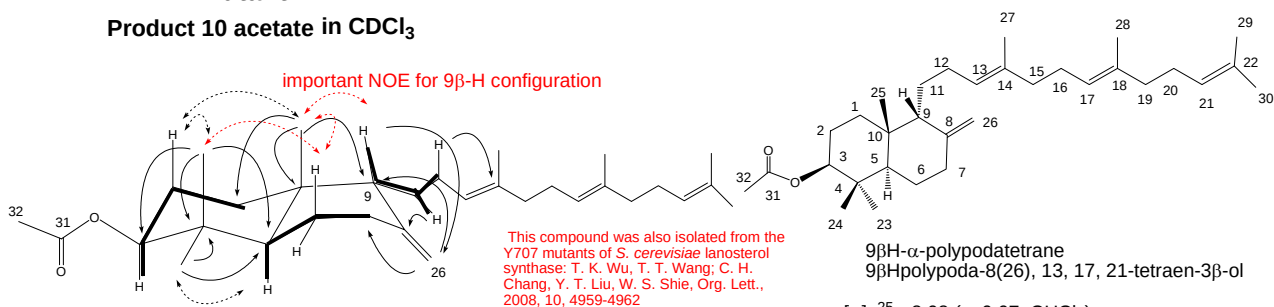
3.2.1. EIMS.



3.2.2. NMR data analyses, $[\alpha]_D$, and HREIMS.

F474A mutant

Product 10 acetate in $CDCl_3$



$[\alpha]_D^{25} = -8.08$ ($c=0.07$, $CHCl_3$)
No report of $[\alpha]_D$ for this compound

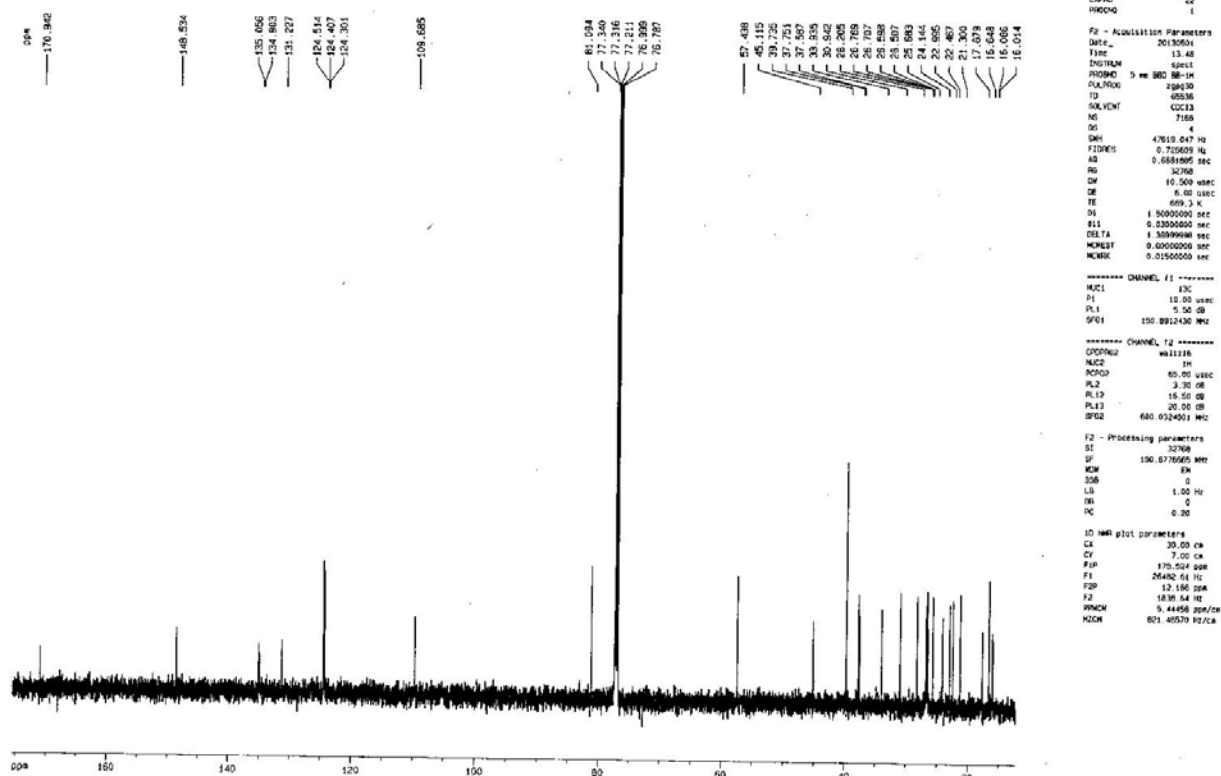
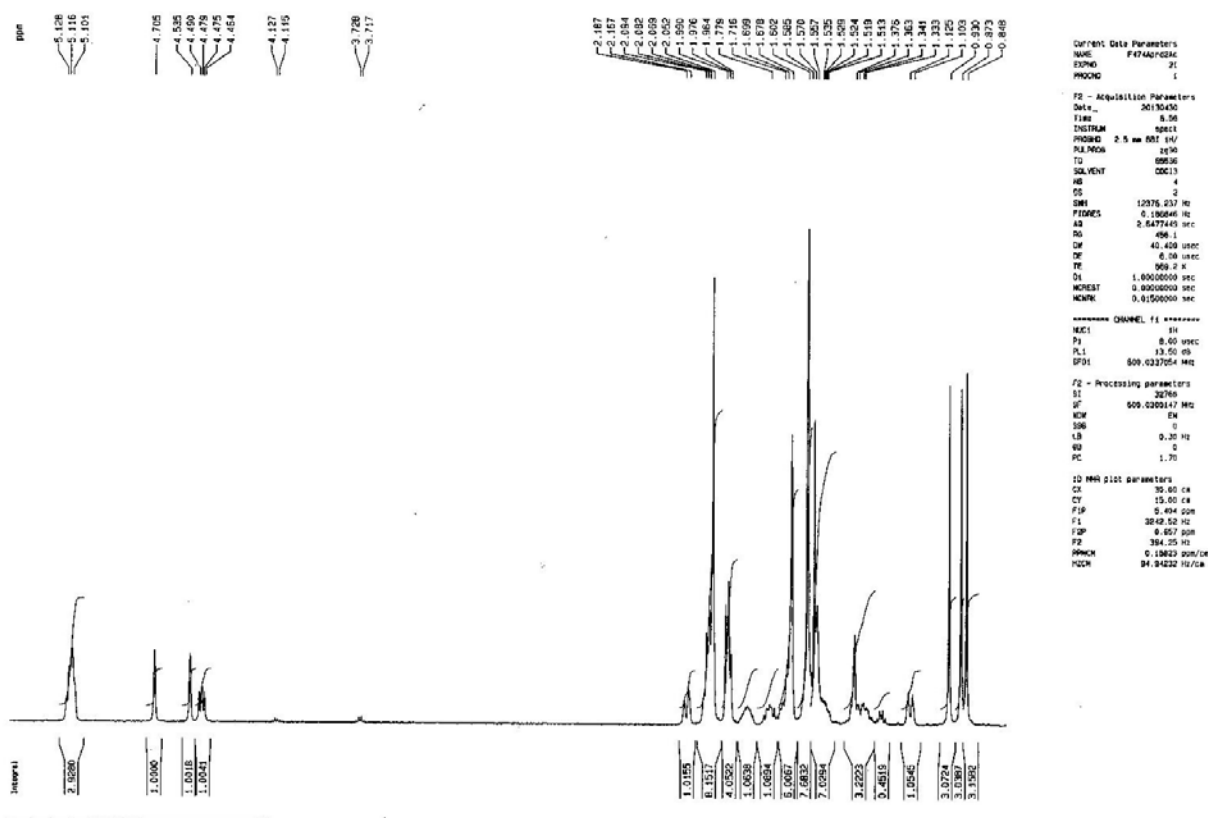
600 MHz in $CDCl_3$
the solvent peak 1H : 7.26 ppm; ^{13}C : 77.0 ppm

NOE: $\longleftrightarrow$ HMBC: $\longrightarrow$
COSY & HONAHAL

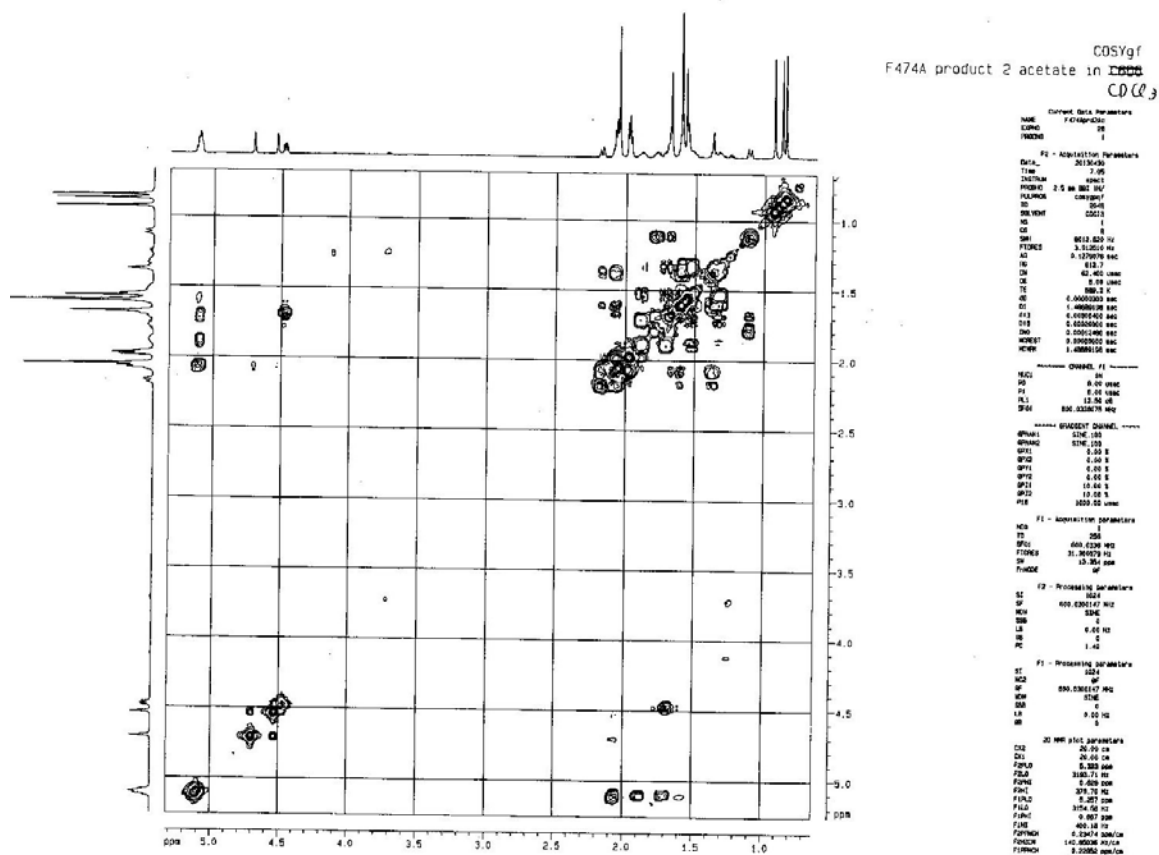
HREIMS
calcd. 468.39673
observed: 468.39627

NO.	1H	^{13}C	NO.	1H	^{13}C	NO.	1H	^{13}C	NO.	1H	^{13}C
1	1.11 (bd, J=12.6 Hz); 1.78 (m)	33.94 (t)	9	1.59 (m)	57.44(d)	17	5.12 (m) ^e	124.4(d) ^f	25	0.930 (3H,s)	22.47 (q)
2	1.68 (2H, m)	24.14 (t)	10	—	37.59(s)	18	—	134.9 (s) ^h	26	4.54(bs); 4.71(bs)	109.7 (t)
3	4.47(dd, 9.0, 6.6 Hz)	81.09 (d)	11	1.33 (m); 1.52(m)	26.60 (t) ⁱ	19	1.98 (2H, m) ⁱ	39.74 (t) ^a	27	1.570 (3H, s) ^c	16.09 (q) ^d
4	—	37.75(s)	12	1.71 (m); 1.88(m)	26.51(t) ^j	20	2.07 (2H, m) ^g	26.71(t) ^b	28	1.602 (3H, s) ^c	16.01 (q) ^d
5	1.38 (m)	45.12(d)	13	5.12 (m) ^e	124.5 (d) ^f	21	5.12 (m) ^e	124.3 (d)	29	1.602 (3H,s)	17.68 (q)
6	1.38 (m); 1.62(m)	22.99(t)	14	—	135.1 (s) ^h	22	—	131.2 (s)	30	1.678 (3H, s)	25.68 (q)
7	2.08(m); 2.17 (bd, J=12.0 Hz)	30.94 (t)	15	1.98 (2H, m) ⁱ	39.74 (t) ^a	23	0.873 (3H,s)	28.20(q)	31	—	170.9 (s)
8	—	148.5 (s)	16	2.07 (2H, m) ^g	26.77(t) ^b	24	0.848 (3H,s)	16.65(q)	32	2.052 (3H, s)	21.30 (q)

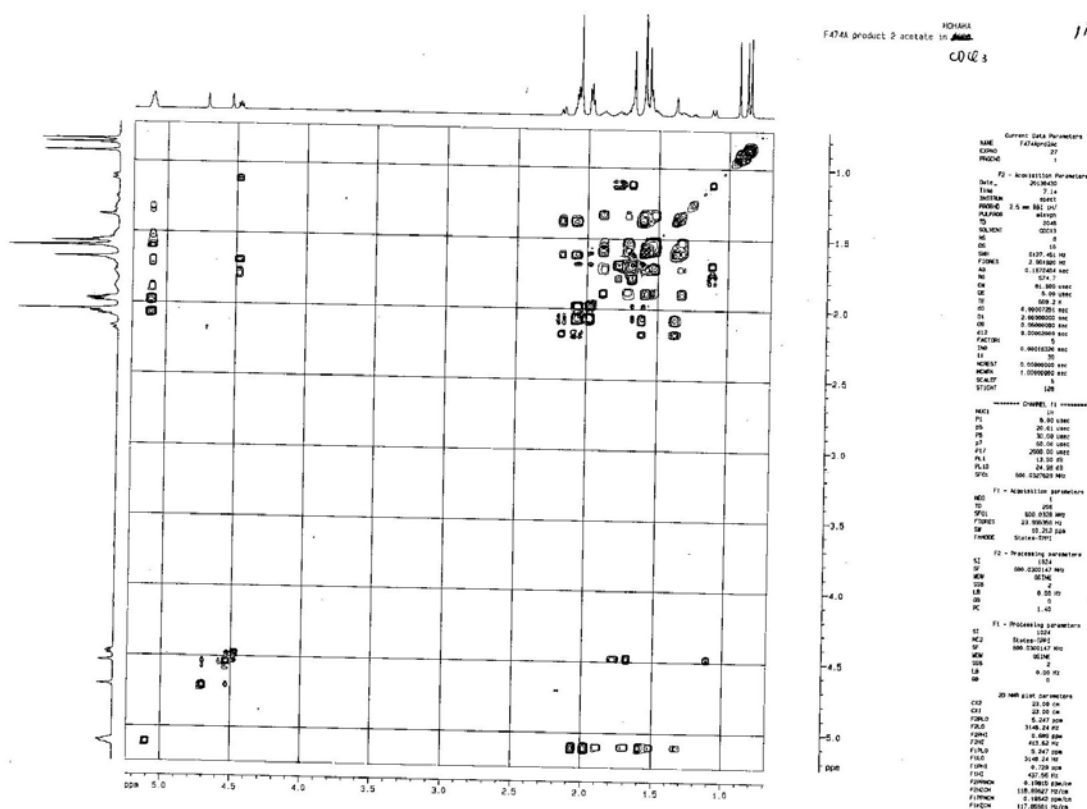
The assignments of the symbols a-i are indistinguishable between the same symbols, due to the very close or identical chemical shifts.
a, i, g: overlapped.



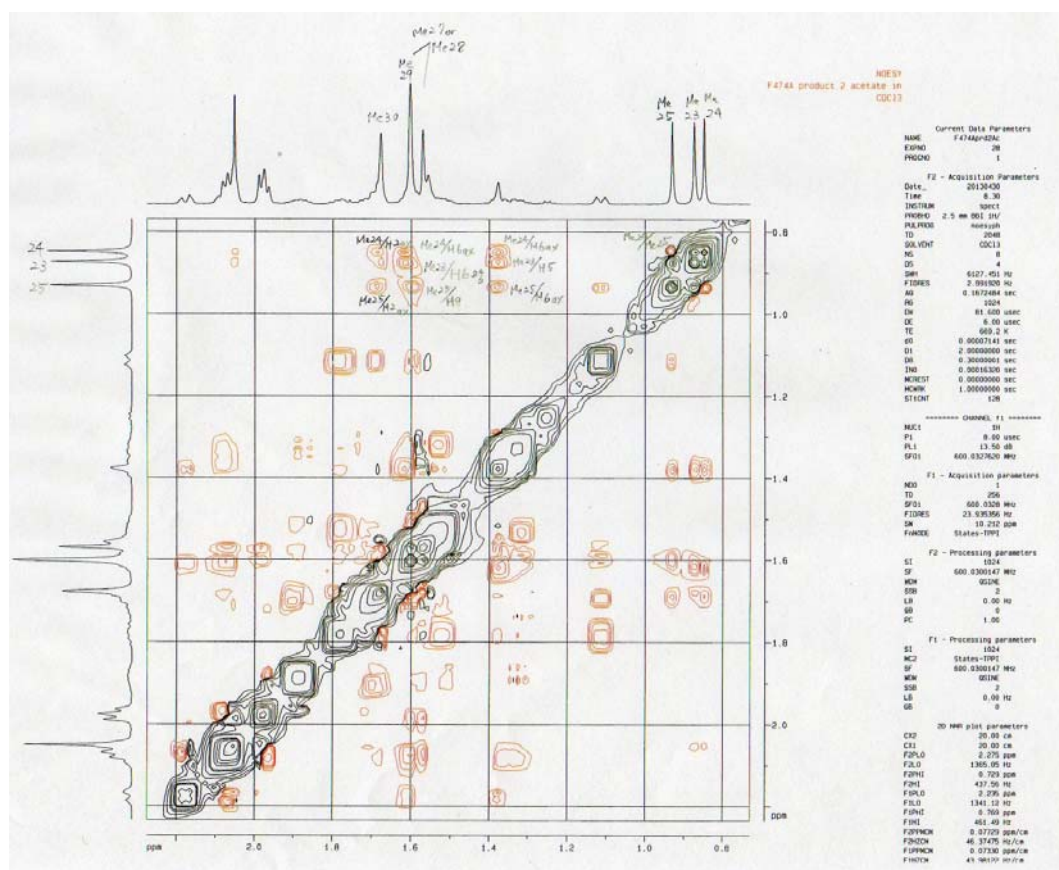
3.2.5. ^1H - ^1H COSY in CDCl_3 (600 MHz)



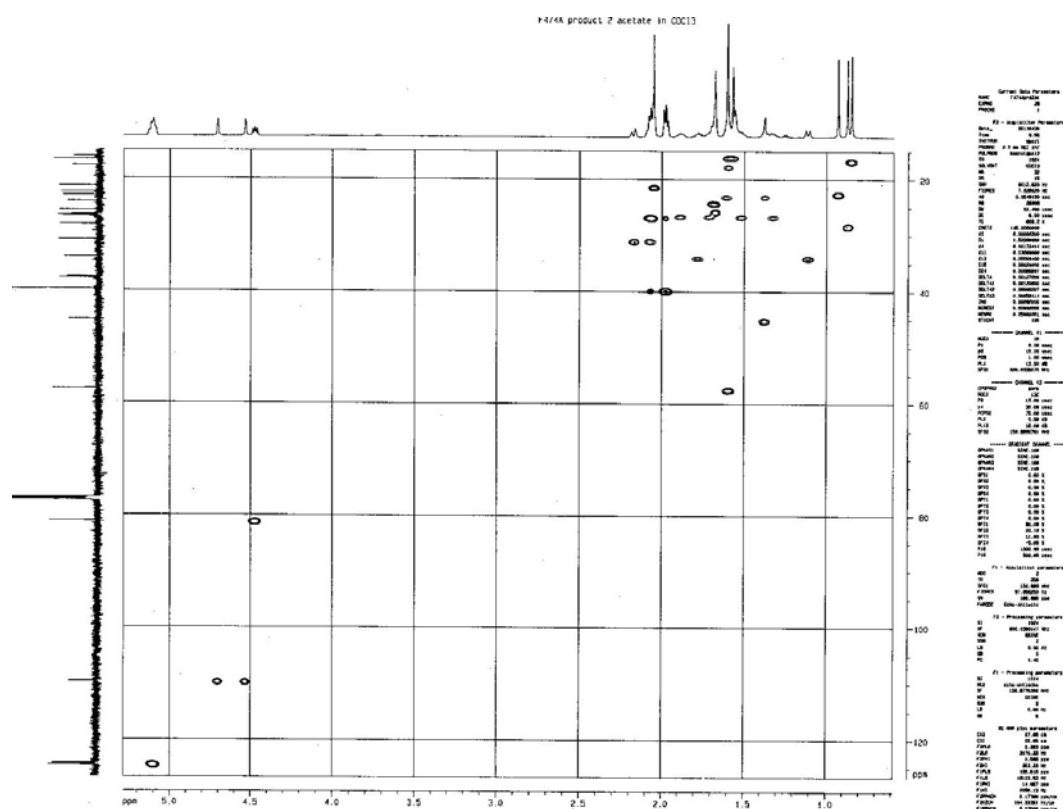
3.2.6. HOHAHA in CDCl₃ (600 MHz)



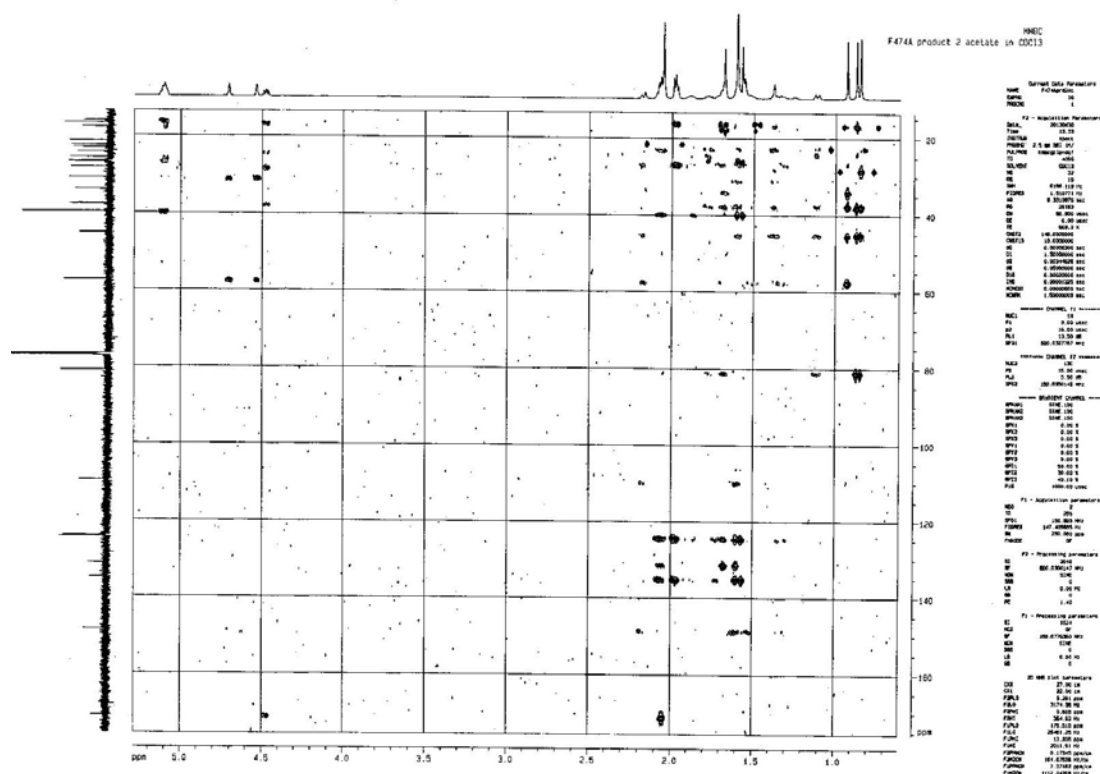
3.2.7. NOESY in CDCl₃ (600 MHz)



3.2.8. HSQC in CDCl₃



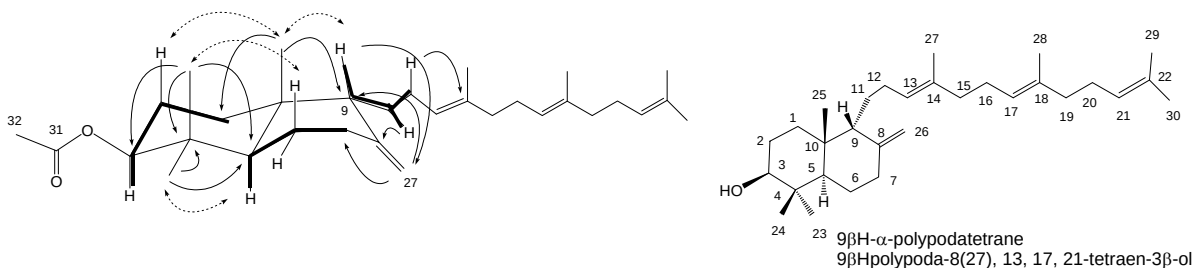
3.2.9. HMBC in CDCl₃



3.2.10. NMR data analyses in C₆D₆ (600 MHz). To confirm the stereochemistry of 9β-H, the NMR spectra of this compound were measured again in the different solvent C₆D₆.

F474A mutant

Product 10 acetate in (600 Hz, C_6D_6)



600 MHz in C₆D₆
the solvent peak ¹H: 7.28 ppm; ¹³C:128.0 ppm

NOE: $\longleftrightarrow$ HMBC: $\longrightarrow$
COSY & HOHAHA: —

NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	1.04 (m); 1.77(m)	34.22 (t)	9	1.68 (m)	57.73(d)	17	5.46 (t, J=7.0 Hz) ^e	124.9(d) ^f	25	1.045 (3H,s)	22.65 (q)
2	1.79(m); 1.93 (m)	24.56 (t)	10	—	37.94(s) ^g	18	—	134.9 (s) ^h	26	4.92(bs); 4.76(bs)	110.0 (t)
3	4.84(dd, 11.4, 3.6 Hz)	80.82 (d)	11	1.44 (m);1.59(m)	26.71 (t)	19	2.28 (2H, m) ⁱ	40.23 (t) ^a	27	1.775 (3H, s) ^c	16.20 (q)
4	—	37.81(s)	12	1.96 (m); 2.18(m)	26.97(t)	20	2.38 (2H, m)	27.11(t) ^b	28	1.752 (3H, s) ^c	16.13 (q)
5	1.40 (m)	45.44(d) ^g	13	5.45 (t, J=7.0Hz) ^e	125.3 (d) ^f	21	5.38 (bt, J=6.8 Hz)	124.8 (d)	29	1.700 (3H, s)	17.73 (q)
6	1.38 (m); 1.56(m)	23.30(t)	14	—	135.1 (s) ^h	22	—	131.1 (s)	30	1.813 (3H, s)	25.83 (q)
7	2.09 (m);2.19 (m)	31.20 (t)	15	2.23 (2H, m) ⁱ	40.25 (t) ^a	23	1.013 (3H,s)	28.38(q)	31	—	169.9 (s)
8	—	148.8 (s)	16	2.32 (2H, m)	27.23(t) ^b	24	1.013 (3H,s)	16.99(q)	32	1.862 (3H, s)	20.81 (q)

The assignments of the symbols a~i are indistinguishable between the same symbols, due to the very close chemical shifts.

3.2.11. NOESY spectrum in C₆D₆ (600 MHz)

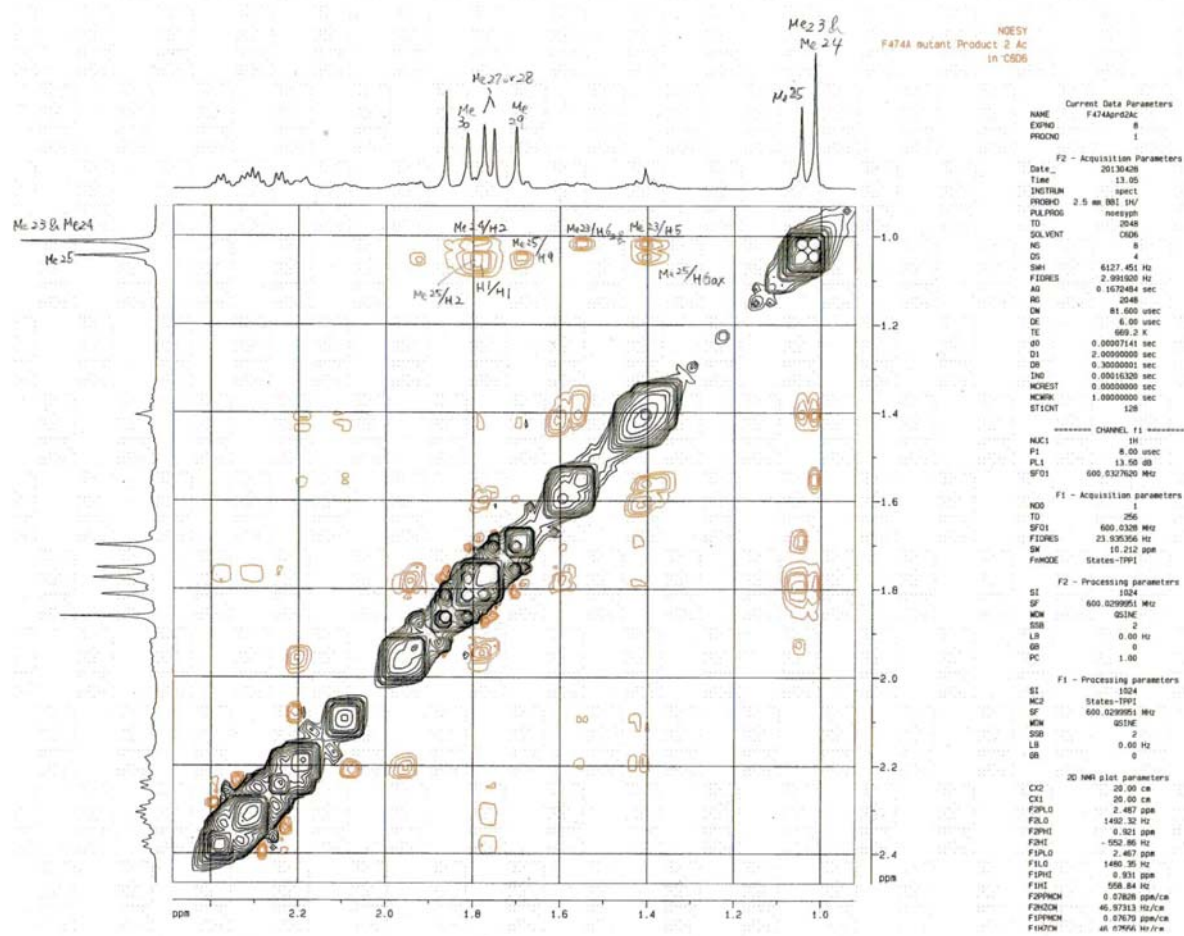
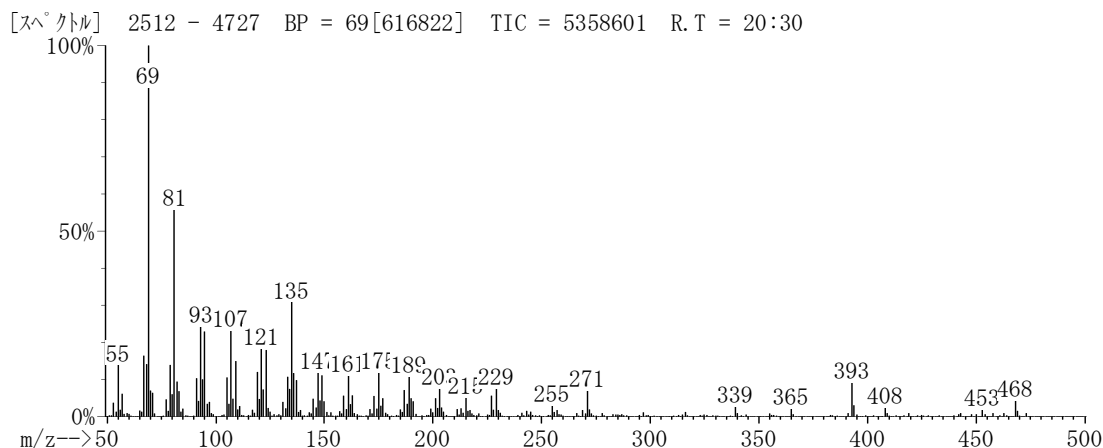


Fig. S3.3. Product 11 acetate produced by F474A mutant

3.3.1 EIMS

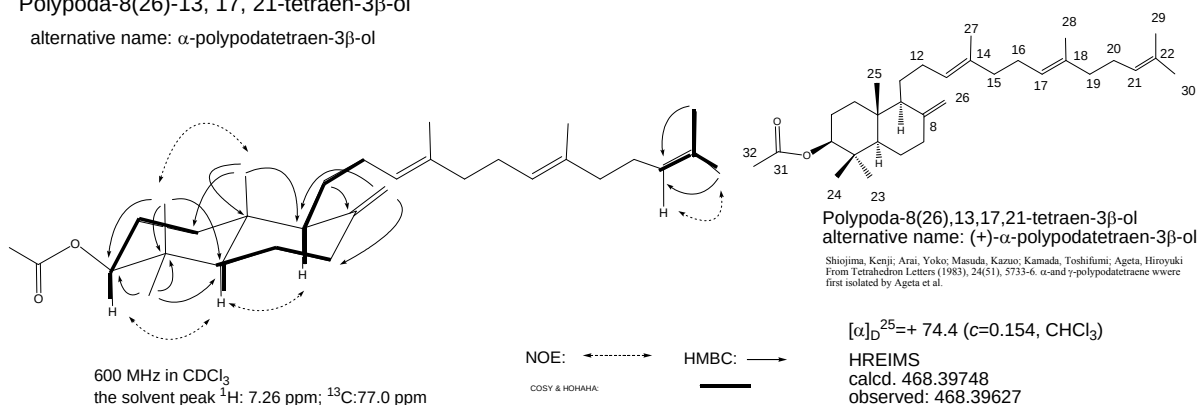


3.3.2. NMR data analyses in CDCl₃, [α]_D, and HREIMS.

F474A mutant Product 11 acetate in CDCl₃

Polypoda-8(26)-13, 17, 21-tetraen-3β-ol

alternative name: α-polypodatetraen-3β-ol



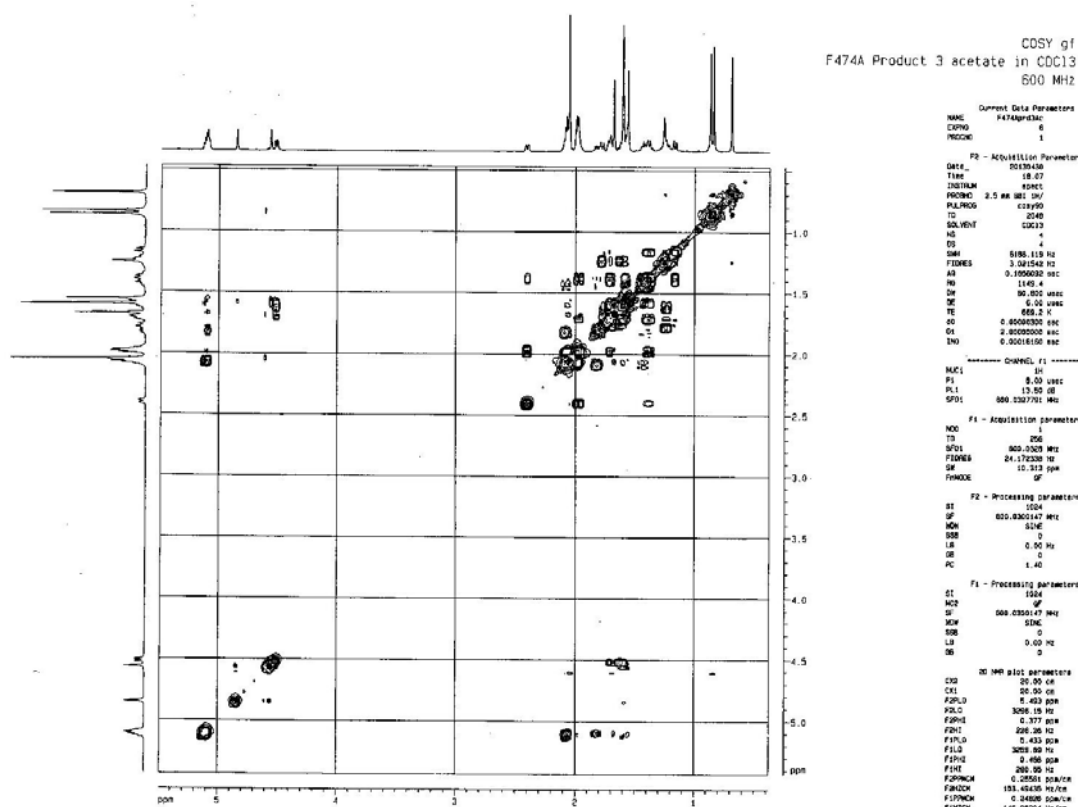
NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	1.22 (m); 1.78(m)	36.73 (t)	9	1.59 (m)	55.79(d)	17	5.11 (m) ^e	124.4(d) ^f	25	0.692(3H, s)	14.53 (t)
2	1.61(m); 1.72 (m)	24.30 (t)	10	—	39.10(s)	18	—	134.9 (s) ^g	26	4.56 (s); 4.85(s)	106.7(t)
3	4.51(dd, 11.4, 4.2 Hz)	80.82 (d)	11	1.42(m); 1.69(m)	23.81 (t) ^b	19	1.98 (2H, m)	39.74 (t) ^a	27	1.601(3H, s) ^d	16.02 (q) ^c
4	—	38.01(s)	12	1.82 (m); 2.08 (m)	27.78 (t) ^f	20	2.07 (2H, m)	26.70(t) ^b	28	1.558 (3H, s) ^d	16.02 (q) ^c
5	1.16 (dd, J=12.6, 2.4 Hz)	54.71(d)	13	5.11 (m) ^e	124.8 (d) ^f	21	5.11 (m) ^e	124.2(d) ^f	29	1.601 (3H, s)	17.68 (q)
6	1.38 (m); 1.69(m)	23.93(t) ^h	14	—	135.2 (s) ^g	22	—	131.2 (s)	30	1.677 (3H, s)	25.69 (q)
7	1.98 (m); 2.40 (bd, J=11.4Hz)	38.06 (t)	15	1.98 (2H, m)	39.74 (t) ^a	23	0.867 (3H, s)	28.22(q)	31	—	169.9 (s)
8	—	147.9 (s)	16	2.07 (2H, m)	26.76(t) ^b	24	0.841 (3H, s)	16.52(q)	32	2.049 (3H, s)	21.31 (q)

The assignments of the symbols a–h are indistinguishable between the same symbols, due to the very close or inseparable chemical shifts.

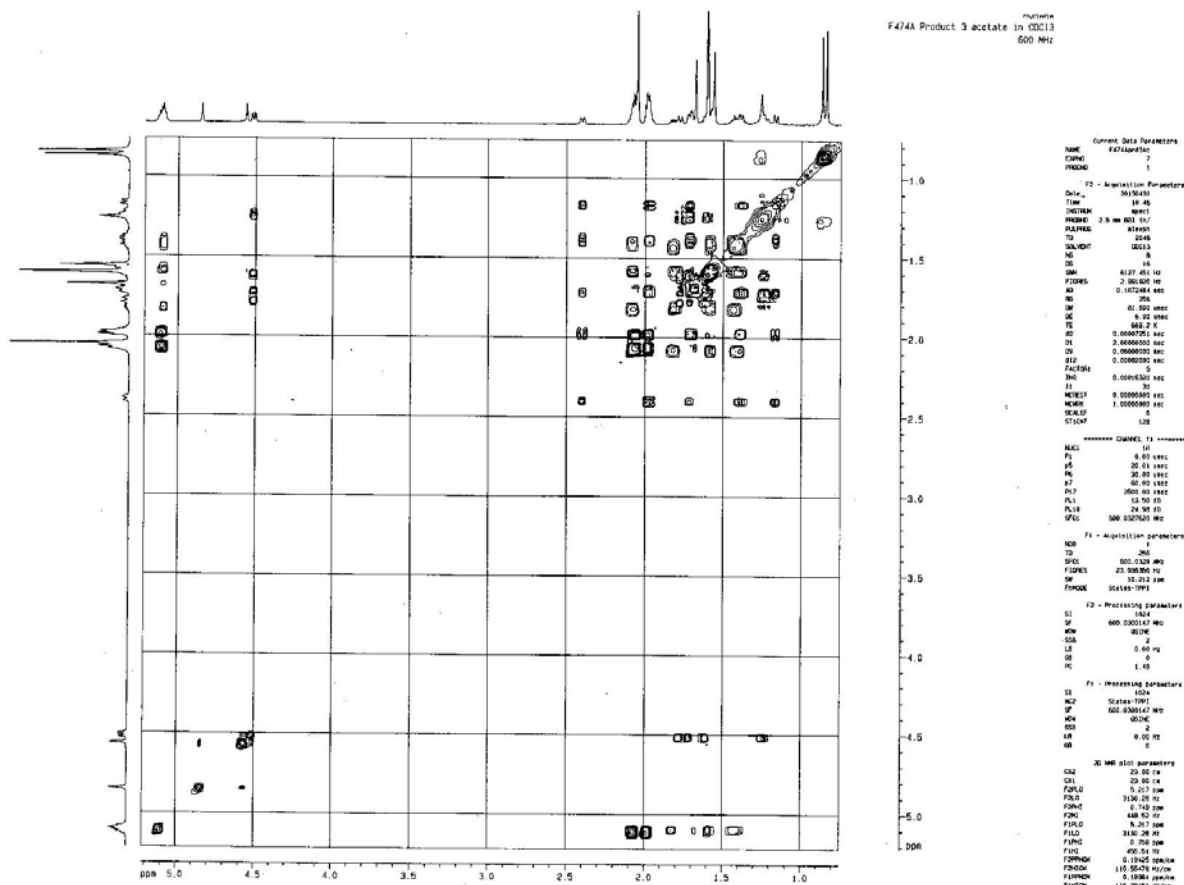
a: The carbon signals of C15 and C19 are overlapped. c: The carbon signals of C-27 and C-28 were overlapped.

d: The proton signals of Me-27 and Me-28 are exchangeable. e and f: signals were indistinguishable.

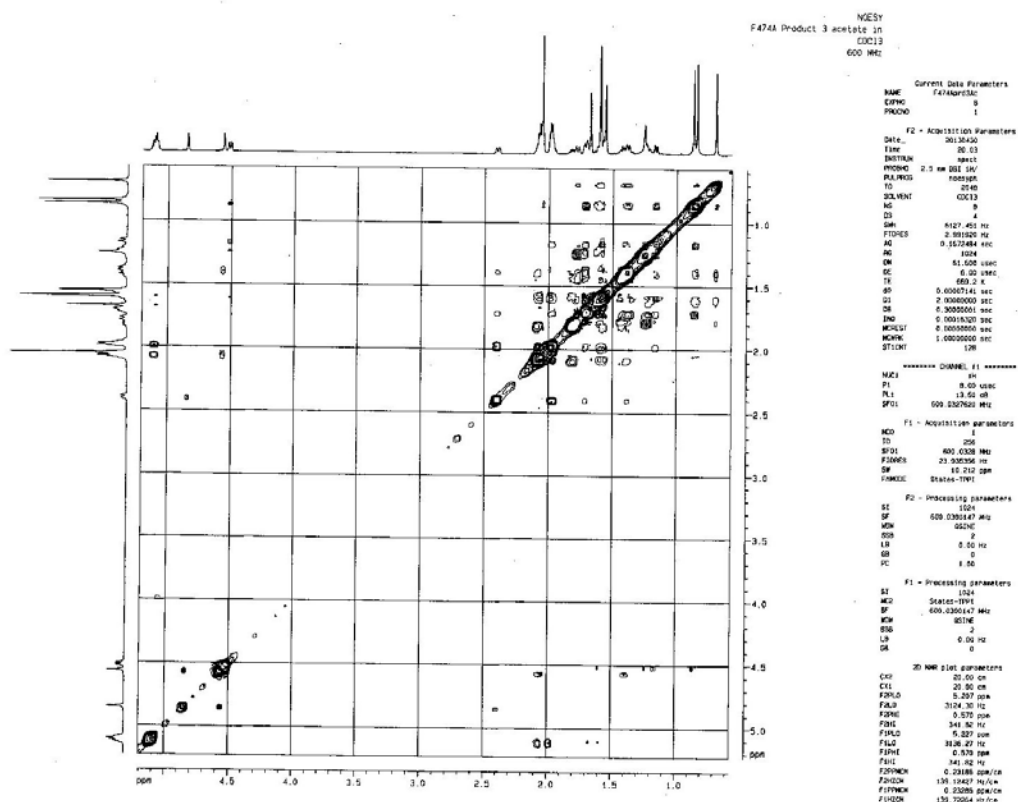
3.3.5. COSY in C₆D₆ (600 MHz)



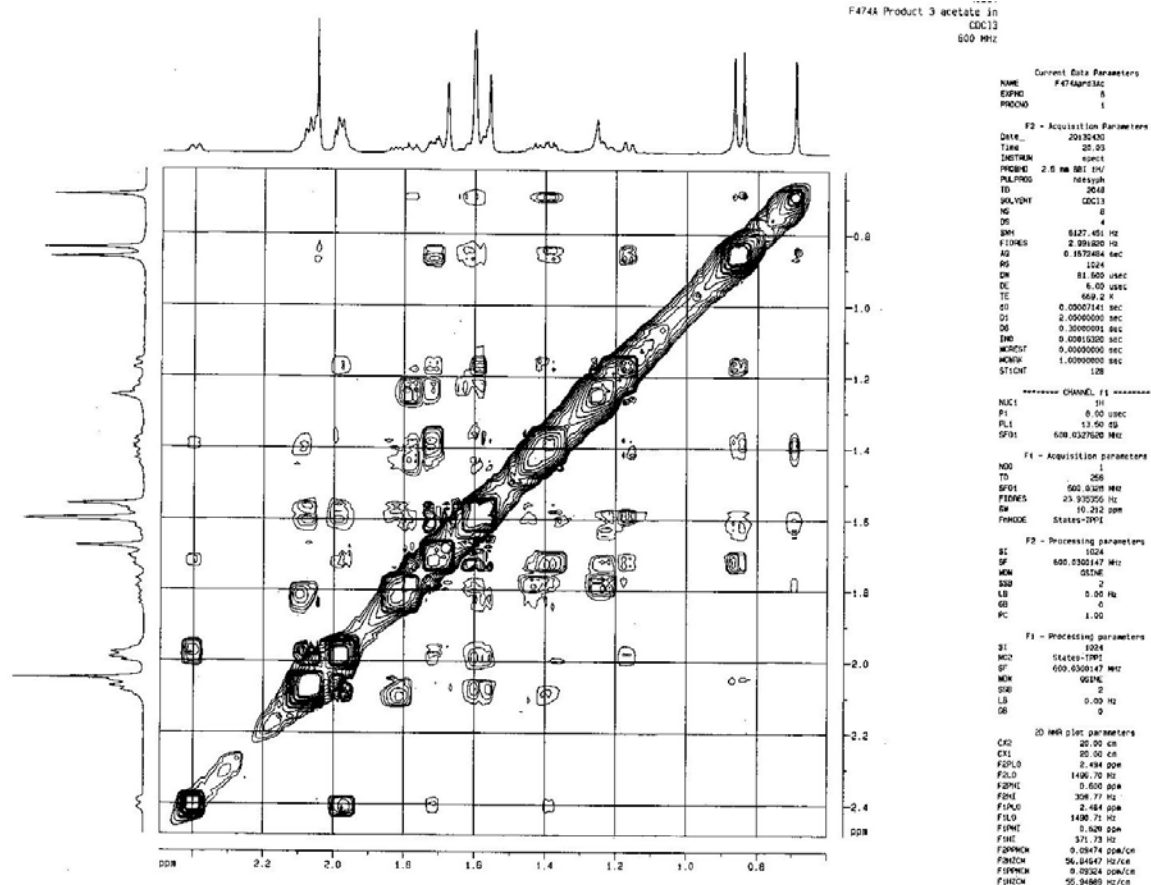
3.3.6 HOHAHA in C₆D₆ (600 MHz)



3.3.7. NOESY in C₆D₆ (600 MHz): Whole region



3.3.8. NOESY in C₆D₆ (600 MHz), expanded region



3.3.10. HMBC in C₆D₆ (600 MHz).

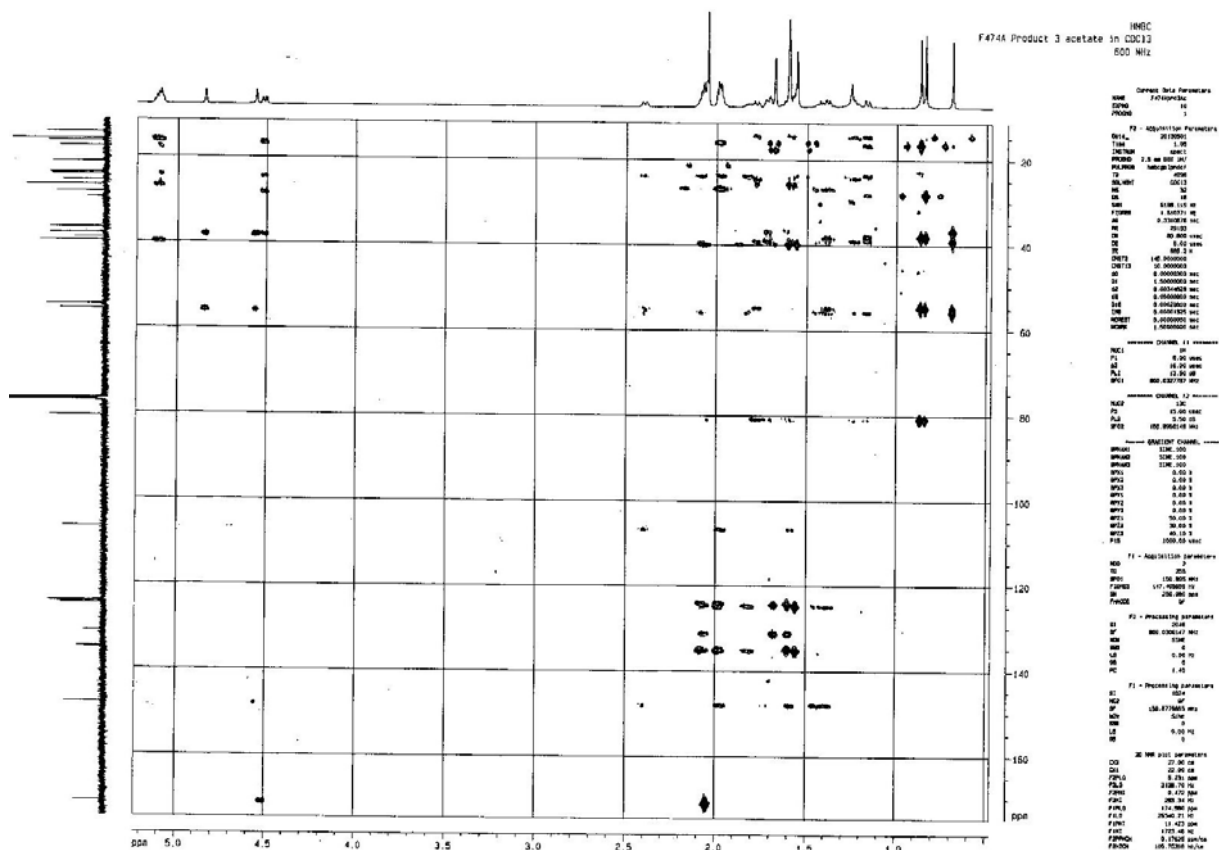
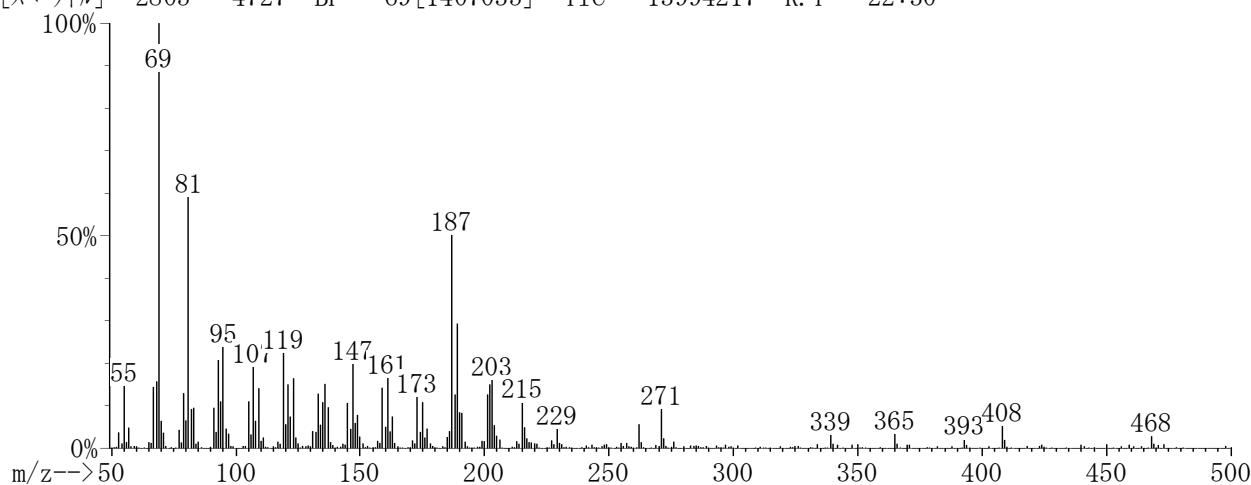


Fig. S.3. 4. Product 12 acetate produced by F474A mutant

3.4.1. EIMS

[スペクトル] 2805 - 4727 BP = 69[1407035] TIC = 13994217 R. T = 22:30

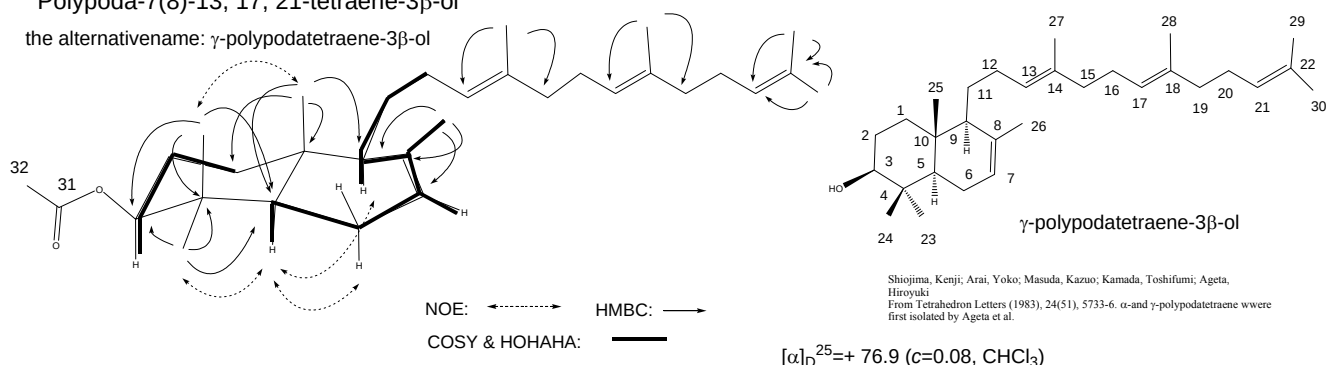


3.4.2. NMR data analyses in C₆D₆, [α]_D, HREIMS

F474A mutant Product 12 acetate in C₆D₆

Polypoda-7(8)-13, 17, 21-tetraene-3β-ol

the alternative name: γ-polypodatetraene-3β-ol



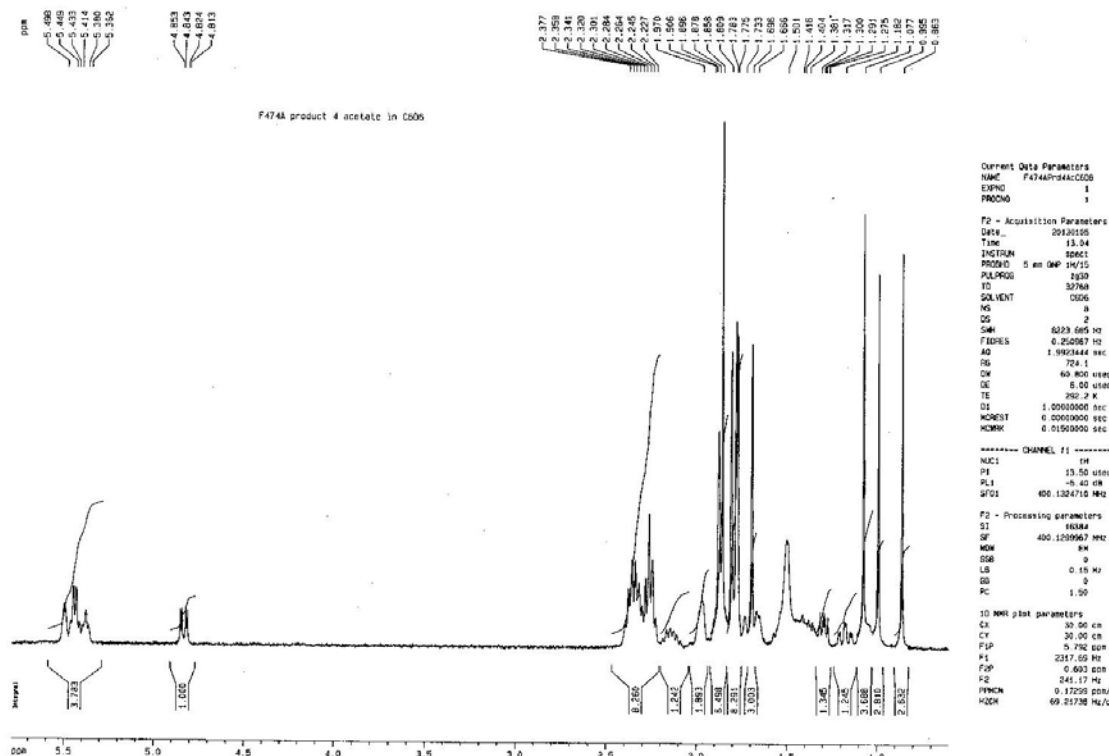
400 MHz in C₆D₆
the solvent peak ¹H: 7.28 ppm; ¹³C: 128.0 ppm

HREIMS
calcd. 468.39686
observed: 468.39627

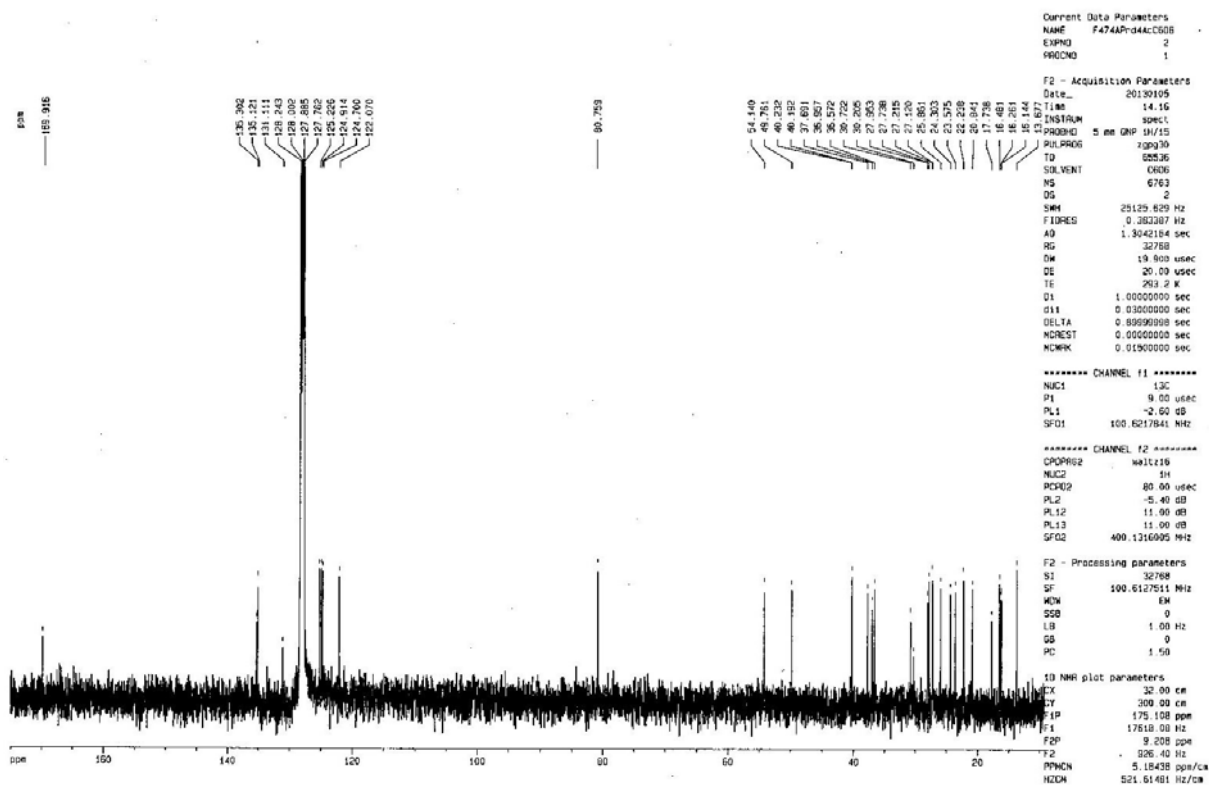
NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	1.18 (ddd, J=12.8, 12.8, 3.2 Hz); 1.83 (m)	36.96 (t)	9	1.66 (m)	54.14 (d)	17	5.43 (t, J=6.4 Hz) ^e	124.7 (d) ^f	25	0.863 (3H, s)	13.68 (q)
2	1.70 (m); 1.82 (m)	24.30 (t)	10	—	36.57 (s)	18	—	135.1 (s)	26	1.878 (3H, bs)	22.24 (q)
3	4.83 (dd, 11.6, 4.0 Hz)	80.76 (d)	11	1.38 (m); 1.52 (m)	27.74 (t)	19	2.26 (2H, m)	40.19 (t) ^a	27	1.787 (3H, s) ^c	16.26 (q) ^d
4	—	37.69 (s)	12	2.15 (m); 2.35 (m)	30.72 (t)	20	2.33 (2H, m)	27.12 (t) ^b	28	1.775 (3H, s) ^c	16.14 (q) ^d
5	1.30 (dd, J=10.4, 6.8 Hz)	49.76 (d)	13	5.44 (t, J=6.4 Hz) ^e	125.2 (d) ^f	21	5.38 (bt, J=7.2 Hz)	124.9 (d)	29	1.696 (3H, s)	17.74 (q)
6	1.97 (2H, m)	23.57 (t)	14	—	135.1 (s)	22	—	131.1 (s)	30	1.809 (3H, s)	25.86 (q)
7	5.50 (1H, brd s)	122.1 (d)	15	2.26 (2H, m)	40.23 (t) ^a	23	0.995 (3H, s)	27.95 (q)	31	—	169.9 (s)
8	—	135.3 (s)	16	2.33 (2H, m)	27.21 (t) ^b	24	1.077 (3H, s)	16.48 (q)	32	1.858 (3H, s)	20.84 (q)

The assignments of the symbols a-f are indistinguishable between the same symbols, due to the very close chemical shifts.

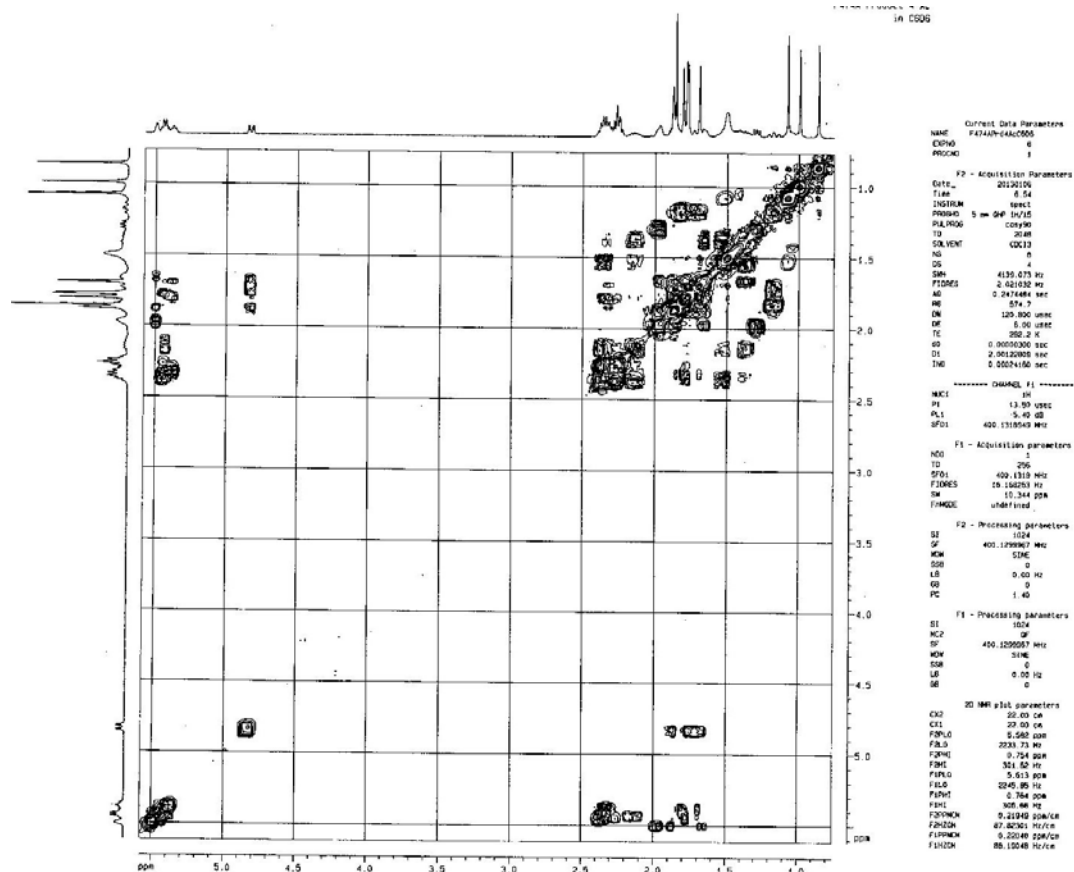
3.4.3. ^1H NMR in C_6D_6 (400 MHz)



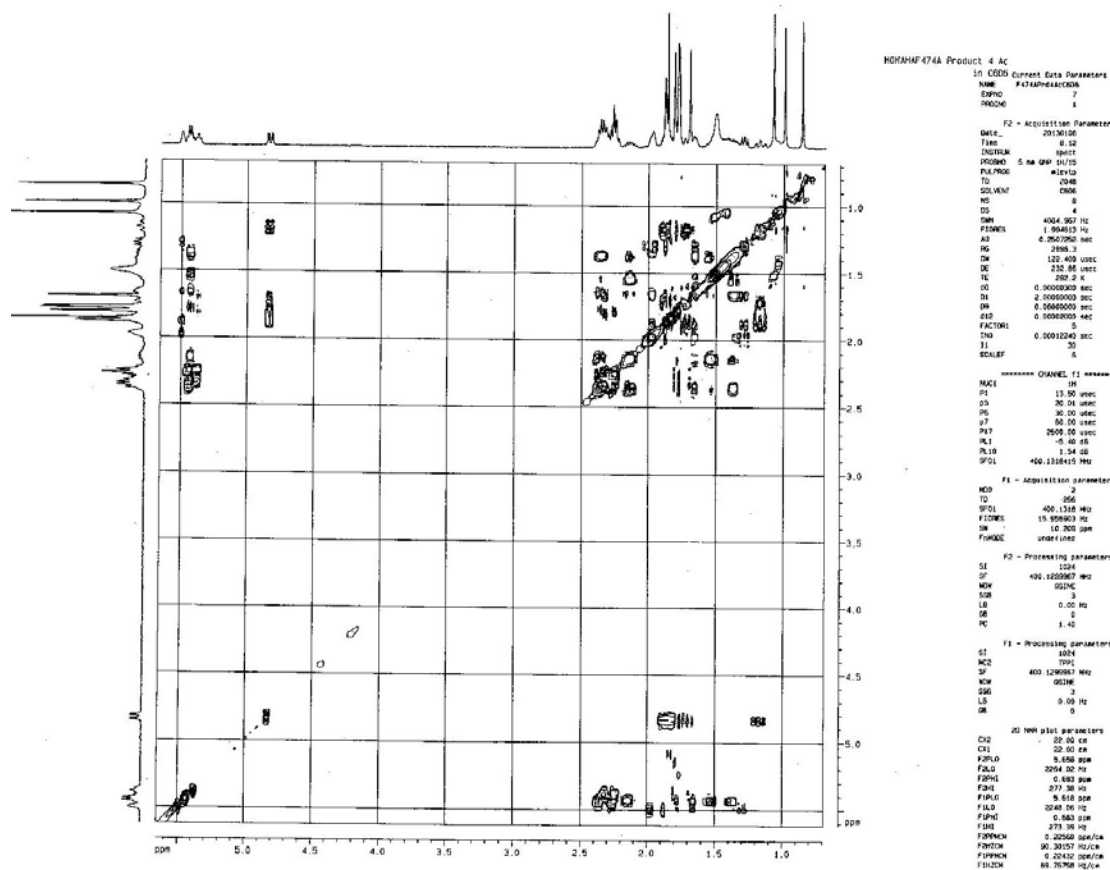
3.4.4. ^{13}C NMR in C_6D_6 (100 MHz)



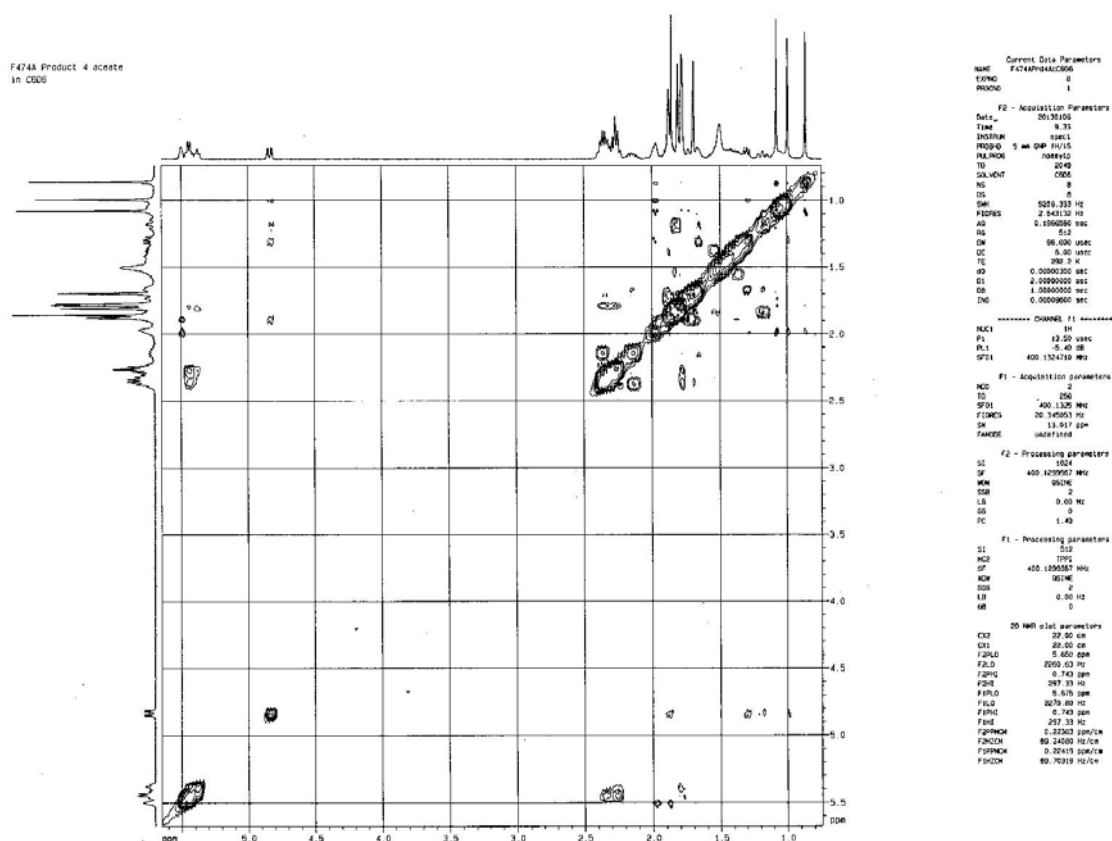
3.4.5. COSY NMR in C₆D₆ (400 MHz)



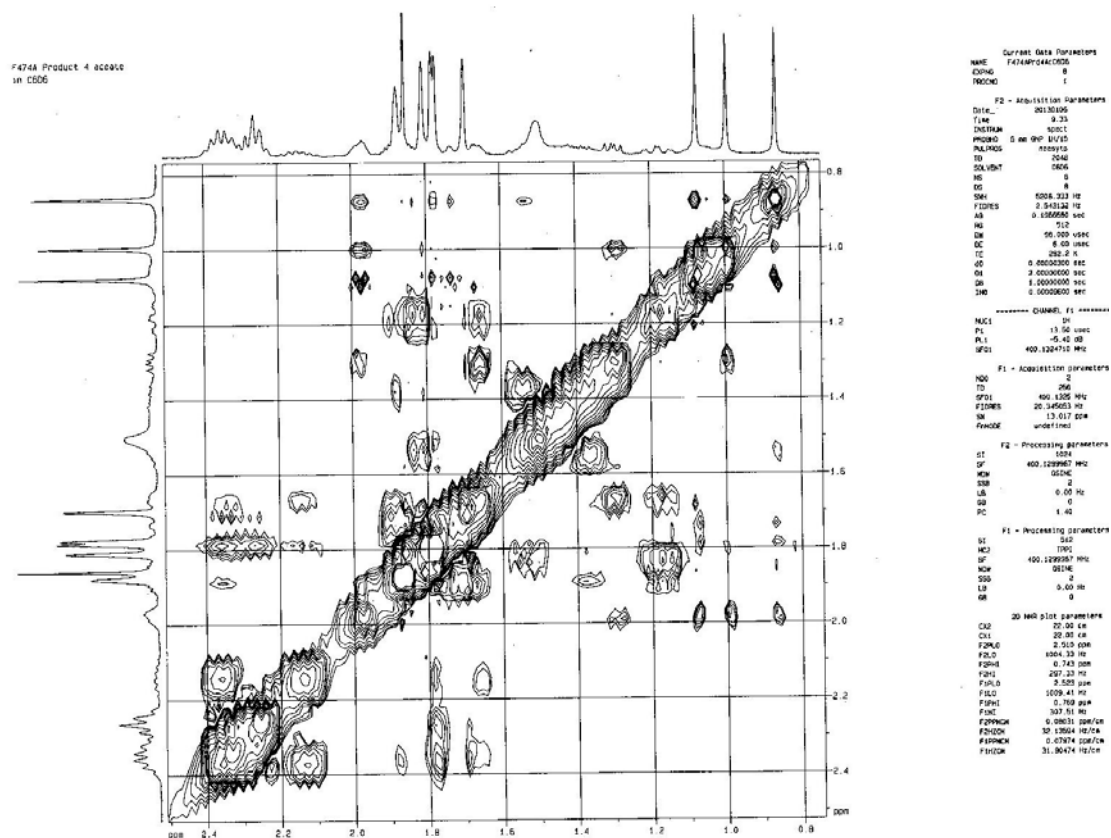
3.4.6. HOHAHA NMR in C₆D₆ (400 MHz)



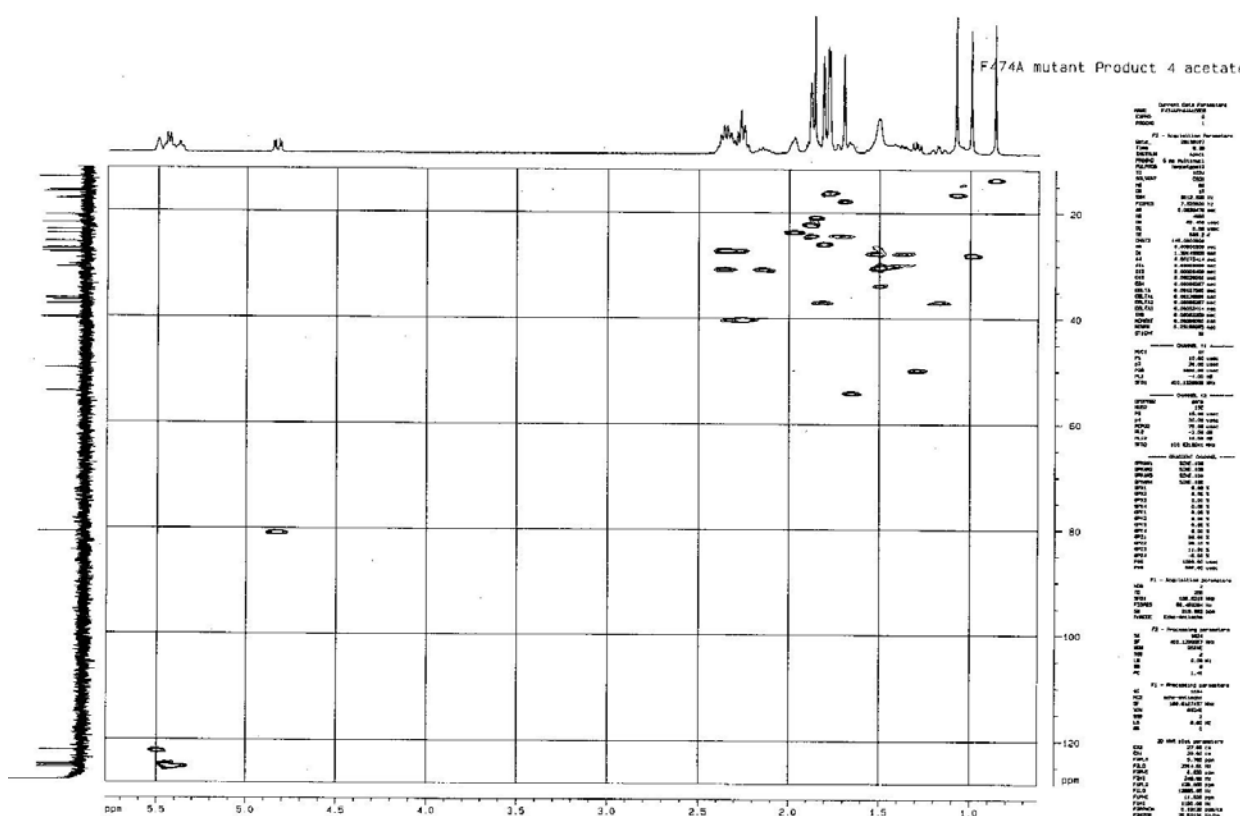
3.4.7. NOESY spectrum in C₆D₆ (400 MHz), whole region



3.4.8. NOESY spectrum in C₆D₆ (400 MHz), expanded region



3.4.9. HSQC spectrum in C₆D₆ (400 MHz).



3.4.10. HMBC spectrum in C₆D₆ (400 MHz).

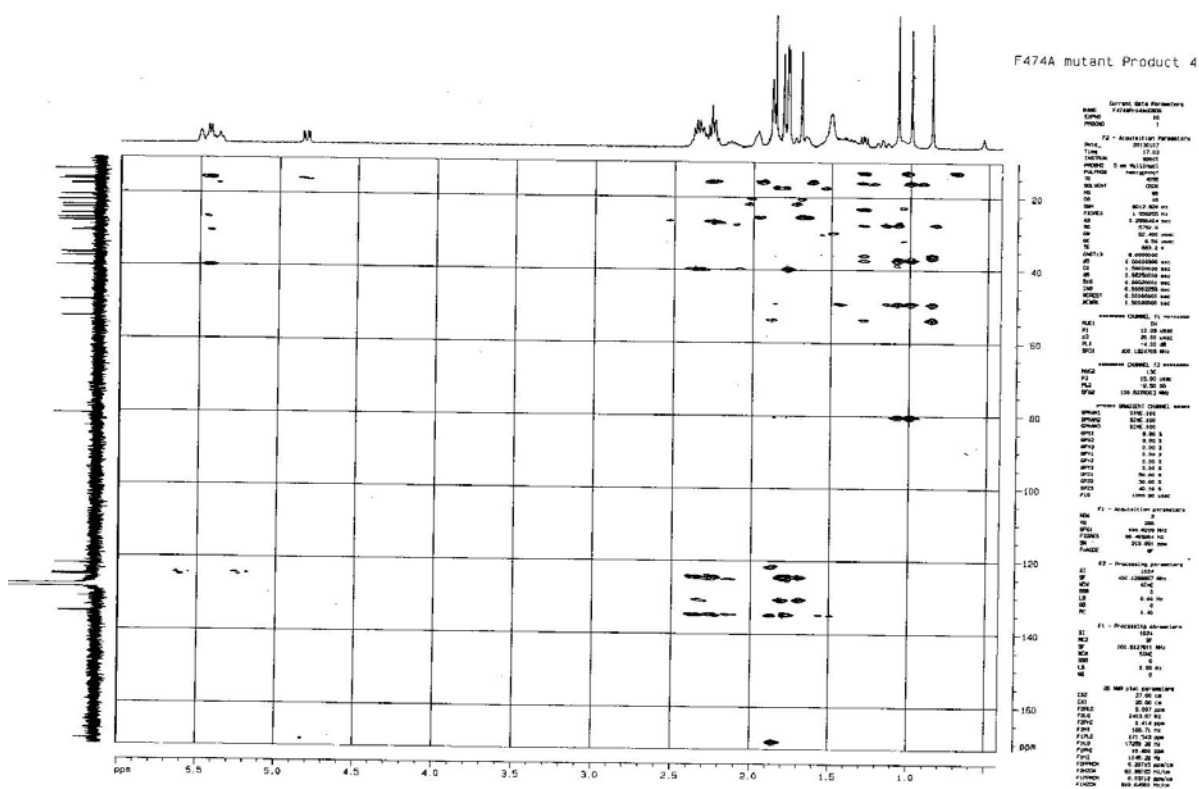
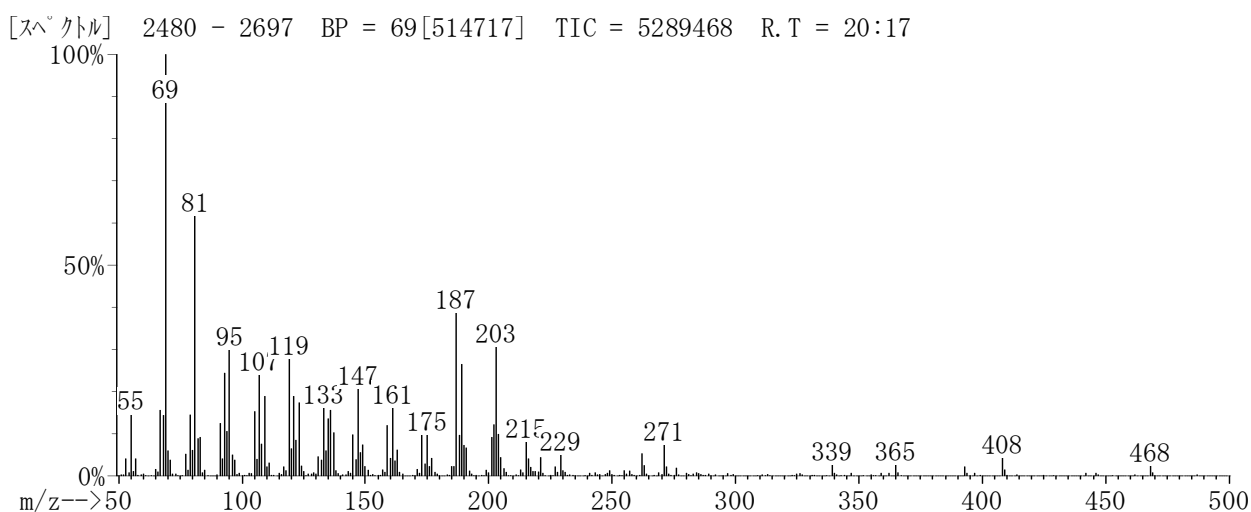


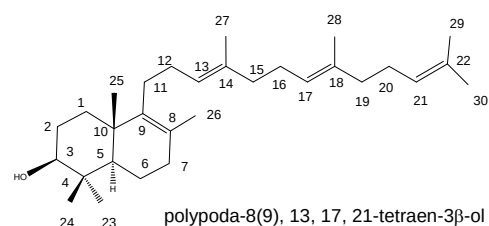
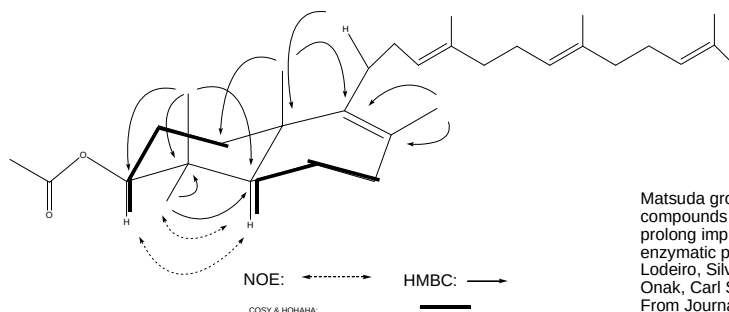
Figure S3. 5. Product 13 acetate from F474A mutant

3.5.1 EIMS



3.5.2. NMR data analyses of **13** in C₆D₆, [α]_D, HREIMS

Product 13 acetate isolated by F474A mutant



Matsuda group reported this compound as a mixture of other triterpene compounds as one of artifacts of 2,3-oxidosqualene which are produced by prolong impregnation in SiO₂, thus they indicated that this compound is not enzymatic product.
Lodeiro, Silvia; Xiong, Quanbo; Wilson, William K.; Kolesnikova, Mariya D.; Onak, Carl S.; Matsuda, Seiichi P. T.
From Journal of the American Chemical Society (2007), 129(36), 11213-11222

$[\alpha]_D^{25} = +13.9$ ($c=0.04$, CDCl₃)

There is not literature for the specific rotation of this compound.

HREIMS

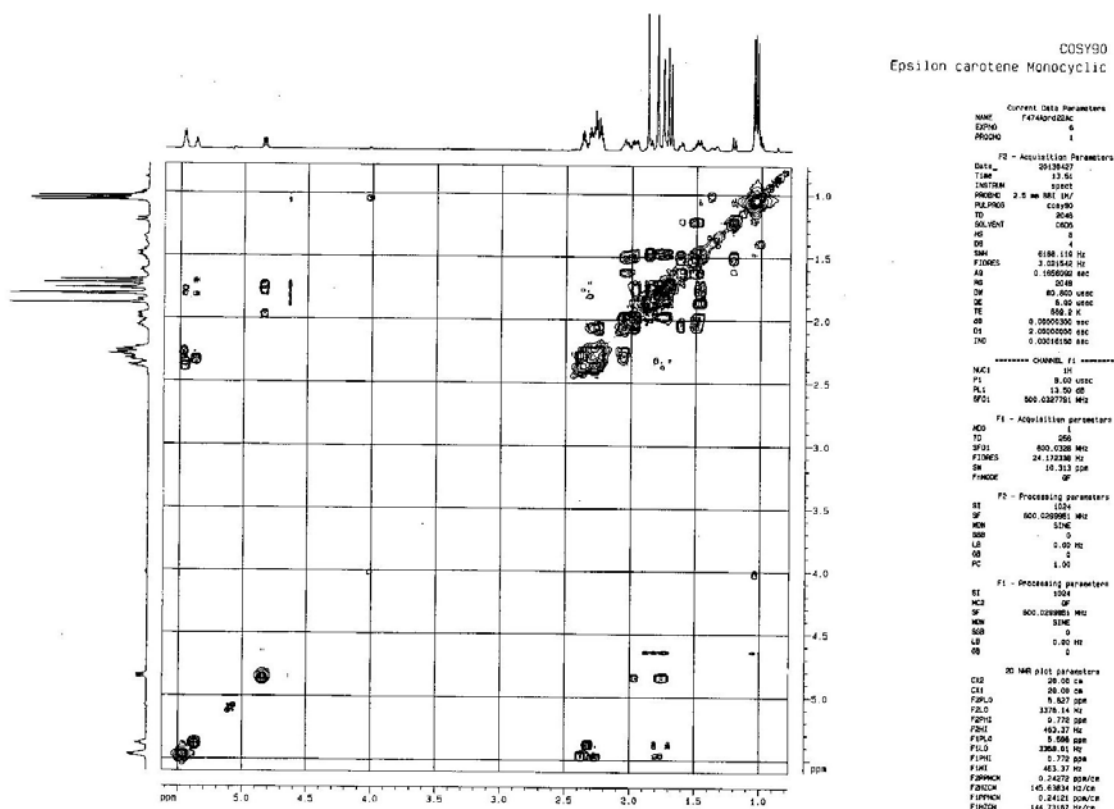
calcd. 468.39673; observed: 468.39657

600 MHz in C₆D₆
the solvent peak ¹H: 7.28 ppm; ¹³C:128.0 ppm

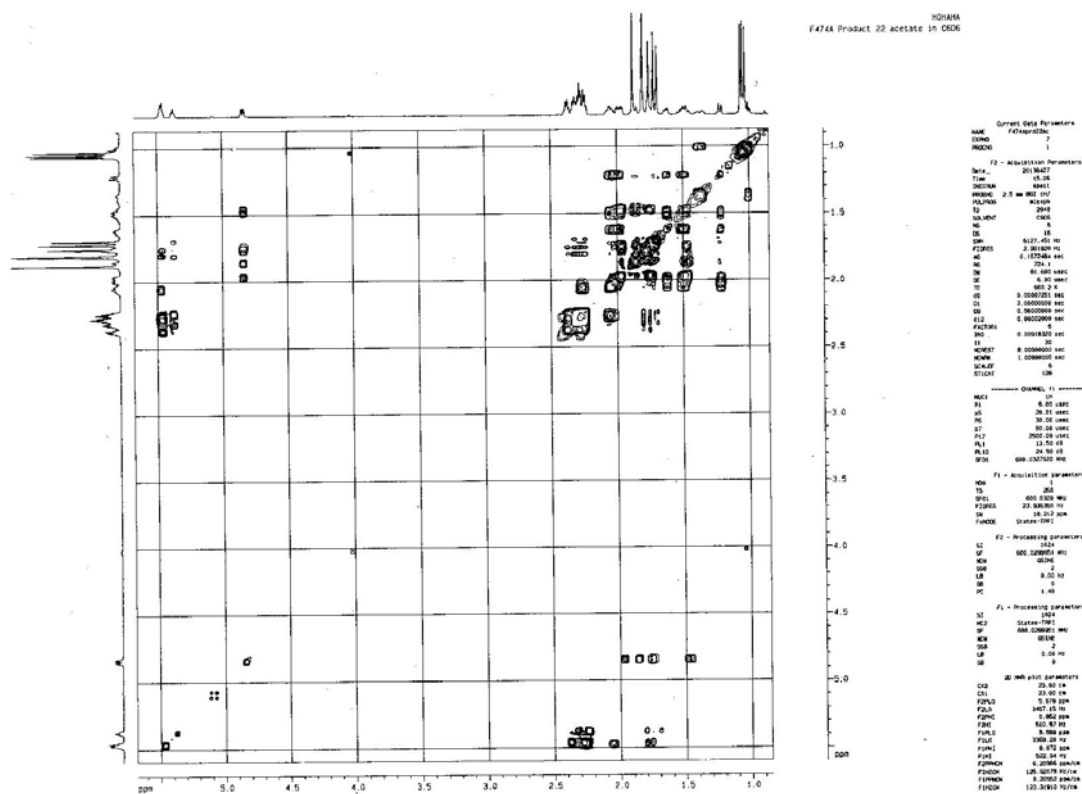
NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	1.47 (m); 1.85 (m)	34.94 (t)	9	—	139.9 (s)	17	5.46 (t, J=6.4 Hz) ^e	124.7 (d) ^f	25	1.064 (3H, s)	20.24 (q)
2	1.76 (m); 1.97 (m)	24.53 (t)	10	—	38.88 (s)	18	—	134.8 (s) ^a	26	1.722 (3H, s)	19.59 (q)
3	4.84 (dd, 11.8, 4.4 Hz)	80.57 (d)	11	2.06 (m); 2.28 (m)	28.80 (t)	19	2.26 (2H, m)	40.20 (t) ^a	27	1.805 (3H, s) ^c	16.23 (q) ^d
4	—	37.94 (s)	12	2.28 (2H, m)	29.54 (t)	20	2.33 (2H, m)	27.10 (t) ^b	28	1.758 (3H, s) ^c	16.14 (q) ^d
5	1.21 (td, J=12.3 Hz)	51.15 (d)	13	5.45 (t, J=6.4 Hz) ^e	125.4 (d) ^f	21	5.38 (bt, J=6.9 Hz)	124.9 (d)	29	1.696 (3H, s)	17.72 (q)
6	1.50 (m); 1.62 (m)	18.97 (t)	14	—	135.1 (s)	22	—	131.1 (s)	30	1.805 (3H, s)	25.83 (q)
7	1.99 (m); 2.02 (m)	33.88 (t)	15	2.26 (2H, m)	40.20 (t) ^a	23	1.031 (3H, s)	28.20 (q)	31	—	169.9 (s)
8	—	126.2 (s)	16	2.33 (2H, m)	27.24 (t) ^b	24	1.049 (3H, s)	16.92 (q)	32	1.881 (3H, s)	20.83 (q)

The assignments of the symbols a-f are indistinguishable between the same symbols, due to the very close chemical shifts.

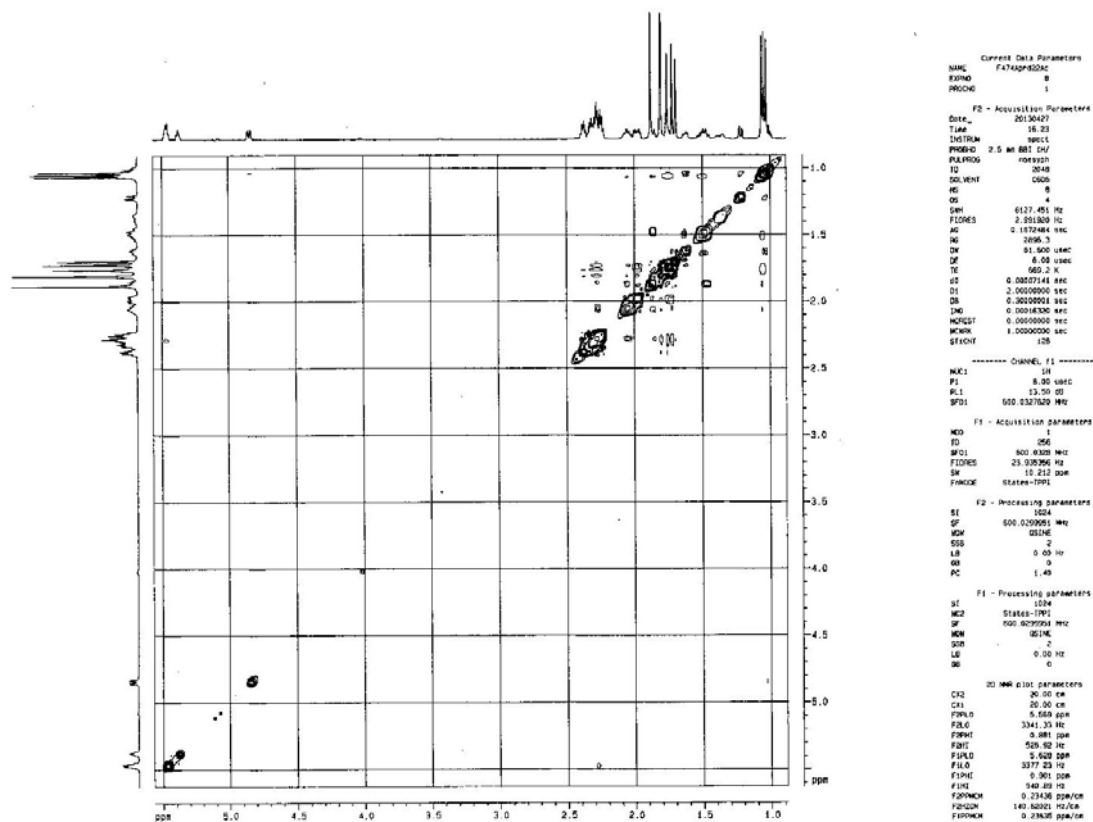
3.5.5. COSY spectrum in C₆D₆ (600 MHz)



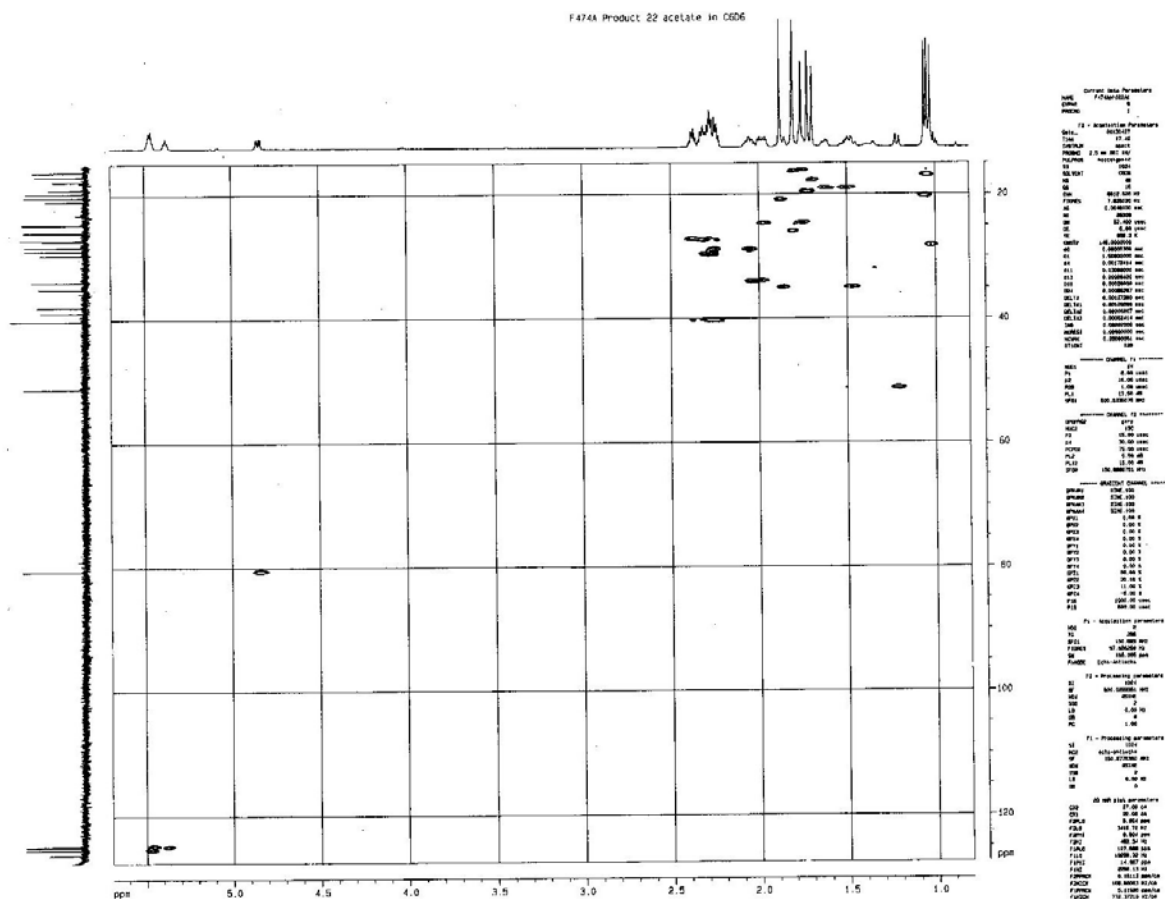
3.5.6. HOHAHA spectrum in C₆D₆ (600 MHz)



3.5.7. NOESY spectrum in C₆D₆ (600 MHz)



3.5.8.. HSQC spectrum in C₆D₆ (600 MHz)



3.5.9.. HMBC spectrum in C₆D₆ (600 MHz)

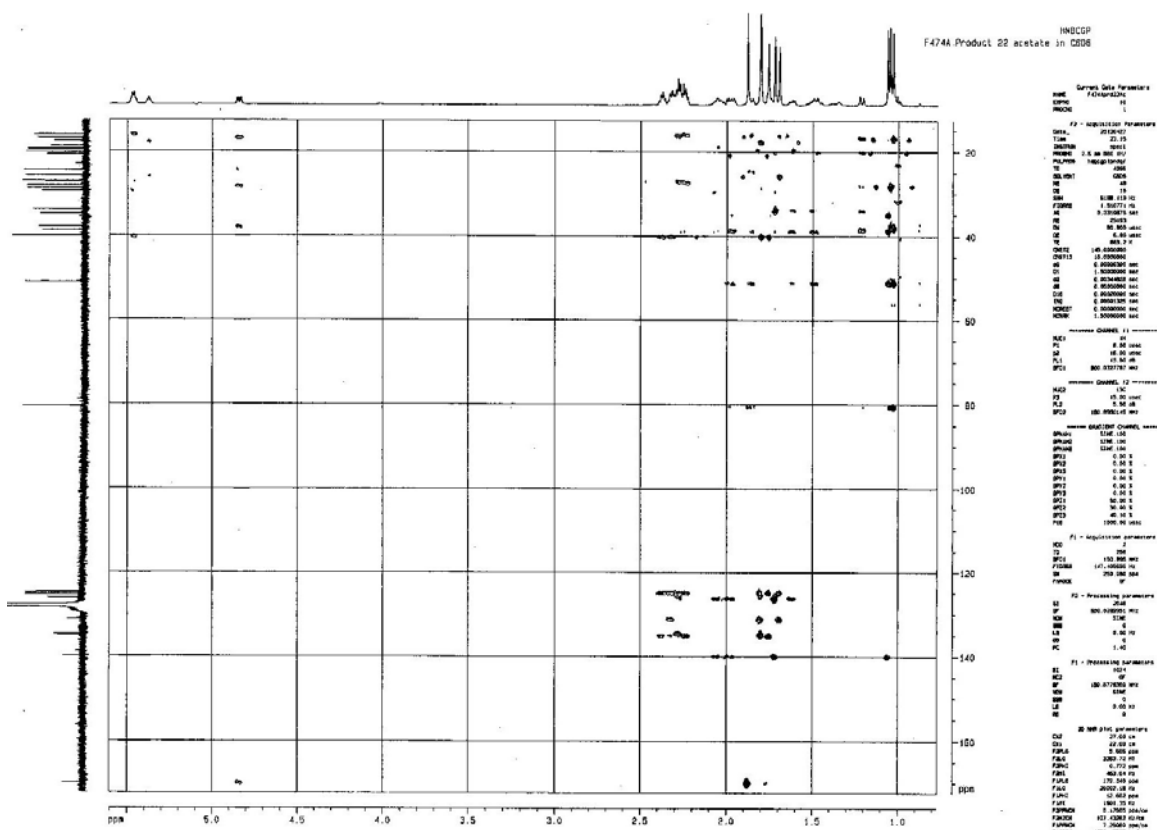


Figure. S.3.6 Product 14 acetate from F474A mutant

(butyrospermol acetate)

EIMS

[スペクトル] 3646 - 5301 BP = 69[32610] TIC = 546798 R.T = 28:11

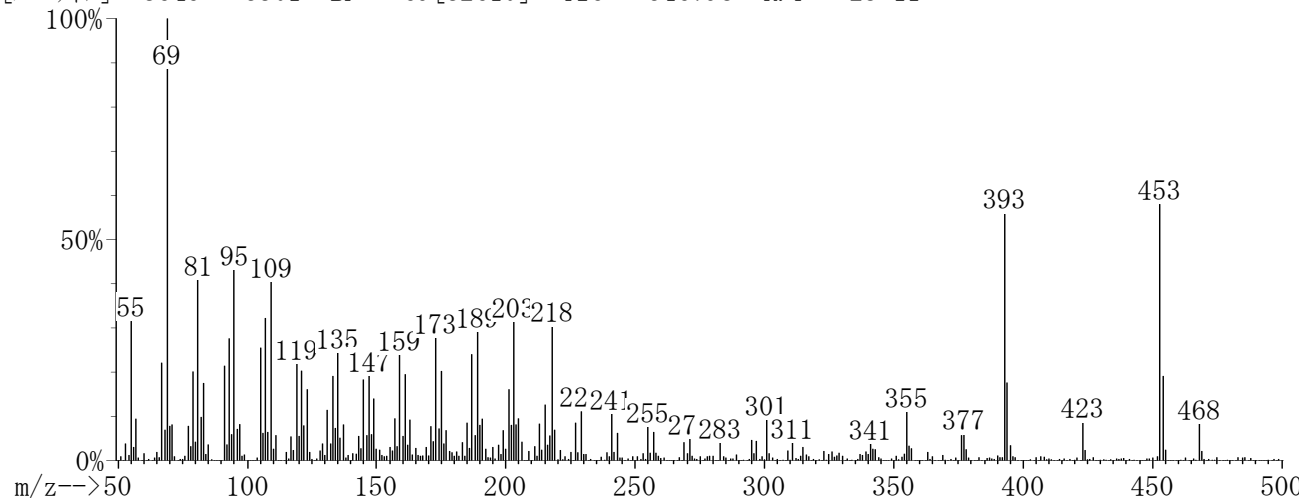


Figure . S.3. 7. Product 15 acetate from F474A mutant

(tirucalla-7,24-dien-3 β -ol acetate)

EIMS

[スペクトル] 3909 - 5301 BP = 69[69671] TIC = 1016963 R.T = 29:58

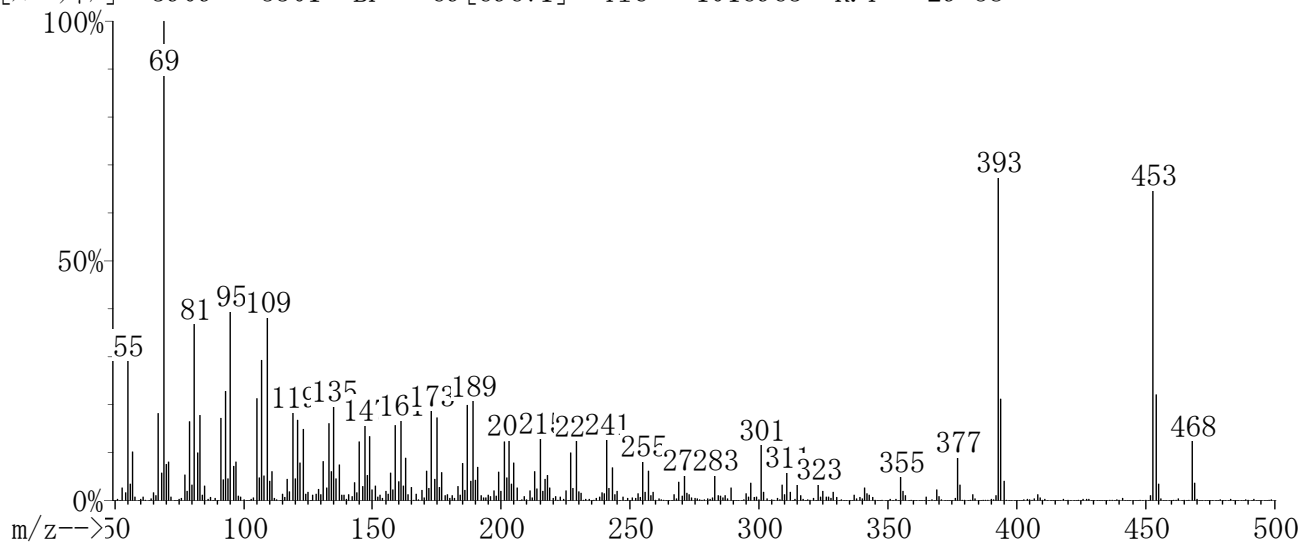
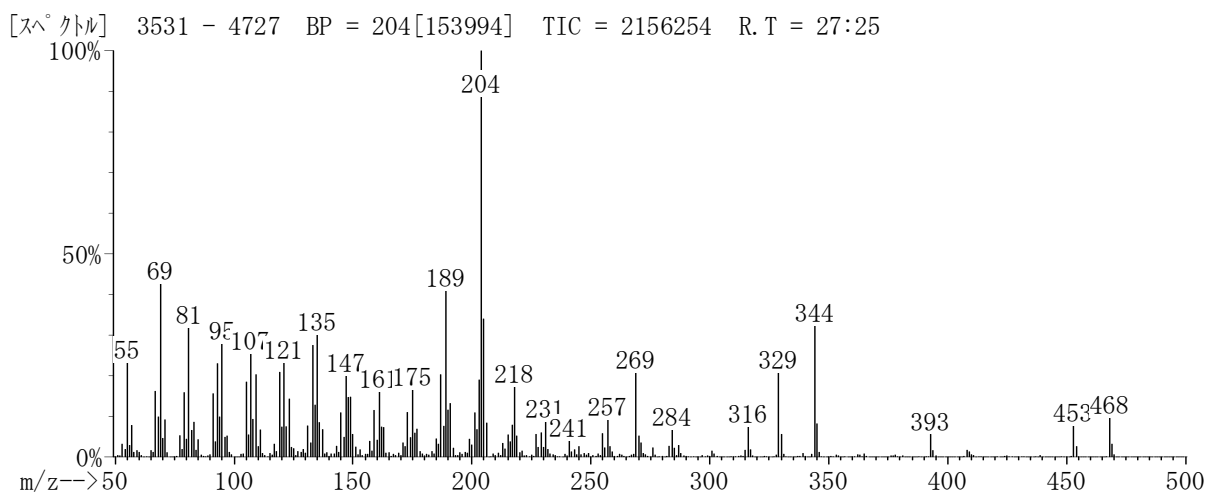


Figure S.3.8. Product 16 acetate from F474A mutant

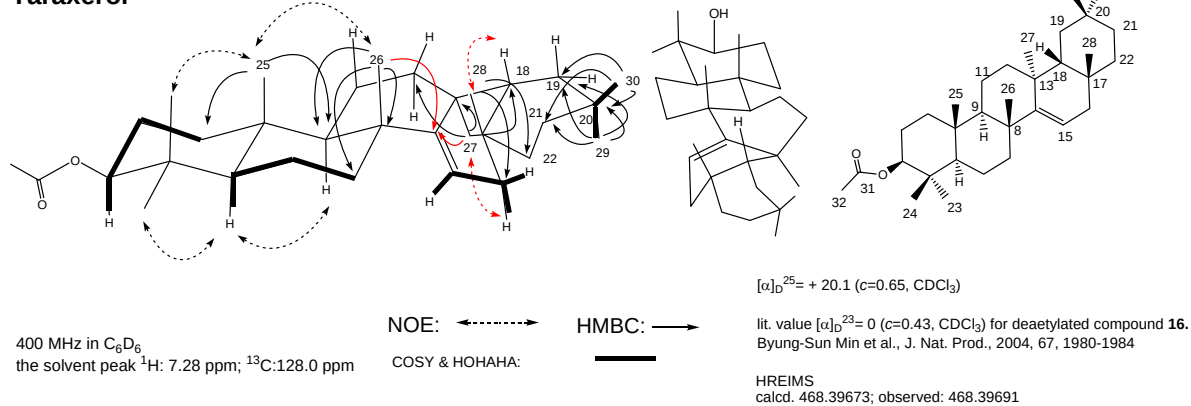
3.8.1. EIMS



3.8.2. NMR data analyses of **16** in C₆D₆ (400 MHz), [α]_D, HREIMS

F474A Product 16 acetate

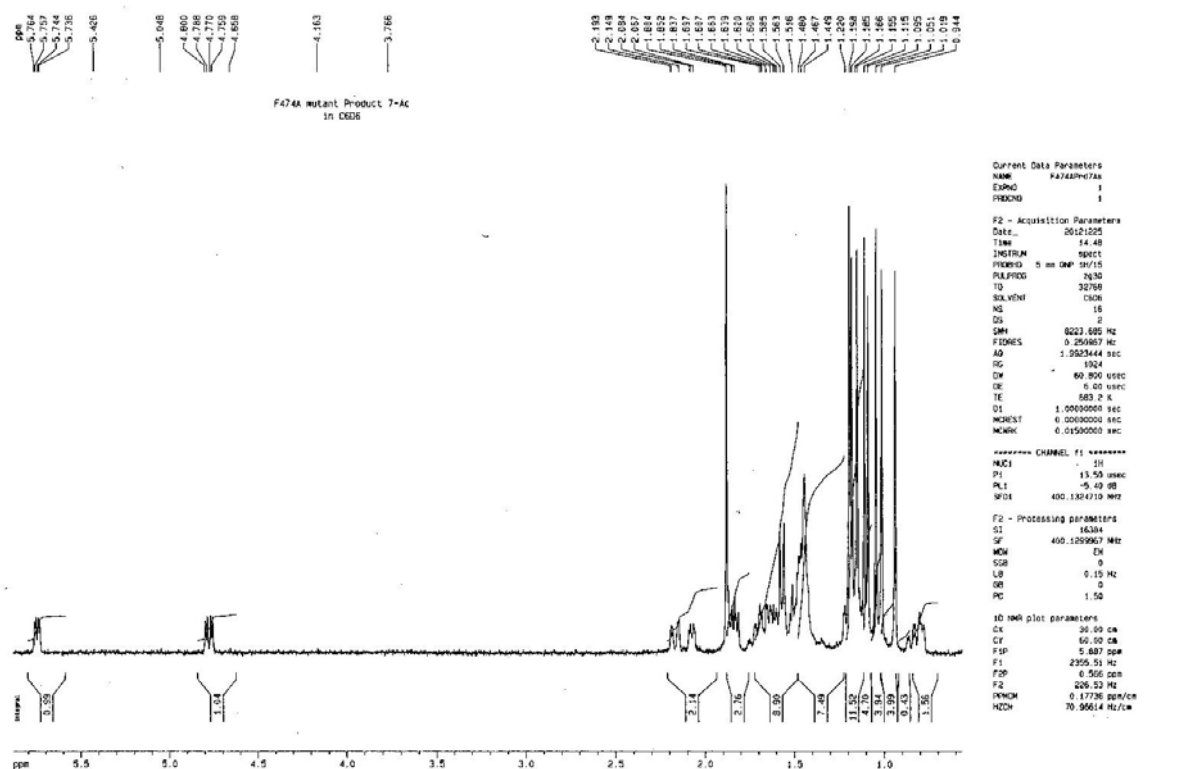
Taraxerol



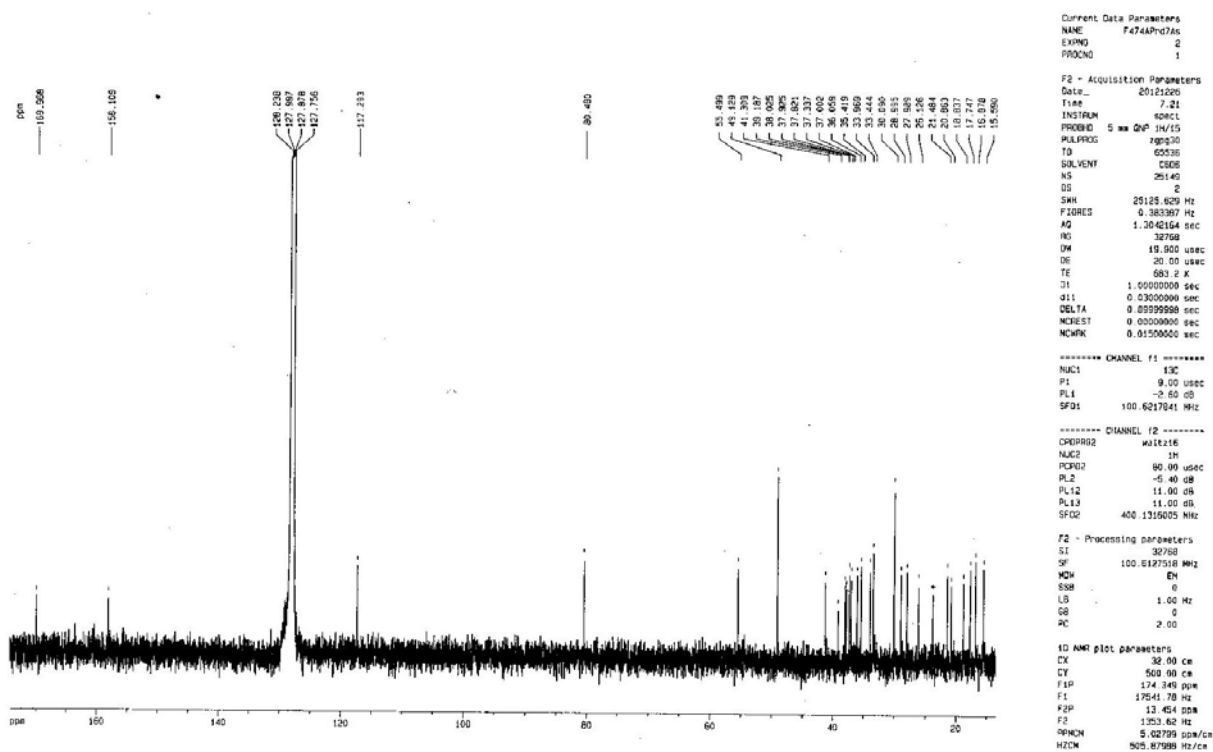
NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	0.83 (ddd, J=13.2, 13.2, 3.6 Hz); 1.48 (m)	37.34(t)	9	1.44 (m)	49.13(d)	17	—	36.06(s)	25	0.944(3H,s)	15.59(q)
2	1.71(m); 1.84 (m)	23.83(t)	10	—	37.76(s) ^a	18	1.17 (m)	49.13(d)	26	1.199 (3H,s)	26.13(q)
3	4.78(dd, 12.0, 4.8 Hz)	81.17 (d)	11	1.44 (m); 1.64(m)	17.75 (t)	19	1.46 (m); 1.59 (m)	33.43 (t)	27	1.115 (3H,s)	21.48 (q)
4	—	37.82(s) ^a	12	1.67(2H, m)	33.97(t)	20	—	28.99 (s)	28	0.933 (3H, s)	30.09(q)
5	0.79 (m)	55.50(d)	13	—	37.92 (s) ^a	21	1.16(m); 1.51(m)	37.00 (t)	29	1.185 (3H, s)	33.97 (q) ^b
6	1.44(m); 1.57(m)	18.84(t)	14	—	158.1 (s)	22	1.20 (m); 1.57 (m)	35.42 (t)	30	1.155 (3H, s)	30.09 (q) ^b
7	1.44 (m); 2.07 (dd, 9.6, 3.2 Hz)	41.31 (t)	15	5.75 (dd, J=8.4, 2.8 Hz)	117.3 (d)	23	1.019 (3H, s)	27.99 (q)	31	—	170.0 (s)
8	—	39.19 (s)	16	1.84(m); 2.17 (dd, J=14.4, 3.2 Hz)	38.02(t)	24	1.051 (3H,s)	16.88(q)	32	1.884 (3H, s)	20.86 (q)

a: The three carbon assignments were indistinguishable due to the close chemical shifts.
b: The assignments of Me-29 and Me-30 may be exchangeable.

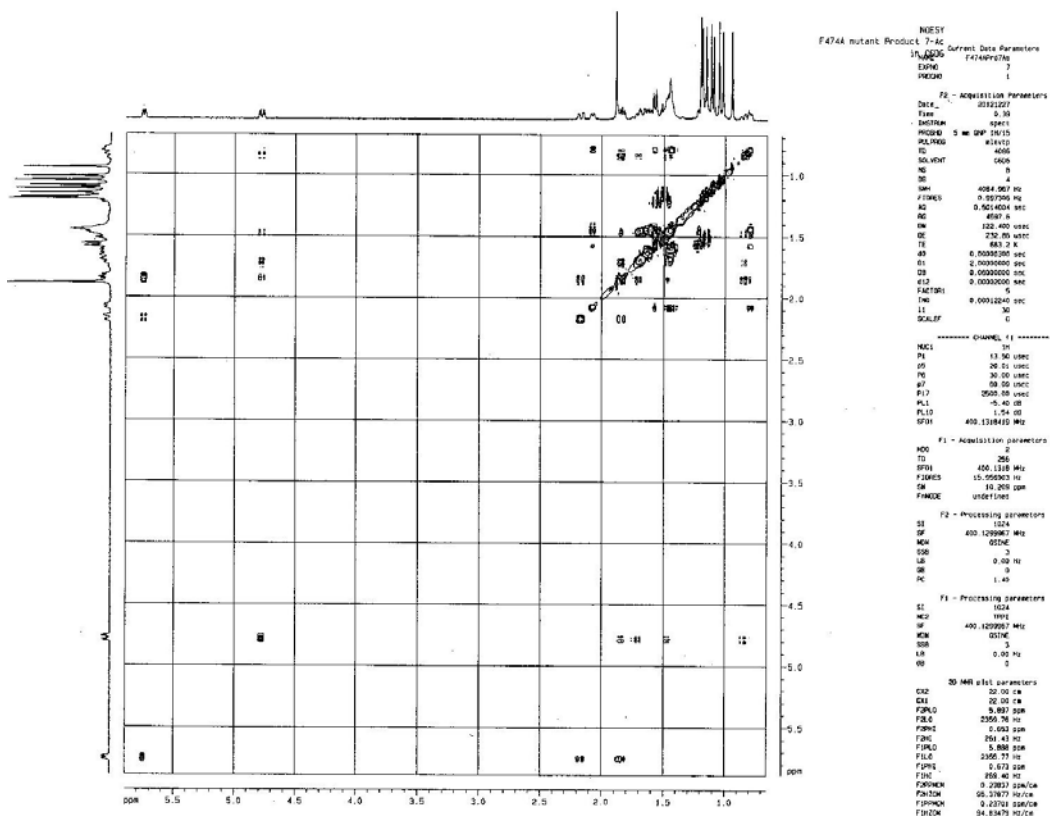
3.8.3. ^1H NMR in C_6D_6 (400 MHz)



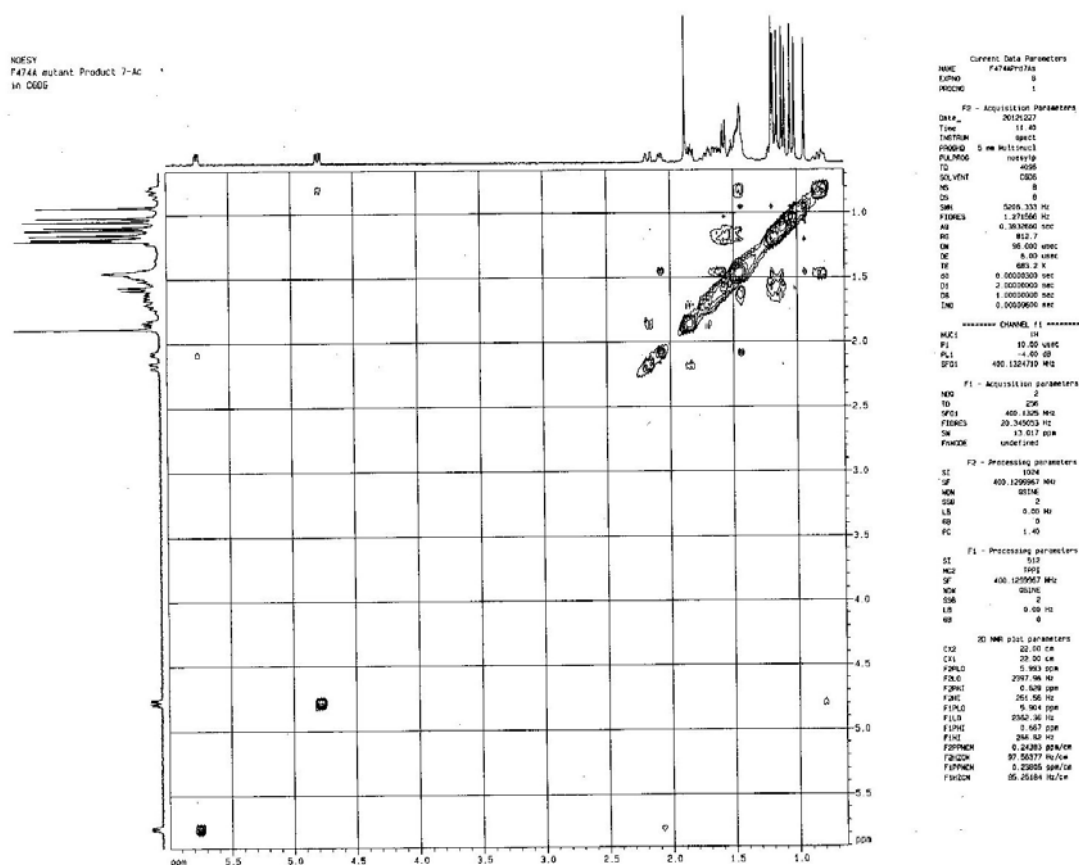
3.8.4. ^{13}C NMR in C_6D_6 (100 MHz)



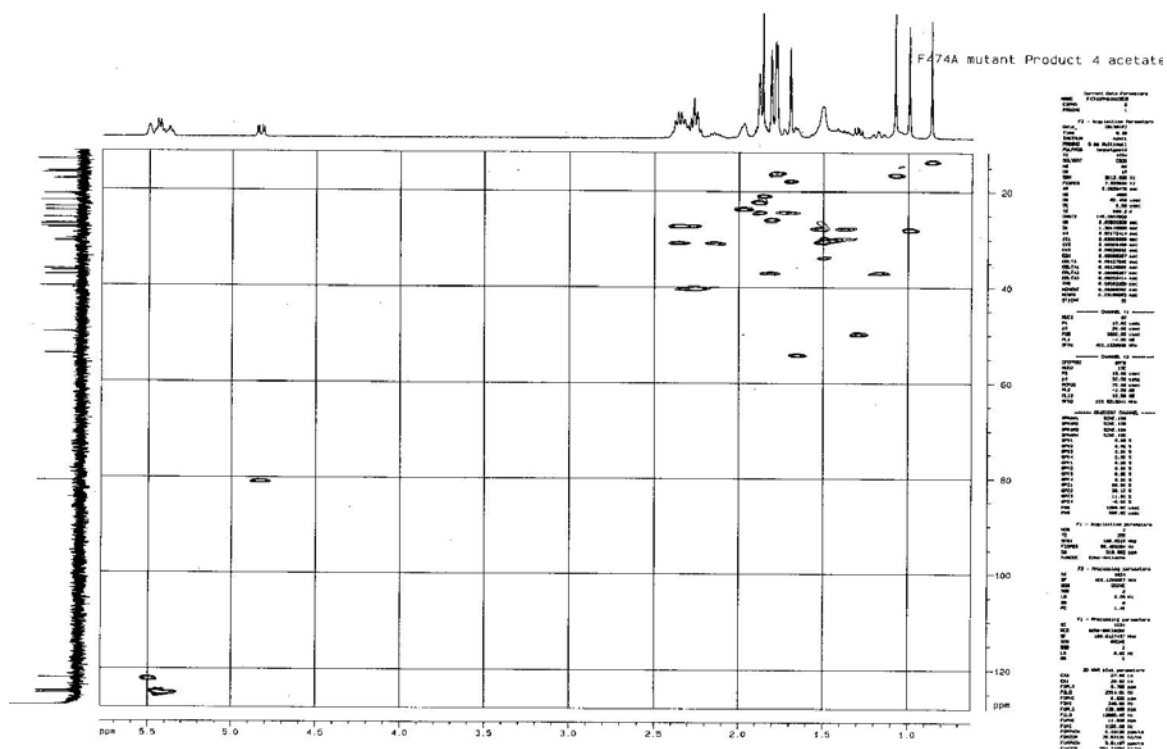
3.8.6 HOHAHA in C₆D₆ (400 MHz)



3.8.7. NOESY in C₆D₆ (400 MHz)



3.8.8. HSQC in C₆D₆ (400 MHz)



3.8.9. HMBC in C₆D₆ (400 MHz)

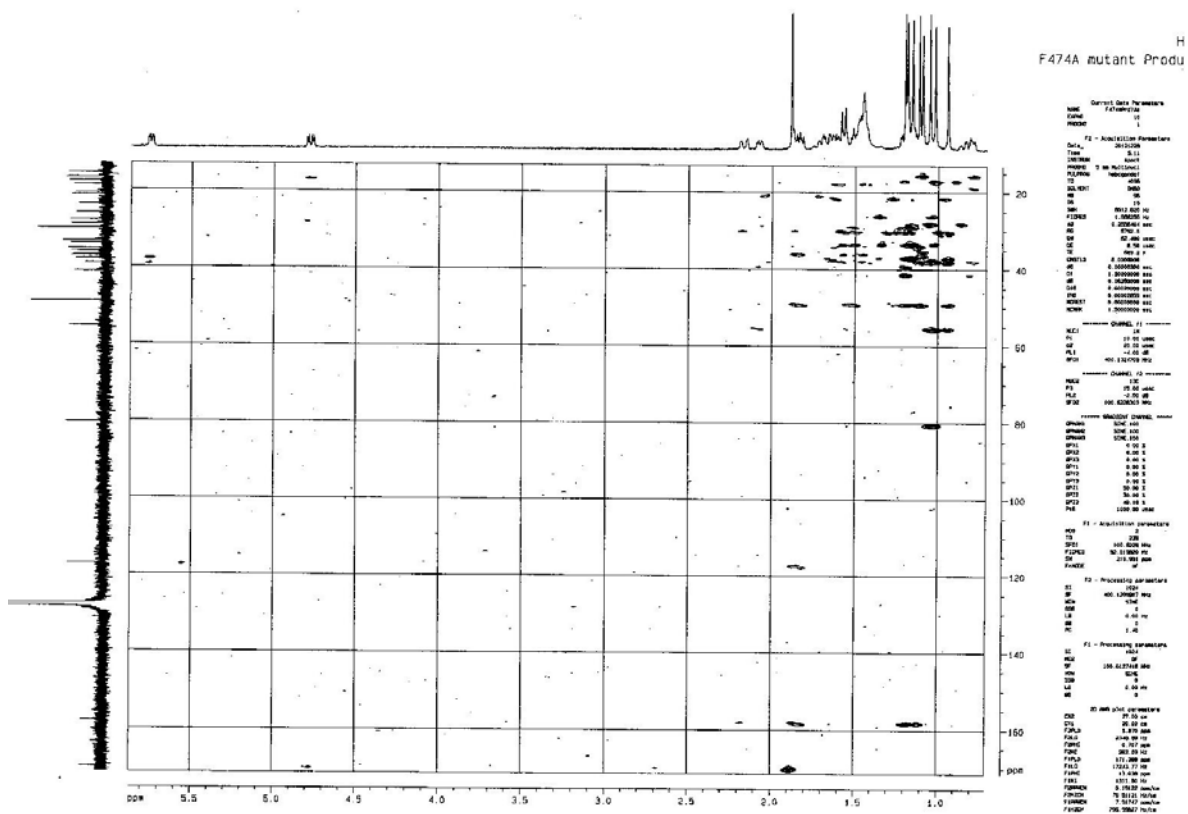
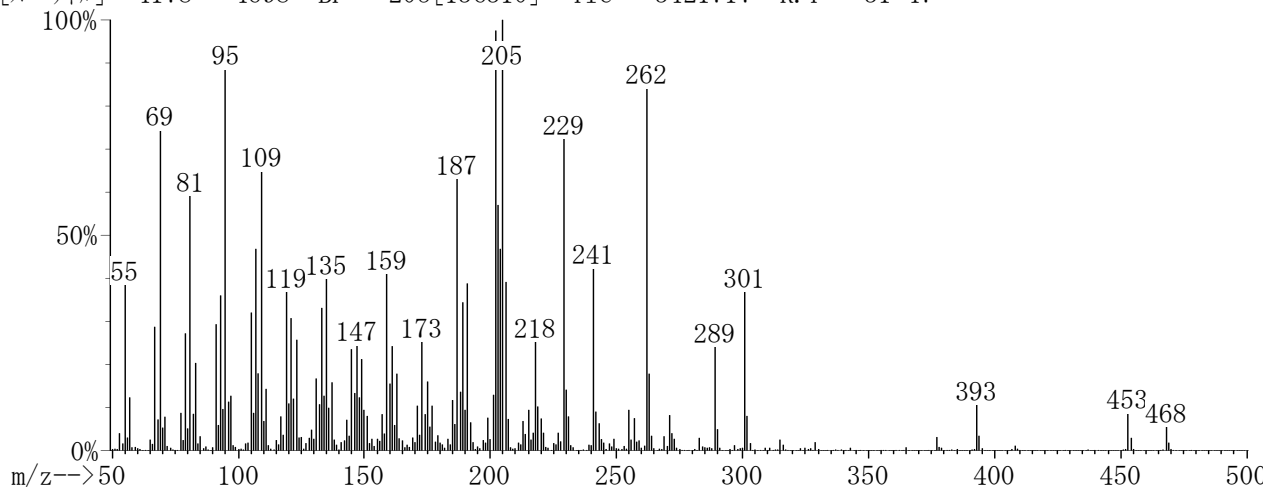


Figure S3.9. Product 17 acetate from F474A mutant

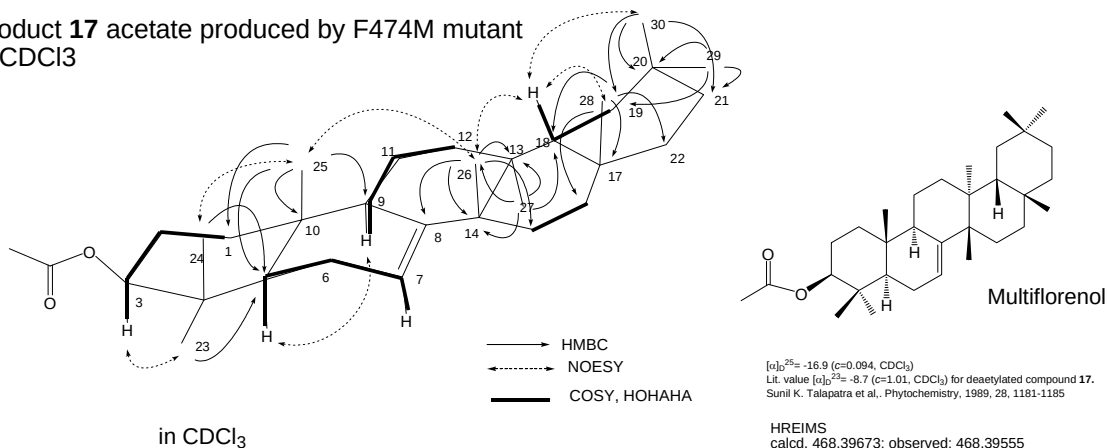
3.9.1. EIMS

[スペクトル] 4178 - 4698 BP = 205[136310] TIC = 3421717 R.T = 31:47



3.9.2. NMR data analyses of **17** in CDCl₃ (600 MHz), [α]_D, and HREIMS

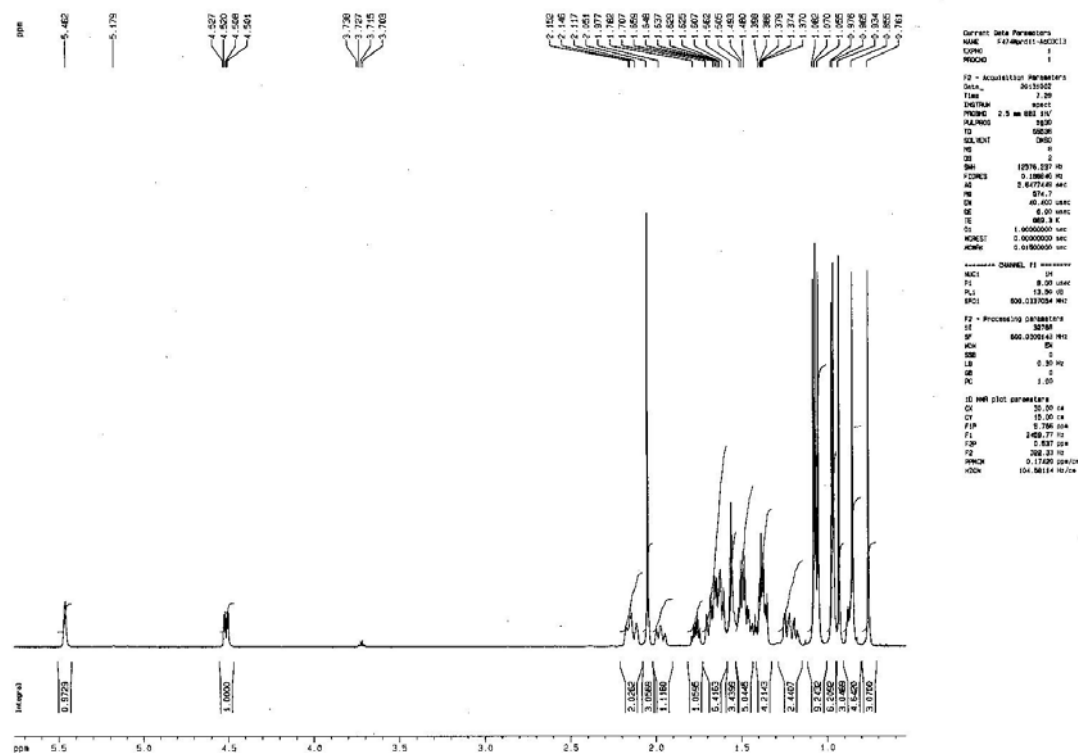
Product **17** acetate produced by F474M mutant in CDCl₃



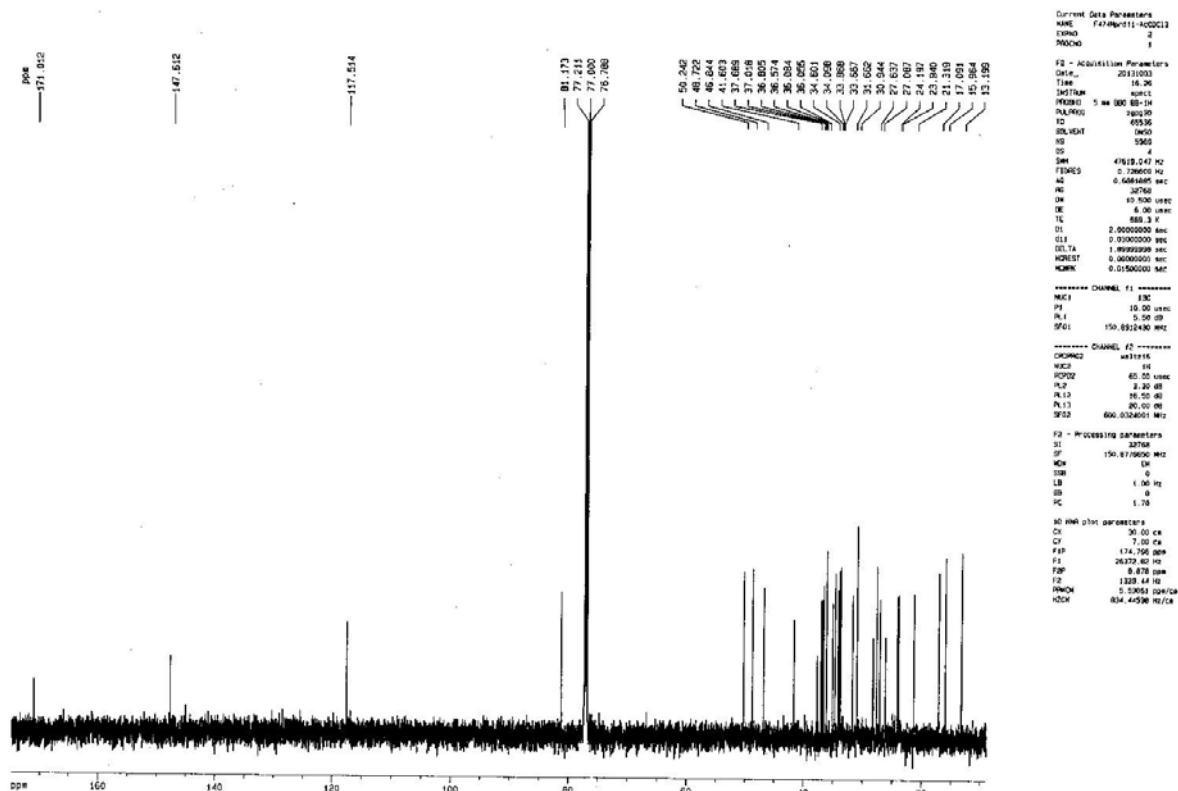
NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C	NO.	¹ H	¹³ C
1	1.19 (m), 1.70(m)	36.80 (t)	9	2.16 (m)	48.72(d)	17	—	30.94 (s) ^b	25	0.761 (3H, s)	13.20 (q)
2	1.64 (2H,m)	24.20(t)	10	—	35.06(s)	18	1.50 (m)	46.84 (d)	26	1.070(3H, s)	27.09(q)
3	4.51(dd, J=11.4; 4.2 Hz)	81.17(d)	11	1.43 (m); 1.54(μ)	17.09 (t)	19	1.38(2H, m)	34.60(t)	27	1.082 (3H, s)	26.16 (q)
4	—	37.69(s)	12	0.87(m); 1.62(m)	36.06(t) ^a	20	—	28.23(s)	28	1.055(3H, s)	30.94(q) ^b
5	1.36 (m)	50.24(d)	13	—	37.02(s)	21	1.24 (m); 1.46(m)	33.87(t)	29	0.976 (3H, s)	33.67(q)
6	1.98 (m); 2.12 (m)	23.94(t)	14	—	41.60(s)	22	1.48(2H,m)	36.57(t)	30	0.965 (3H, s)	34.09(q)
7	5.46(bs)	111.5(d)	15	1.64(m); 1.77(m)	31.66 (t)	23	0.855(3H, s)	27.64(q)	31	—	171.0(s)
8	—	147.6 (s)	16	1.37 (m);1.62 (m)	36.08(t) ^a	24	0.934 (3H, s)	15.96(q)	32	2.051(3H, s)	21.32(q)

^a These carbon signals may be exchangeable between the same letters
^b : Me-28 nd C-17 were overlapped..

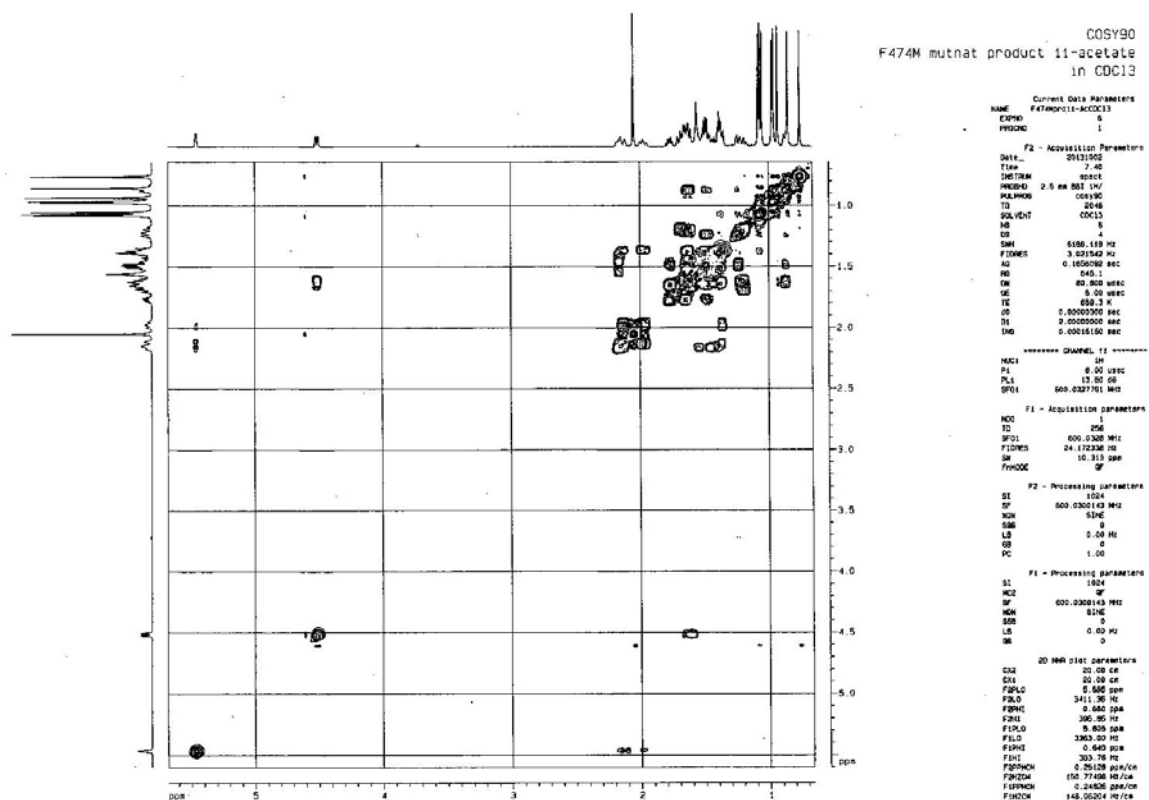
3.9.3. ^1H NMR in CDCl_3 (600 MHz)



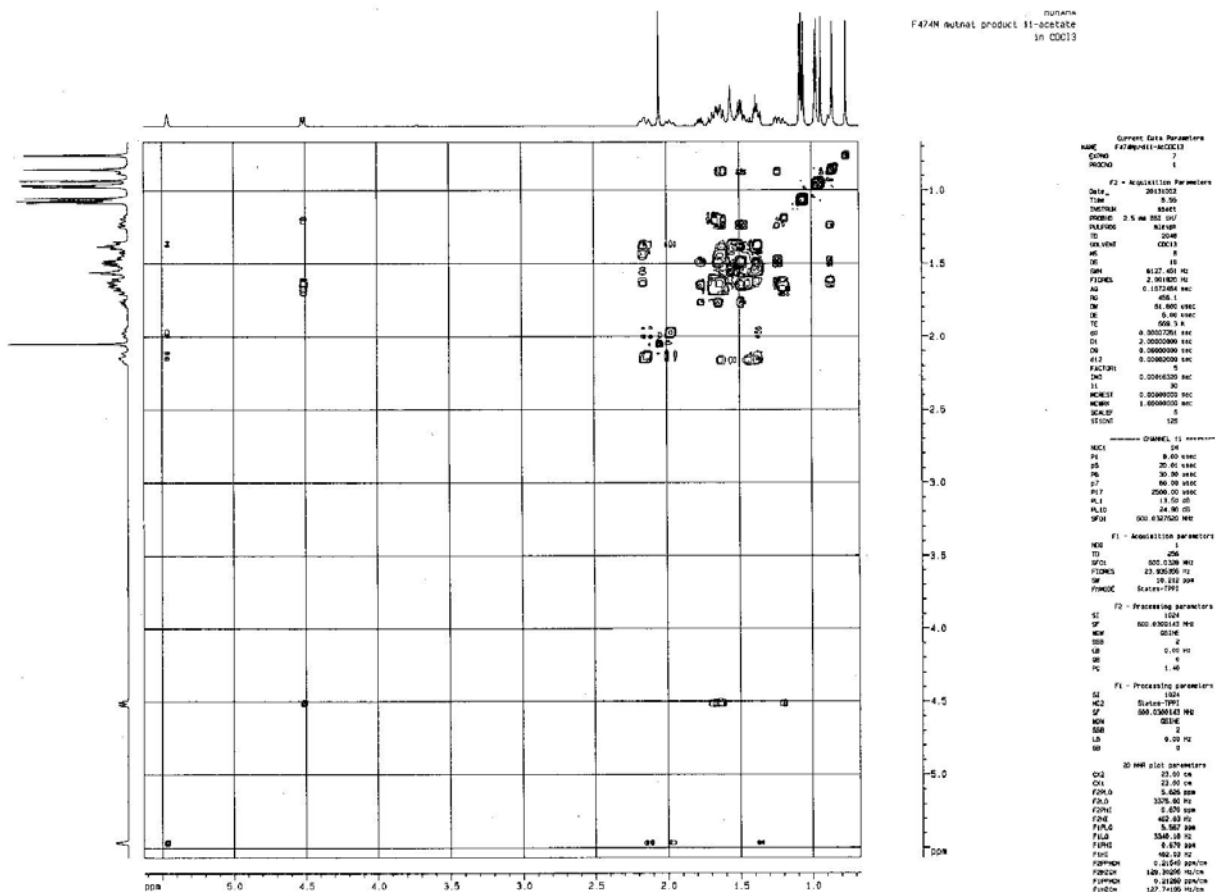
3.9.4. ^{13}C NMR in CDCl_3 (150 MHz)



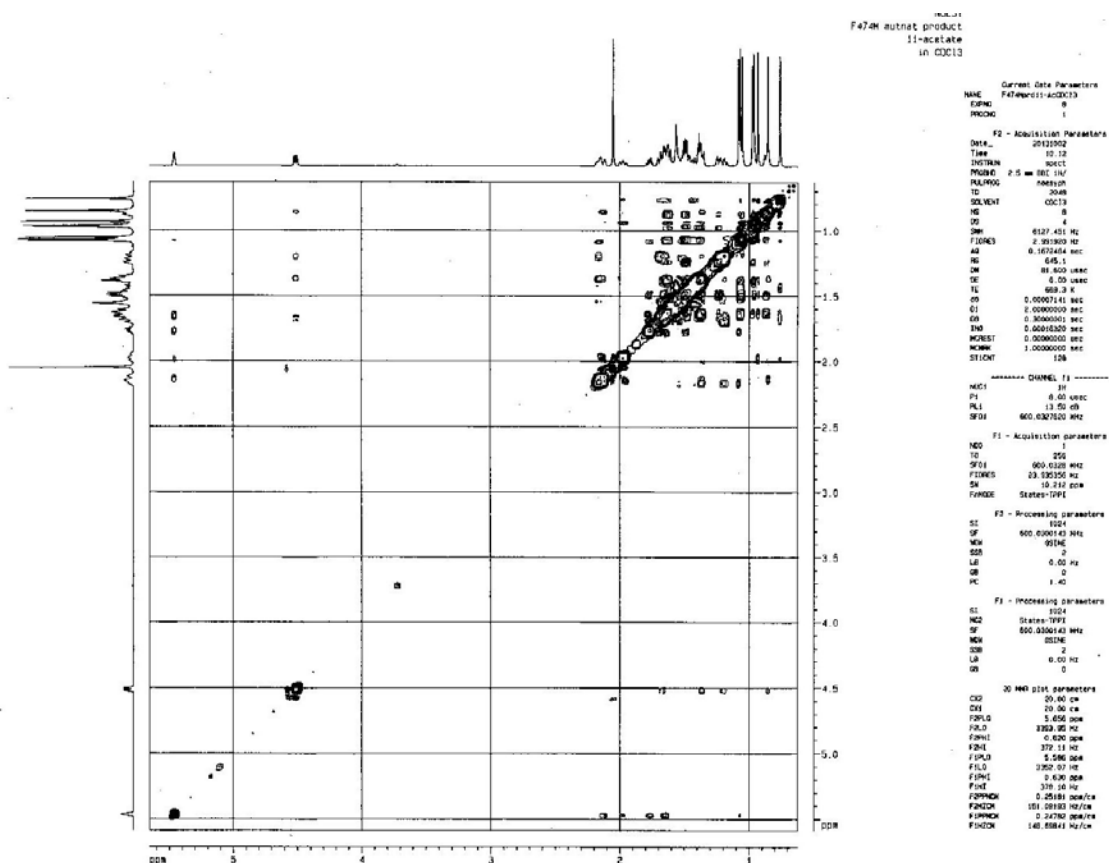
3.9.5. COSY in CDCl₃ (600 MHz)



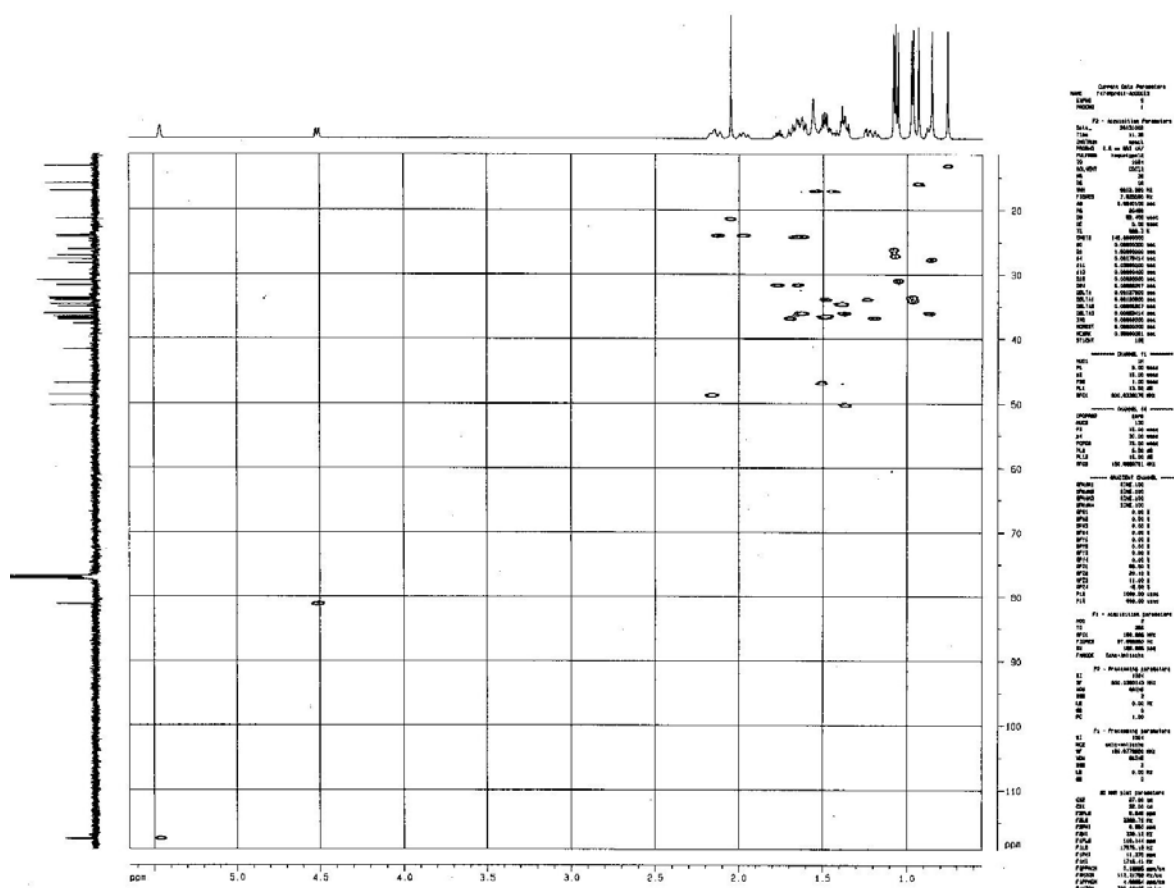
3.9.6. HOHAHA in CDCl₃ (600 MHz)



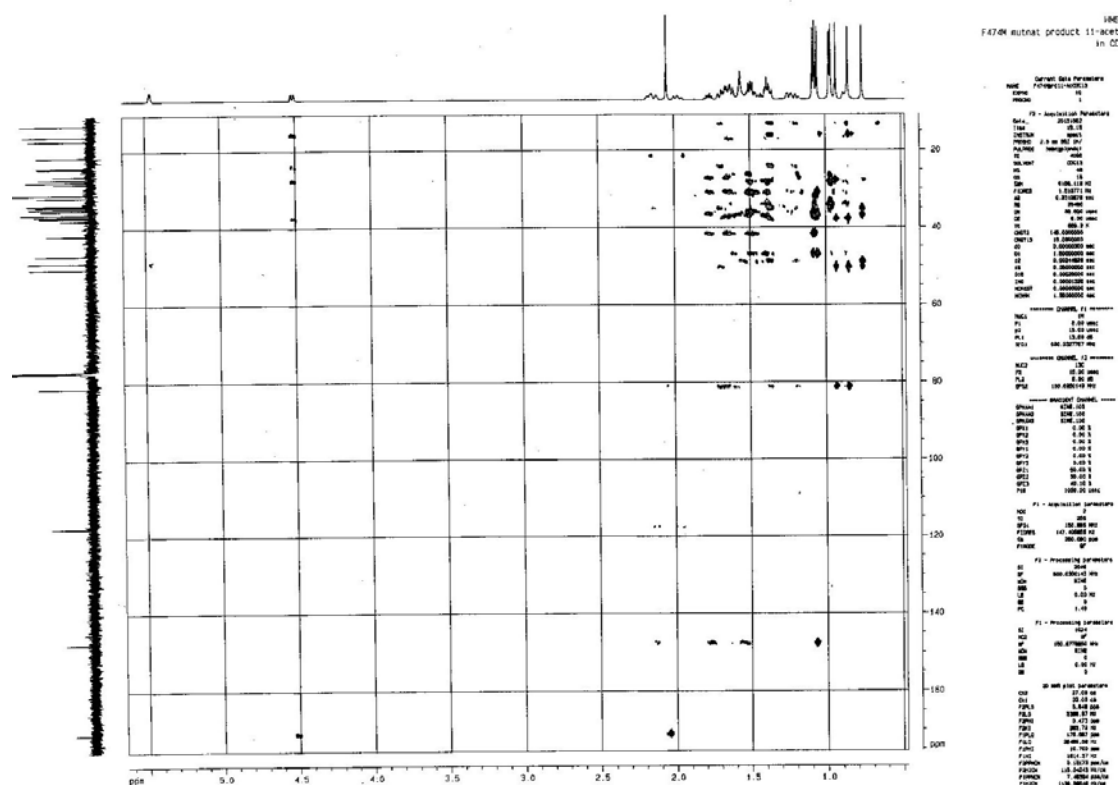
3.9.7. NOESY in CDCl₃ (600 MHz)



3.9.8. HMBC in CDCl₃ (600 MHz)

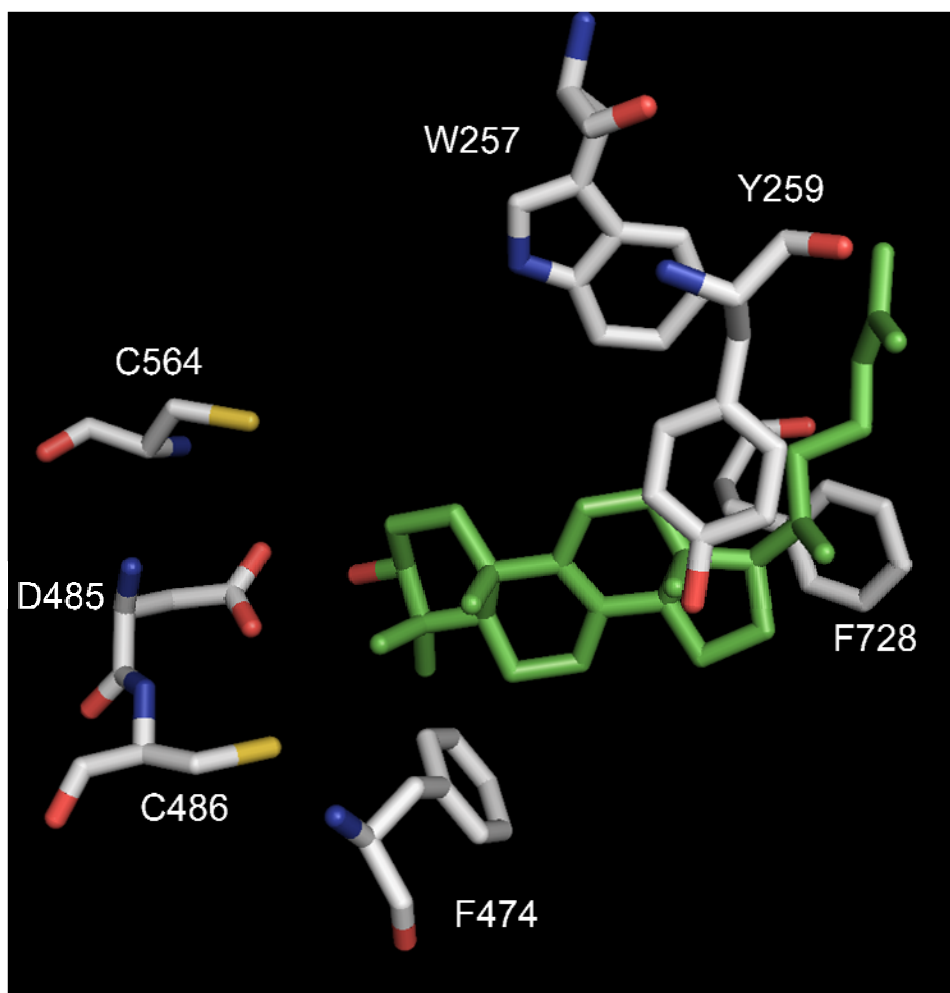


3.9.9. HMBC in CDCl₃ (600 MHz)



Supplementary Figure S4.

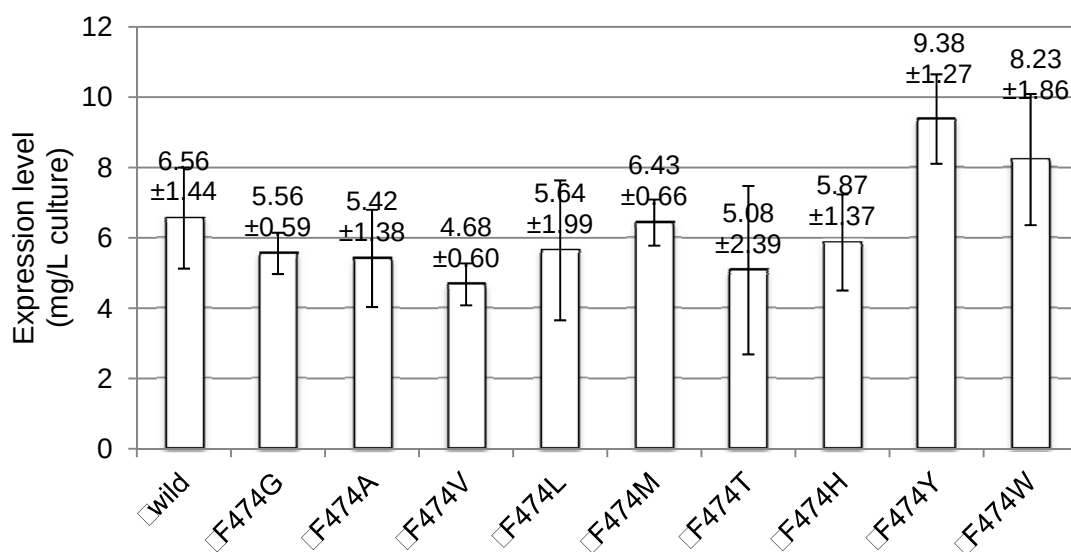
Homology modeling of β -amyrin synthase prepared with Pymol (<http://www.pymol.org>). This model was constructed by ESyPred3D (<http://www.unamur.be/sciences/biologie/urbm/bioinfo/esypred/>), based on the X-ray crystal structure of human lanosterol cyclase (pdb ID: 1w6k, *Nature* **432**, 118-122, 2004). Lambert C, Leonard N, De Bolle X & Depiereux E (2002) ESyPred3D: Prediction of proteins 3D structures *Bioinformatics*. **18**, 1250-1256.



Lanosterol molecule is shown with green color. F728 residue is located in approximate to the D/E ring. We have reported the detailed functional analysis of F728 in the previous paper (R. Ito, I. Hashimoto, Y. Masukawa and T. Hoshino, *Chem. Eur. J.* **2013**, *19*, 17150-17158). By the mutagenesis experiments, we found that D⁴⁸⁵C⁴⁸⁶TA motif triggers the polycyclization reaction and the C564 is involved in hydrogen bond formation with the carboxyl residue of D485, resulting in enhancement of the acidity (R. Ito, Y. Masukawa and T. Hoshino, *FEBS J.*, **2013**;280:1267-1280). F474 is situated in the vicinity to B-ring formation site. Y259, corresponding to Y261 of PNY β -amyrin synthase, is also shown. It was reported that the Y261H mutant of PNY gave the teracyclic products (see the Text and the ref. T. Kushihiro, M. Shibuya, K. Masuda, Y. Ebizuka, *J. Am. Chem. Soc.*, **2000**, *122*, 6816-6824).

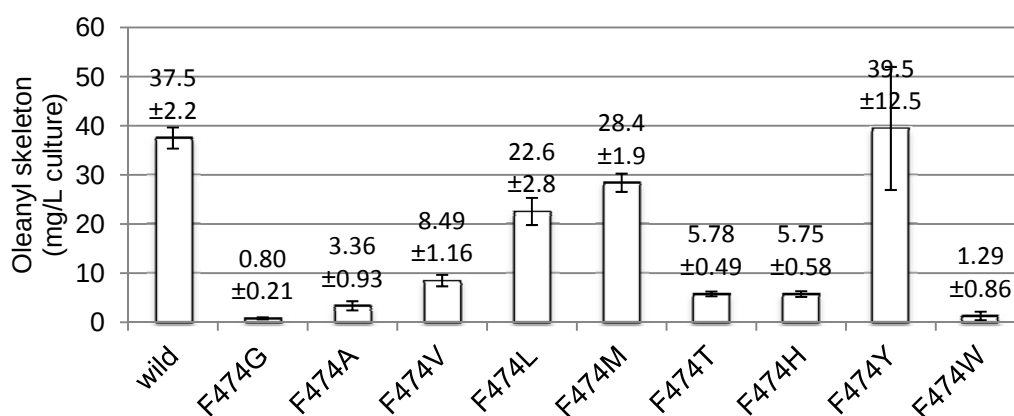
Supplementary Figure S5.

Estimation of the EtAS enzyme activities for the wild-type and the mutants.



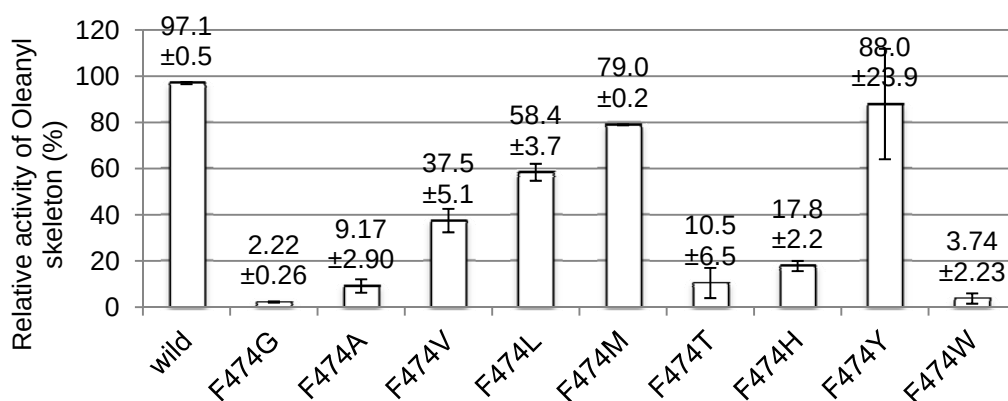
<Expression level of β -amyrin synthase (EtAS)>

Figure S5.1. Expression level (mg/L) of the EtAS enzymes of the wild-type and the site-directed mutant. The yeast cells grown in 1L-medium were collected and the protein amounts (mg) were quantified by Western blot analysis.



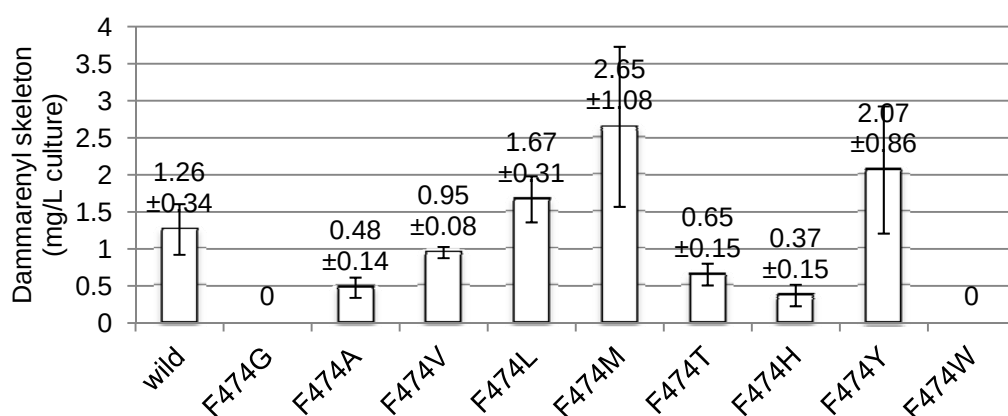
<GC analyses for the quantities of oleanane-type triterpenes>

Figure S5.2. The quantities of products **2**, **16** and **17** (oleanane skeleton) produced by 1L-culture of each of the mutant strains that were determined by GC analyses using GGOH (geranylgeraniol) as an internal standard.



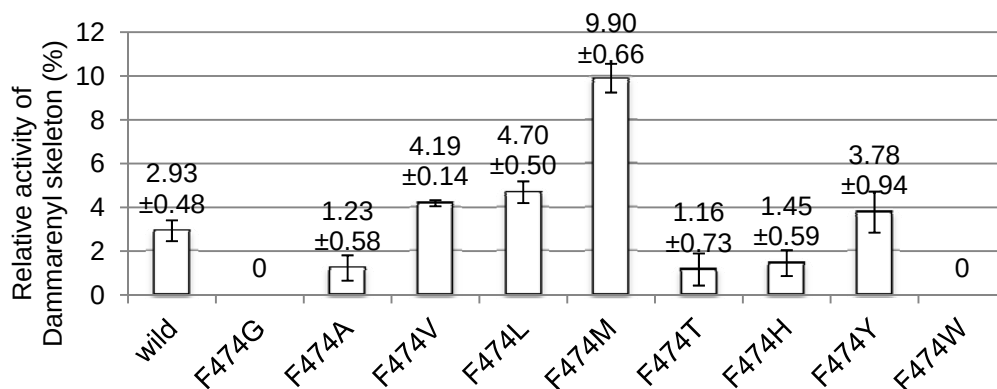
<Enzyme activities for the production of oleanane-type triterpenes>

Figure S5.3. Enzyme activities of the mutants relative to that of the wild-type. The wild-type activity (100%) indicates the sum of the relative activities shown in Fig. S5.2 and Fig. S5.4. These enzyme activities were estimated by dividing the amounts of oleanane-type products (**2**, **16** and **17**) by the expressed quantities of EtAS enzymes. This means that the values of Fig. S5.2 were divided by those of Fig. S5.1. The wild-type did not show 100% activity, i.e., 97.1 ± 0.5 %, because the wild-type produced the tetracycles **14** and **15** in a small amount (1.26 ± 0.34 %), see Fig. S5.4.



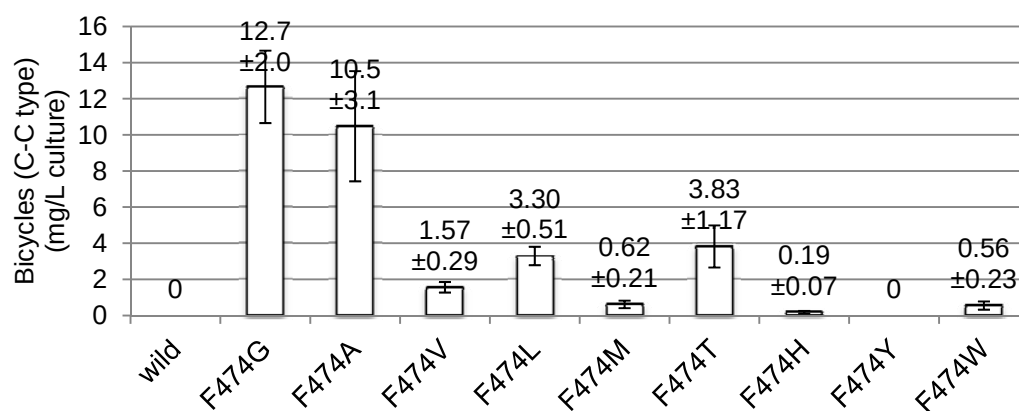
< GC analyses for the quantities of Dammarane-types>

Figure S5.4. The quantities of products **14** and **15** (dammarane skeleton) produced by 1L-culture of each of the mutant strains that were determined by GC analyses using GGOH (geranylgeraniol) as an internal standard. As shown in this Figure, the quantities of the tetracyclic products were significantly small.



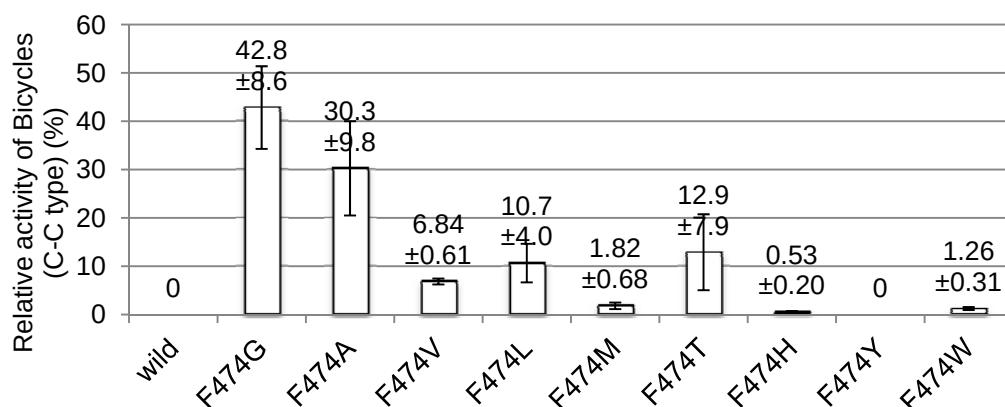
< Enzyme activities for the production of Dammarane-types >

Figure S5.5. Enzyme activities of the mutants relative to that of the wild-type. The wild-type activity (100%) indicates the sum of the relative activities shown in Fig. S5.2 and Fig. S5.4. These enzyme activities were estimated by dividing the amounts of dammarane-type products (**14** and **15**) by the expressed quantities of EtAS enzymes, that is, the values of Fig. S5.4 were divided by those of Fig. S5.1. The activity of the wild-type for the production of **14** and **15** was very low, i.e., $2.93 \pm 0.48\%$, thus, the relative activity does not correspond to 100%. The total values of Fig.S5.3 and Fig. S5.5 for the wild-type corresponds to 100%.



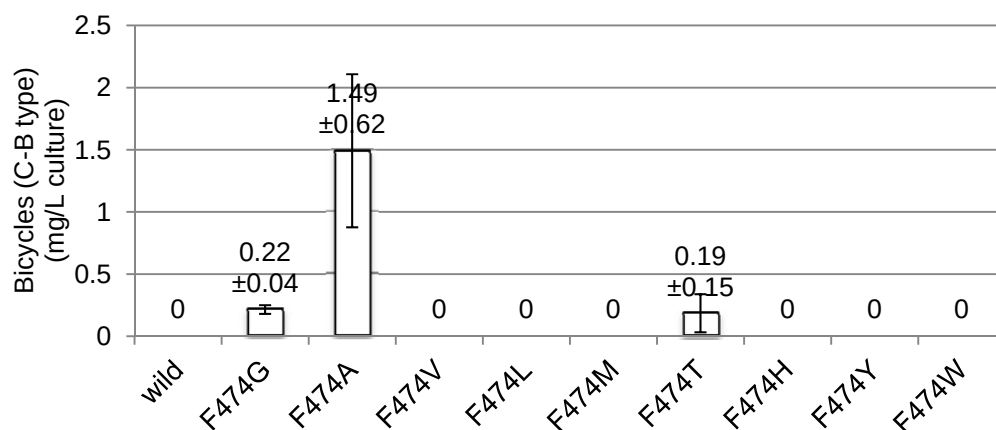
<GC analyses for the quantities of Bicycles (C-C type) >

Figure S5.6. The quantities of products **11** and **12** (polypodatetraene-type skeleton) produced by 1L-culture of each of the mutant strains that were determined by GC analyses using GGOH (geranylgeraniol) as an internal standard. As shown in this figure, none of the C-C (chair-chair) type bicyclic products were produced for the wild-type. The quantities of these bicyclic products are dependent on the mutant strain. The Gly and Ala mutants, the size of which is small, gave the substantial amounts of the bicyclic **11** and **12**.



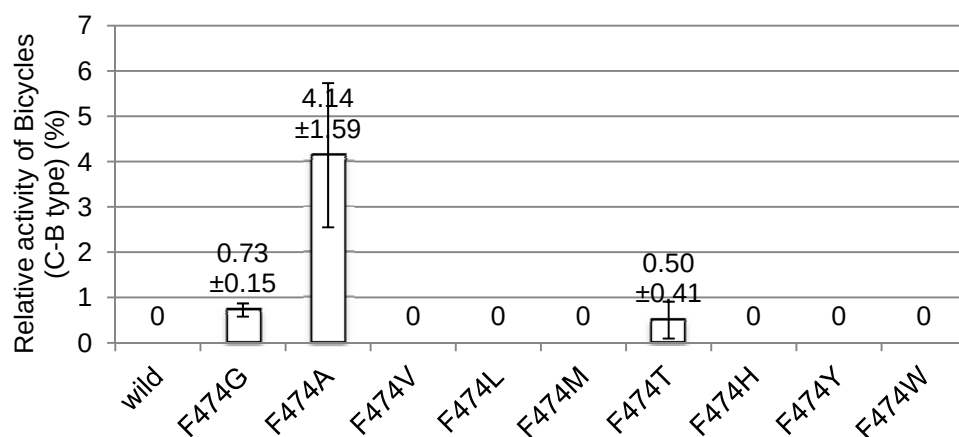
<Enzyme activities for the production of Bicycles (C-C type)>

Figure S5.7. Enzyme activities of the mutants relative to that of the wild-type. The wild-type activity (100%) indicates the sum of the relative activities shown in Fig. S5.2 and Fig. S5.4. These enzymatic activities were estimated by dividing the amounts of C-C (chair-chair) type products (**11** and **12**) by the expressed quantities of EtAS enzymes, that is, the values of Fig. S5.6 were divided by those of Fig. S5.1. The Gly and Ala mutants, the size of which is small, possessed high activities for the production of **11** and **12** (ca 43 % and 30 %, respectively).



<GC analyses for the quantities of Bicycles (C-B type)>

Figure S5.8. The quantities of products **9** and **10** ((9βH)-polypodatetraene compound) produced by 1L-culture of each of the mutant strains that were determined by GC analyses using GGOH (geranylgeraniol) as an internal standard. As shown in this figure, none of the C-B (chair-boat) type bicyclic products were produced for the wild-type. The quantities of these bicyclic products are dependent on the mutant strain. The Gly, Ala and Thr mutants, the size of which is small, gave the trivial amounts of bicyclic **9** and **10**.

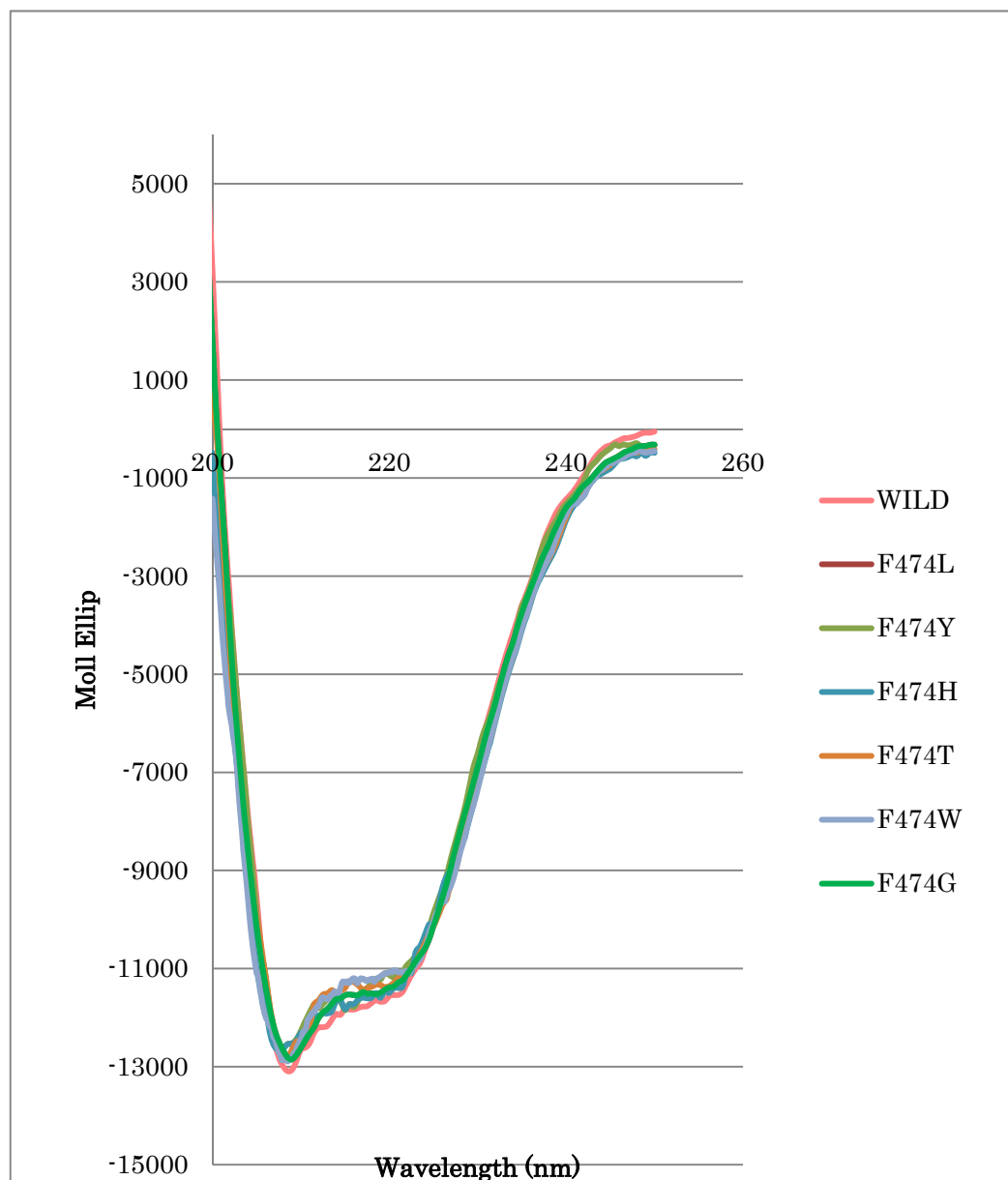


<Enzyme activities for the production of Bicycles (C-B)>

Figure S5.9. Enzyme activities of the mutants relative to that of the wild-type. The wild-type activity (100%) indicates the sum of the relative activities shown in Fig. S5.2 and Fig. S5.4. These enzymatic activities were estimated by dividing the amounts of C-B (chair-boat) type products (**9** and **10**) by the expressed quantities of EtAS enzymes, that is, the values of Fig. S5.8 were divided by those of Fig. S5.1. Other mutants with larger size, such as the Val, Leu, Met, His, Tyr and Trp variants, showed no activity for the production of **9** and **10**, but the Gly and the Ala mutants with small side chain had a little activity for the production of **9** and **10**. This finding suggests that a decreased steric bulk (but with an appropriate size) at this position could confer a boat conformation during B-ring construction.

Supplementary Figure S6. CD spectra of the wild and the mutated EtAS.

The proteins of the wild-type and the mutants were purified in potassium phosphate buffer (pH 7.0, 50 mM) containing 0.01% Brij35, and the final protein concentration was adjusted to 0.1 mgmL^{-1} for CD measurements (the details are reported in our paper, R. Ito, Y. Masukawa and T. Hoshino, *FEBS J.*, **2013**;280:1267-1280). The following CD spectra were measured at 30°C.



Supplementary Table S1

The Triterpene Cyclase Engineering Database (abbreviated as TTCED)

<http://www.ttcged.uni-stuttgart.de/>

This Table shows the list of OSCs, in which the amino acid corresponding to Phe474 of EtAS is substituted with Leu.

family	TTCED number	a.a length	function	origin	monocot or dicot
OSC_1	168699173	650	probable squalene-hopene cyclase	<i>Gemmata obscuriglobus</i>	
OSC_8	50896403	764	cucurbitadienol synthase ^[1]	<i>Cucurbita pepo</i>	dicot
	64310763	415	cycloartenol synthase	<i>Dioscorea zigerensis</i>	monocot
OSC_9	108743267	761	oxidosqualane cyclase ^[2]	<i>Lotus japonicus</i>	dicot
	218198199	410	hypothetical	<i>Oryza sativa</i>	monocot
	47834395	757	β -amyrin synthase (AY698696) ^[3]	<i>Avena longiglumis</i>	monocot
	15866696	757	β -amyrin synthase (AY618699) ^[3,4]	<i>Avena strigosa</i>	monocot
	47834397	757	β -amyrin synthase (AY618698) ^[3]	<i>Avena prostrata</i>	monocot
	47834401	757	β -amyrin synthase (AY618700) ^[3]	<i>Avena ventricosa</i>	monocot
	47834391	757	β -amyrin synthase (AY618695) ^[3]	<i>Avena clauda</i>	monocot
	242096958	649	hypothetical	<i>Sorghum bicolor</i>	monocot
	195614202	760	cycloartenol synthase	<i>Zea mays</i>	monocot
	115485121	756	hypothetical (OsOSC5) ^[5]	<i>Oryza sativa</i>	monocot
	108864253	617	putative cycloartenol synthase	<i>Oryza sativa</i>	monocot
	218185593	551	hypothetical	<i>Oryza sativa</i>	monocot
	218185587	406	hypothetical	<i>Oryza sativa</i>	monocot
	6734726	548	similar to β -amyrin synthase	<i>Oryza sativa</i>	monocot
	115485113	760	OsOSC6 ^[5]	<i>Oryza sativa</i>	monocot
	242084188	733	hypothetical	<i>Sorghum bicolor</i>	monocot
	218190027	656	hypothetical	<i>Oryza sativa</i>	monocot
	115485919	762	hypothetical (OsOSC4) ^[5]	<i>Oryza sativa</i>	monocot
	115444143	495	hypothetical	<i>Oryza sativa</i>	monocot

	222622141	663	hypothetical	<i>Oryza sativa</i>	monocot
	115444147	429	hypothetical	<i>Oryza sativa</i>	monocot
	226533427	762	unknown	<i>Zea mays</i>	monocot
	242060472	529	hypothetical	<i>Sorghum bicolor</i>	monocot
OSC_10	1193452	763	pentacyclic triterpene synthase ^[6]	synthetic construct	
			LUP2 (multifunctional		
	30699377	763	(β -amyrin)	<i>Arabidopsis thaliana</i>	dicot
			(At1g78960) ^[6]		
	15219789	763	LUP5 (multifunctional	<i>Arabidopsis thaliana</i>	dicot
			(6/6/6/5/-)) ^[7]		
	224077636	728	hypothetical	<i>Populus trichocarpa</i>	dicot
	224113075	755	hypothetical	<i>Populus trichocarpa</i>	dicot
	224076232	760	hypothetical	<i>Populus trichocarpa</i>	dicot

The functions of some OSCs listed above were established in the following papers.

[1] M. Shibuya, S. Adachi, and Y. Ebizuka, *Tetrahedron Lett.*, **2004**, 33, 6996-7003.

[2] S. Sawai, T. Akashi, N. Sakurai, H. Suzuki, D. Shibata, S. Ayabe, and T. Aoki, *Plant Cell Physiol.*, **2006**, 47, 673-677.

[3] X.Qi, S. Bakht, M. Leggett, C. Maxwell, R. Melton, and A. Osbourn, *Proc. Natl. Acad. Sci. USA.*, **2004**, 101, 8233-8238.

[4] Z. Xue, L. Duan, D. Liu, J. Guo, S. Ge, J. Dicks, P. OMaille, A. Osbourn, and X. Qi, *New Phytologist*, **2012**, 193, 1022-1038.

[5] R. Ito, K. Mori, I. Hashimoto, C. Nakano, T. Sato, and T. Hoshino, *Org. Lett.*, **2011**, 13, 2678-2681.

[6] T. Husselstein-M., H. Schaller, and P. Benveniste, *Plant Mol. Biol.*, **2001**, 45, 75-92.

[7] Y. Ebizuka, Y. Katsube, T. Tsutsumi, T. Kushiro, and M. Shibuya, *Pure Appl. Chem.*, **2003**, 75, 369-374.