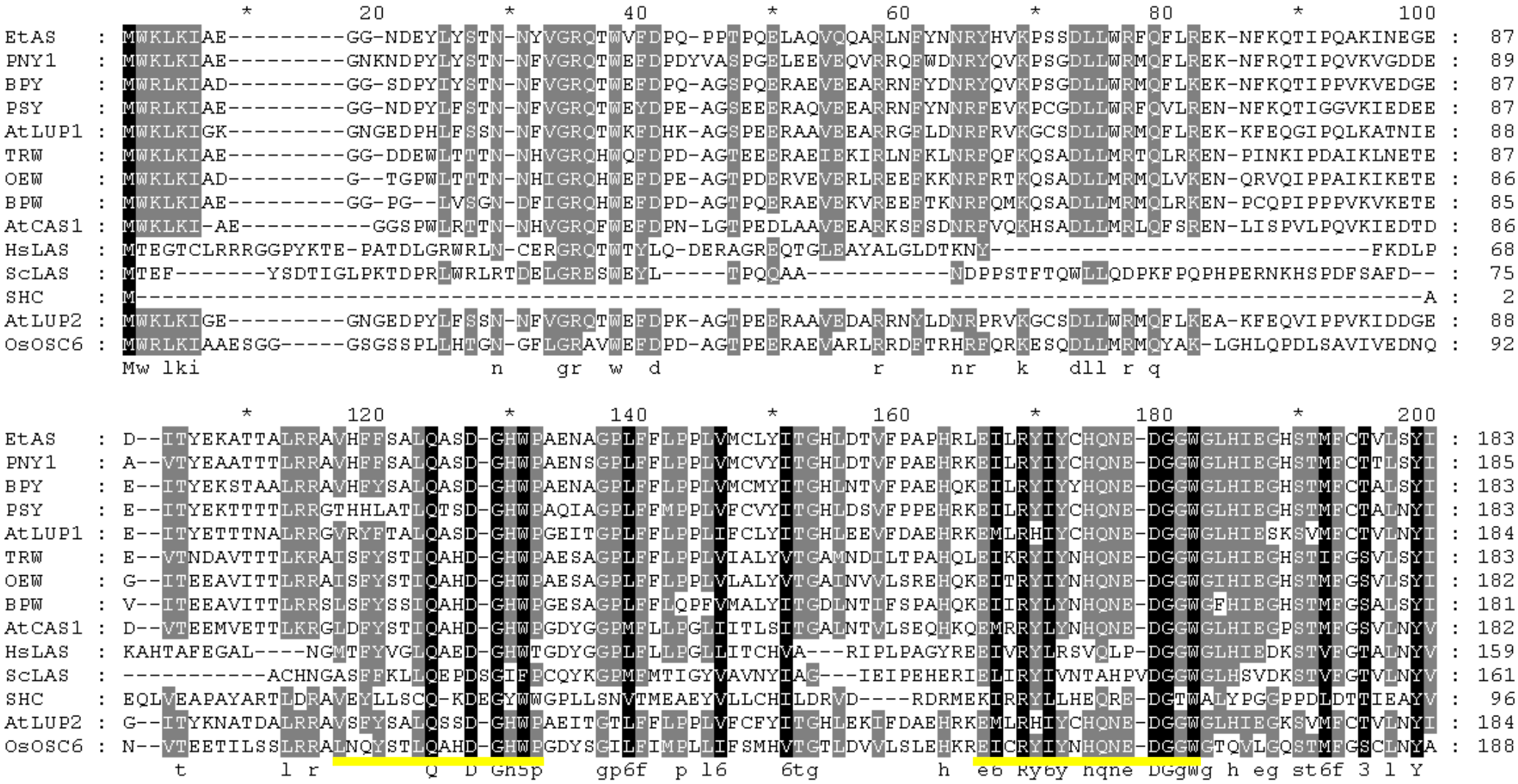
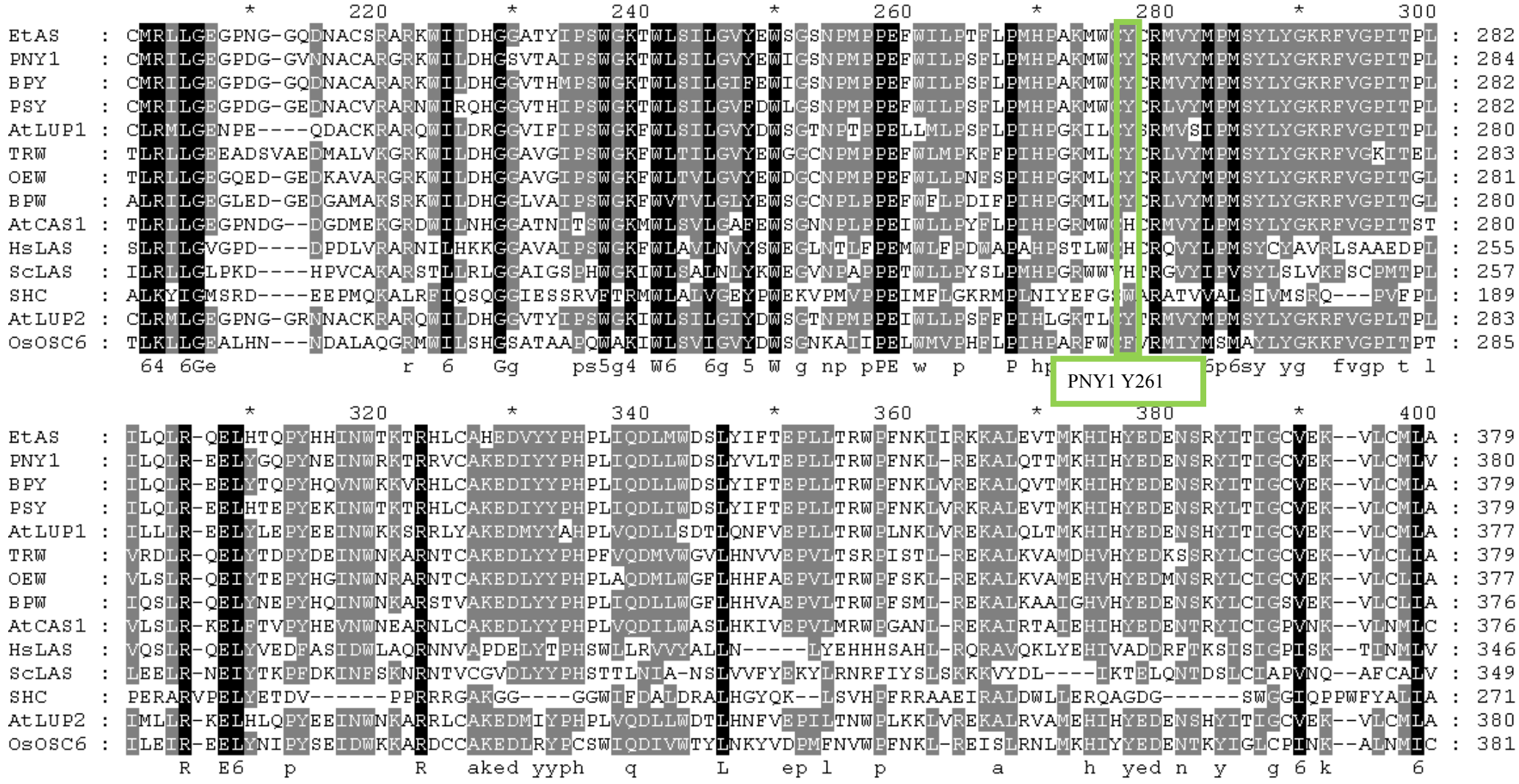
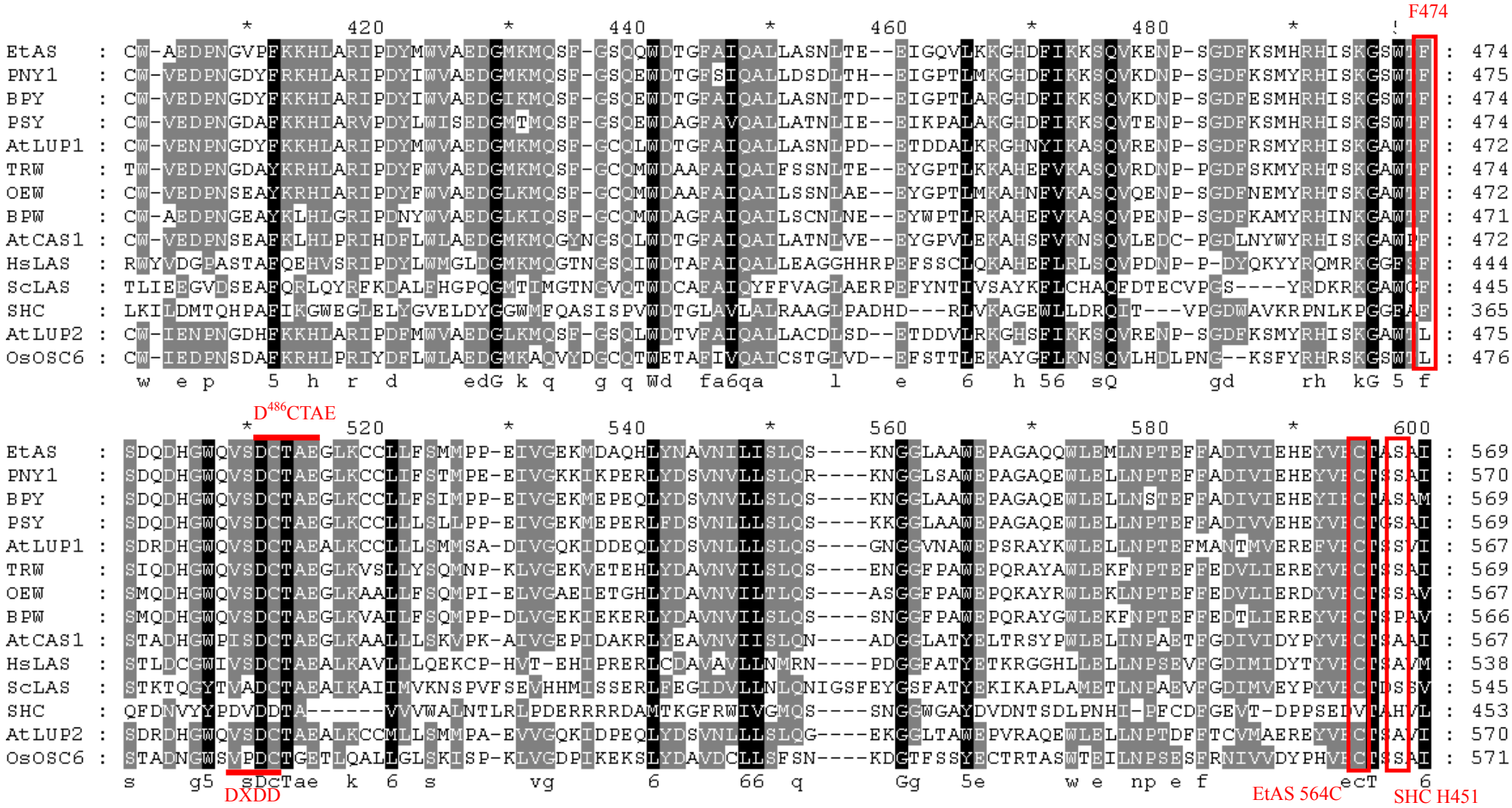
<Electronic Supporting Information>

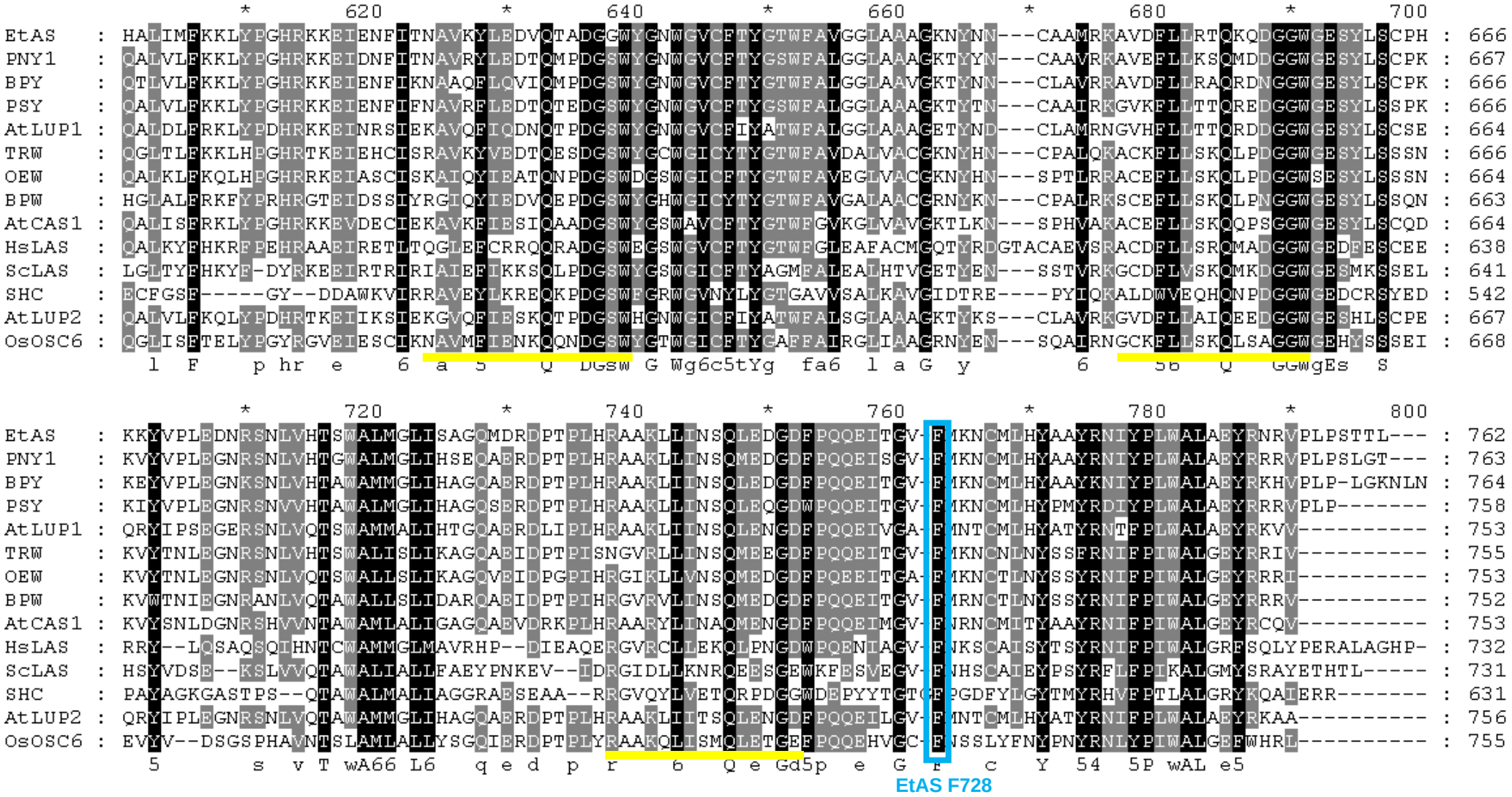
Supplementary Figure S1. Amino acid alignment of various oxidosqualenes

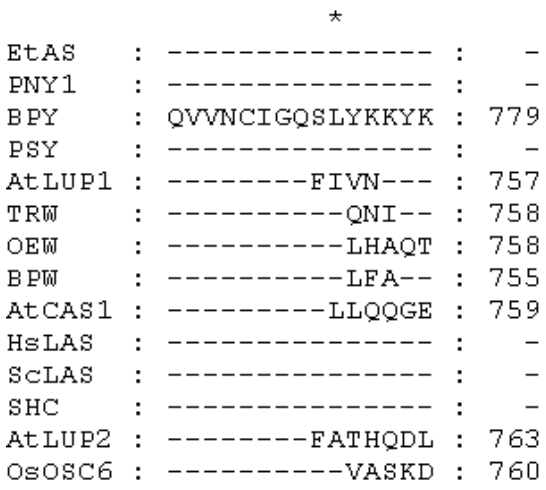



PNY1 Y261



D485C486TAE involved in EtAs β-amyrin synthase


EtAS F728



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* cycloarternol 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 D^{486}CTAE 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).

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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).

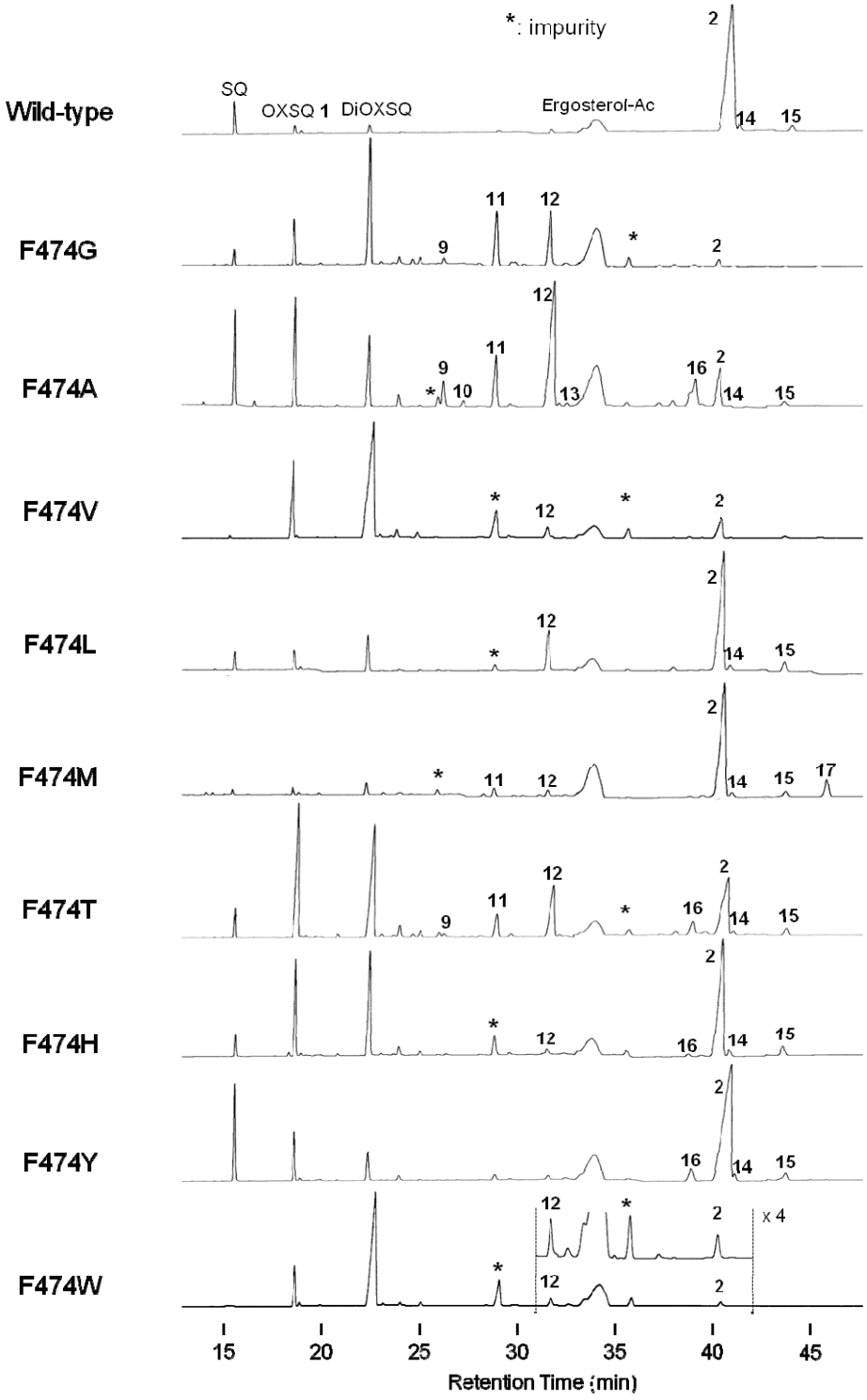
11) D. Ochs, C. Kaletta, K.-D. Entian, A. Beck-Sickinger, and K. Poralla, *J. Bacteriol.*, **174**(1), 298-302 (1992).

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 Ac_2O/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).

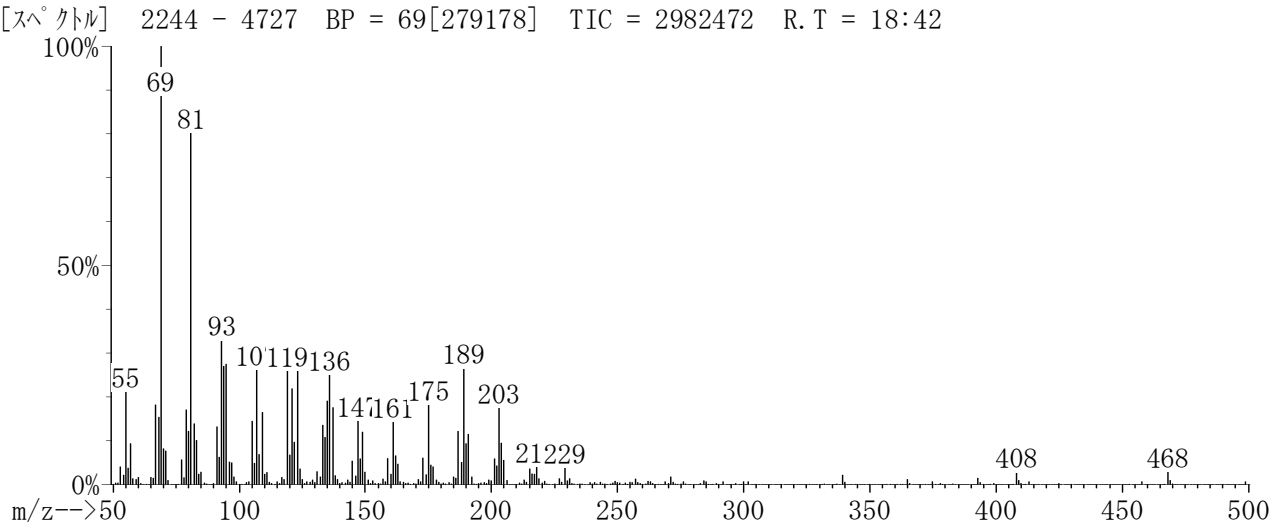


Supplementary Figure S3.

Spectroscopic Data of Triterpene products accumulated in the Mutagenesis Experiments.

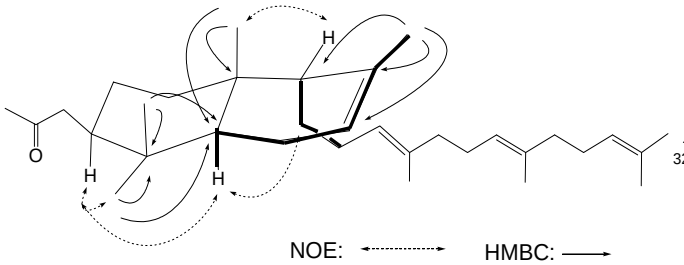
Fig. S3. 1. Product 9 acetate produced by F474A mutant

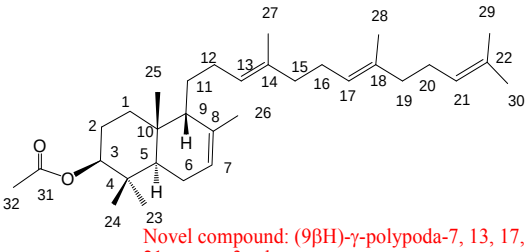
3.1.1 EIMS



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

F474A product 9 acetate
in $CDCl_3$ (600 MHz)





Novel compound: (9βH)-γ-polypoda-7, 13, 17, 21-tetraen-3 -ol

NOE: ⇢ HMBC: →

COSY & HOHAHA: ——

$[\alpha]_D^{25}$= -30.0 (c=0.05, $CHCl_3$)

No report of $[\alpha]_D$for this compound

Produced by a chair-boat folding conformation

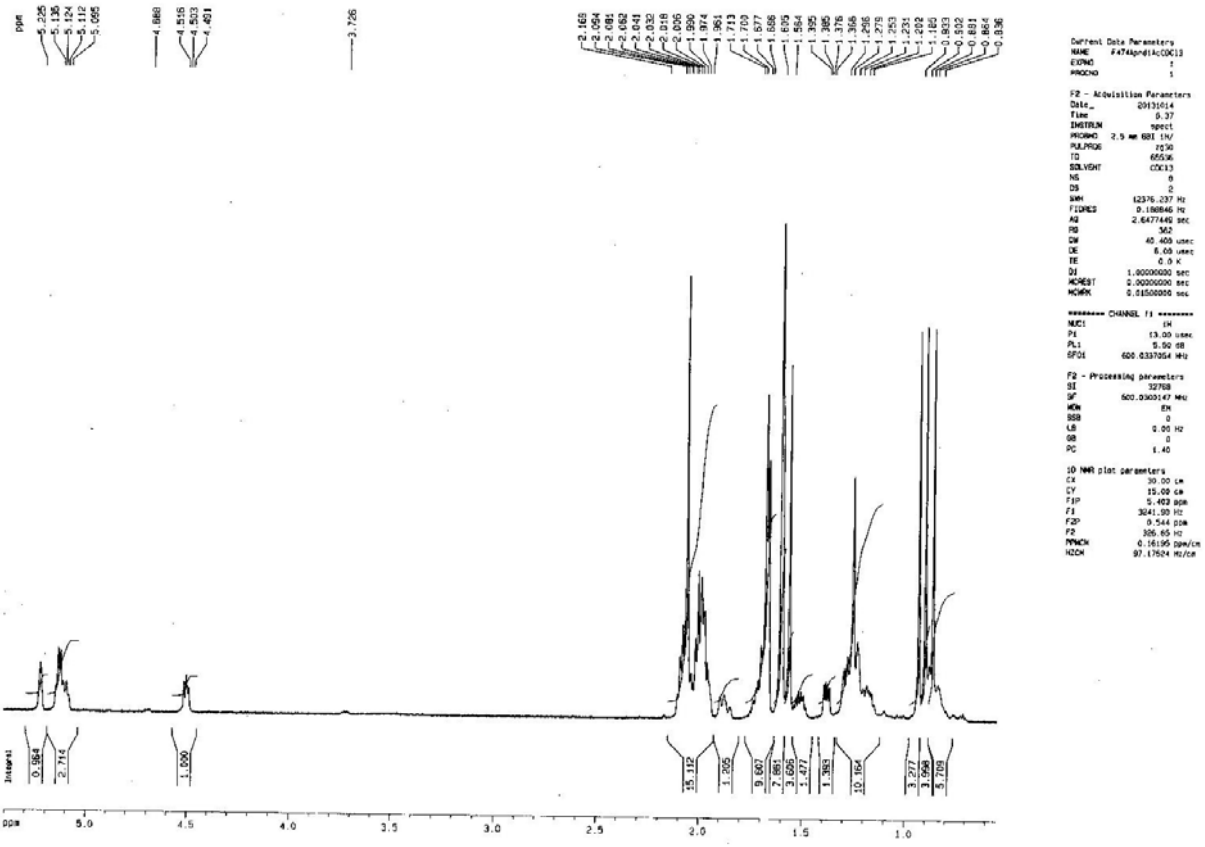
HREIMS
calcd. 468.39673
observed: 468.39715

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

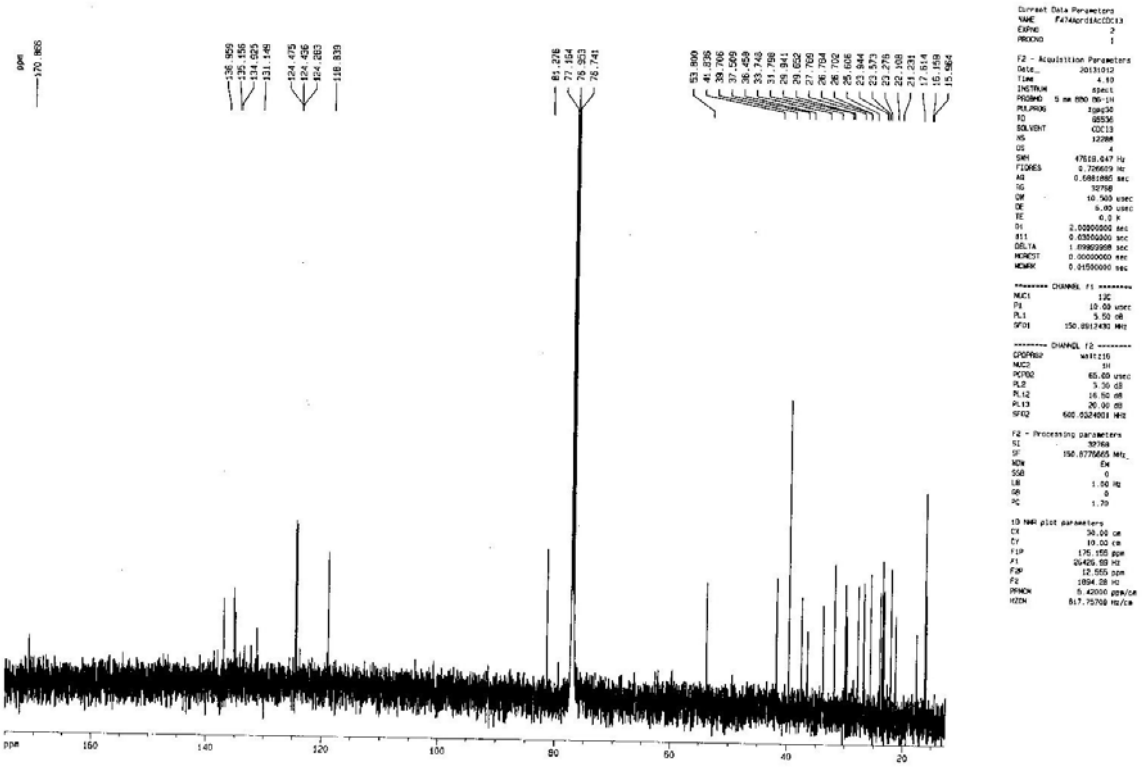
NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C
1	1.28 (m));1.71(m)	**33.75 (t)**	**9**	1.23 (m)	**53.80(d)**	**17**	5.12 (m) *e*	**1244(d)** *f g*	**25**	0.902 (3H,s)	**22.11 (q)**
2	1.70 (2H,m)	**23.94 (t)**	**10**	——	**36.46(s)**	**18**	——	**134.9 (s)**	**26**	1.666 (3H,bs)	**23.28 (q)**
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)**	**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.

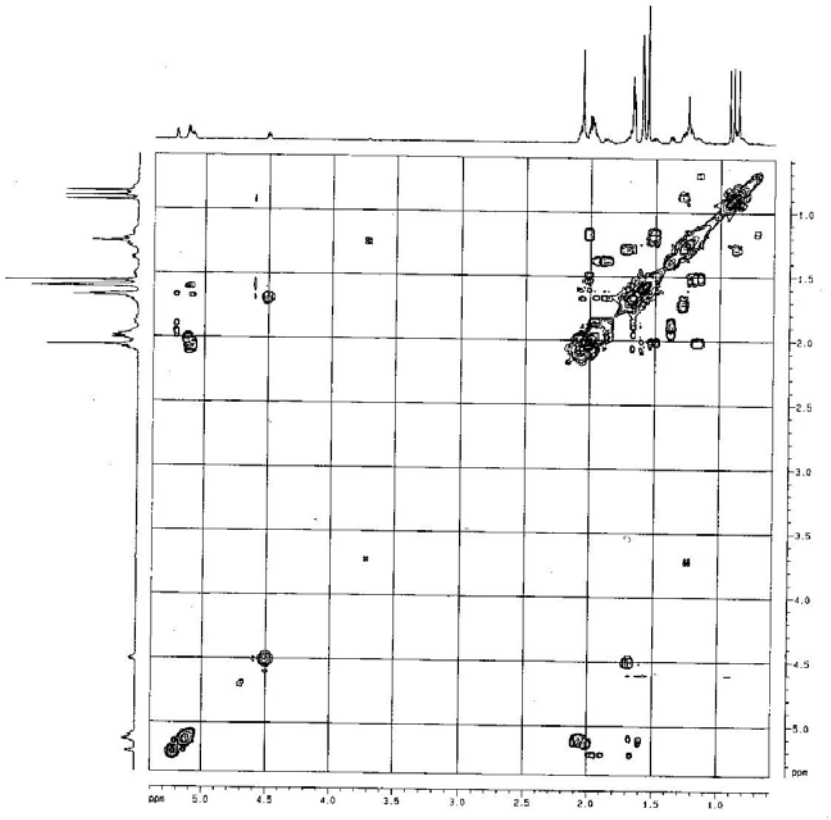
^{1}H-NMR in $CDCl_3$(600 MHz)



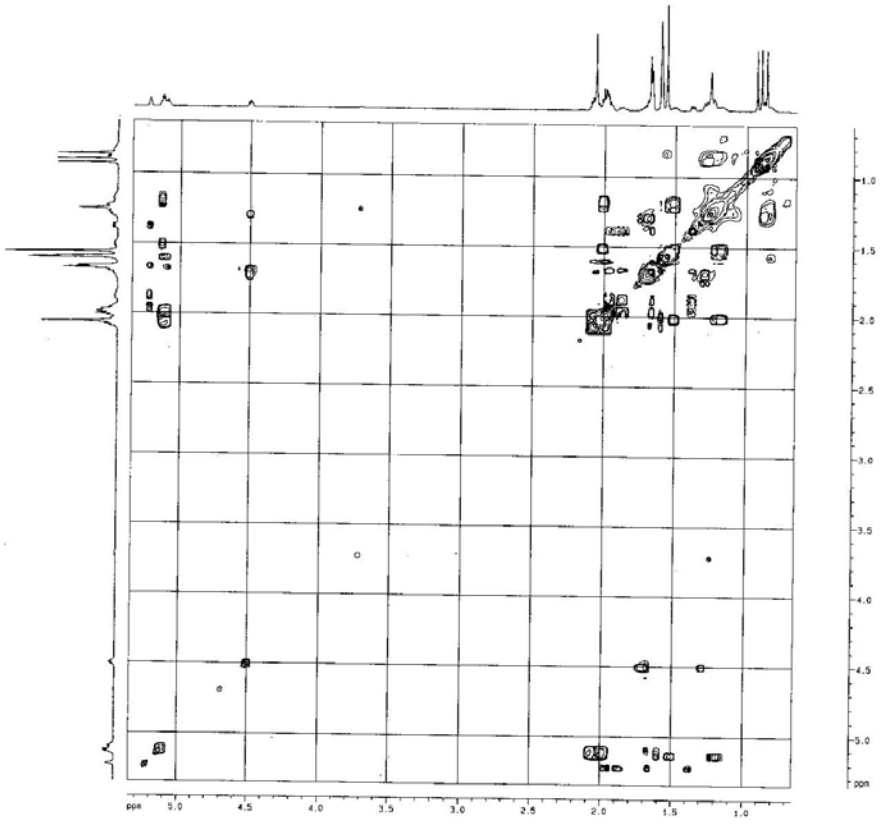
3.1.4 ^{13}C NMR in $CDCl_3$(150 MHz)



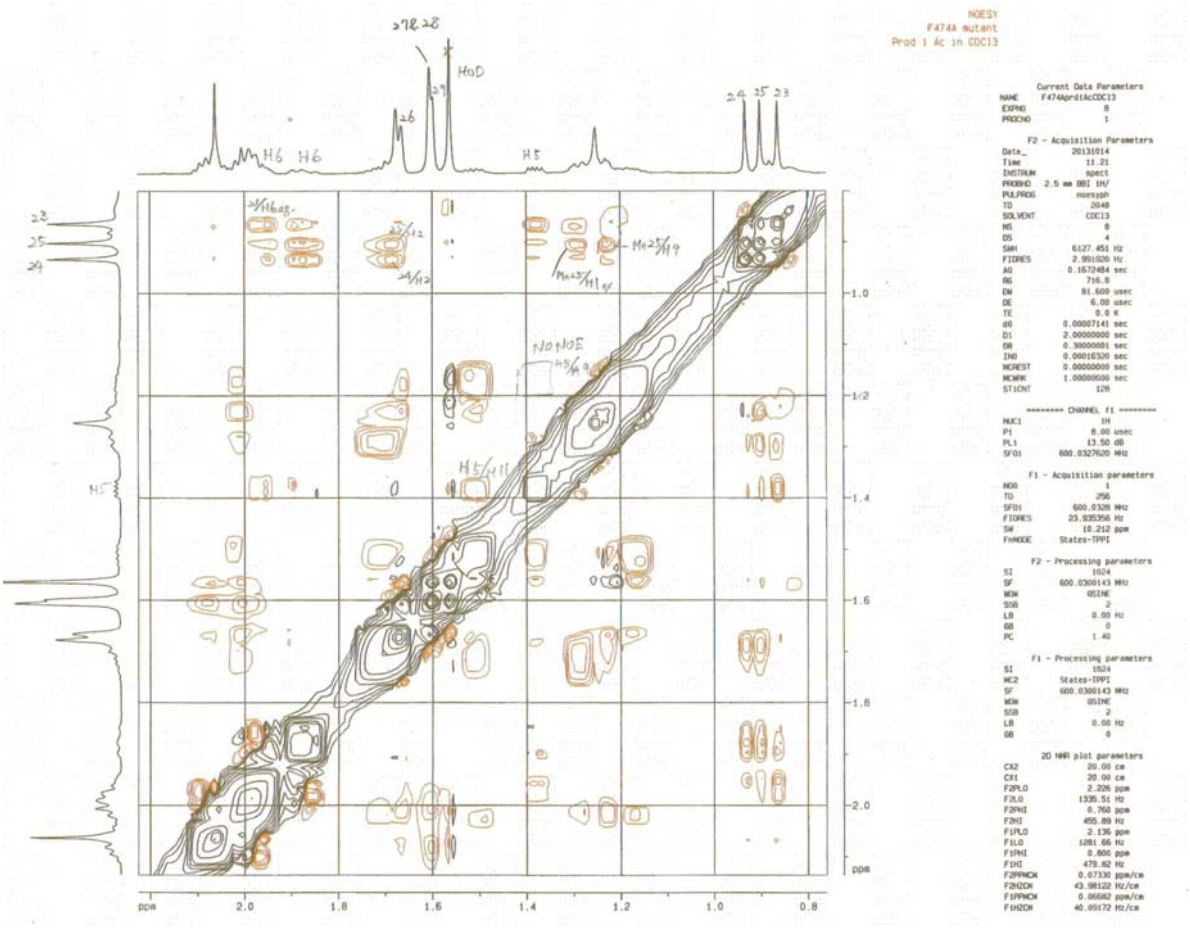
3.1.5. ^{1}H-^{1}H COSY in $CDCl_3$



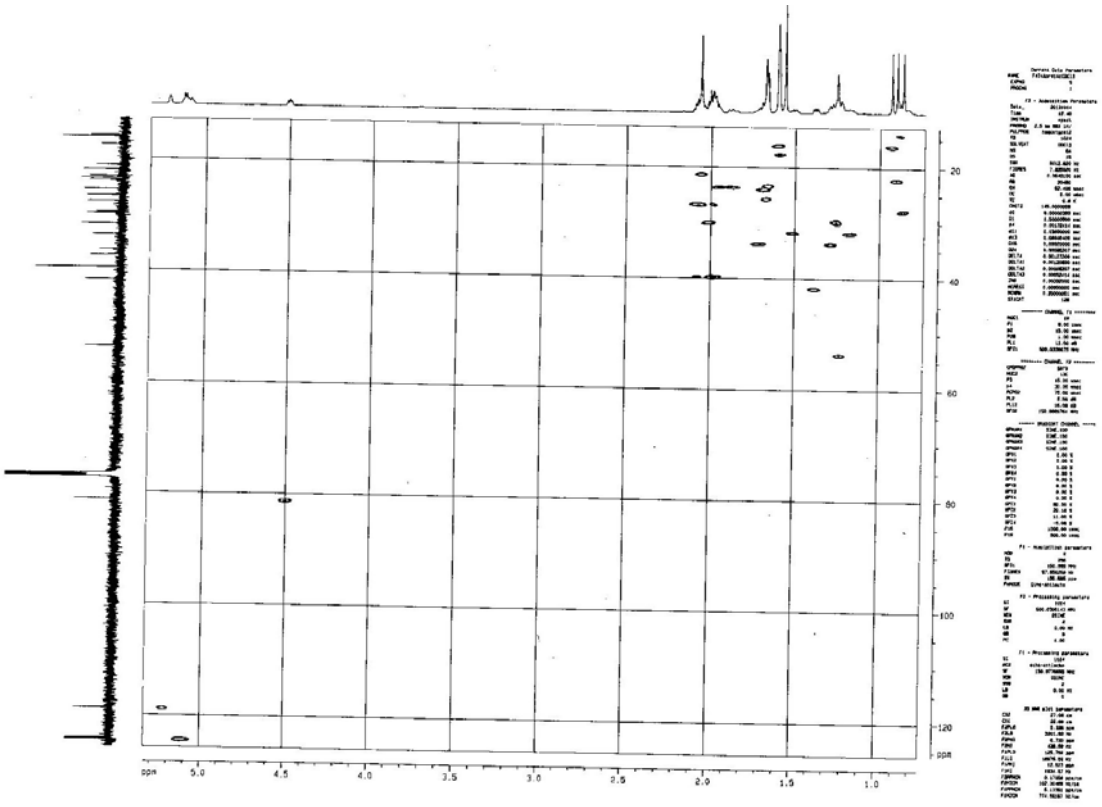
3.1.6. HOHAHA in $CDCl_3$



3.1.7 NOESY in $CDCl_3$



3.1.8 HSQC in $CDCl_3$



3.1.9. HMBC in $CDCl_3$

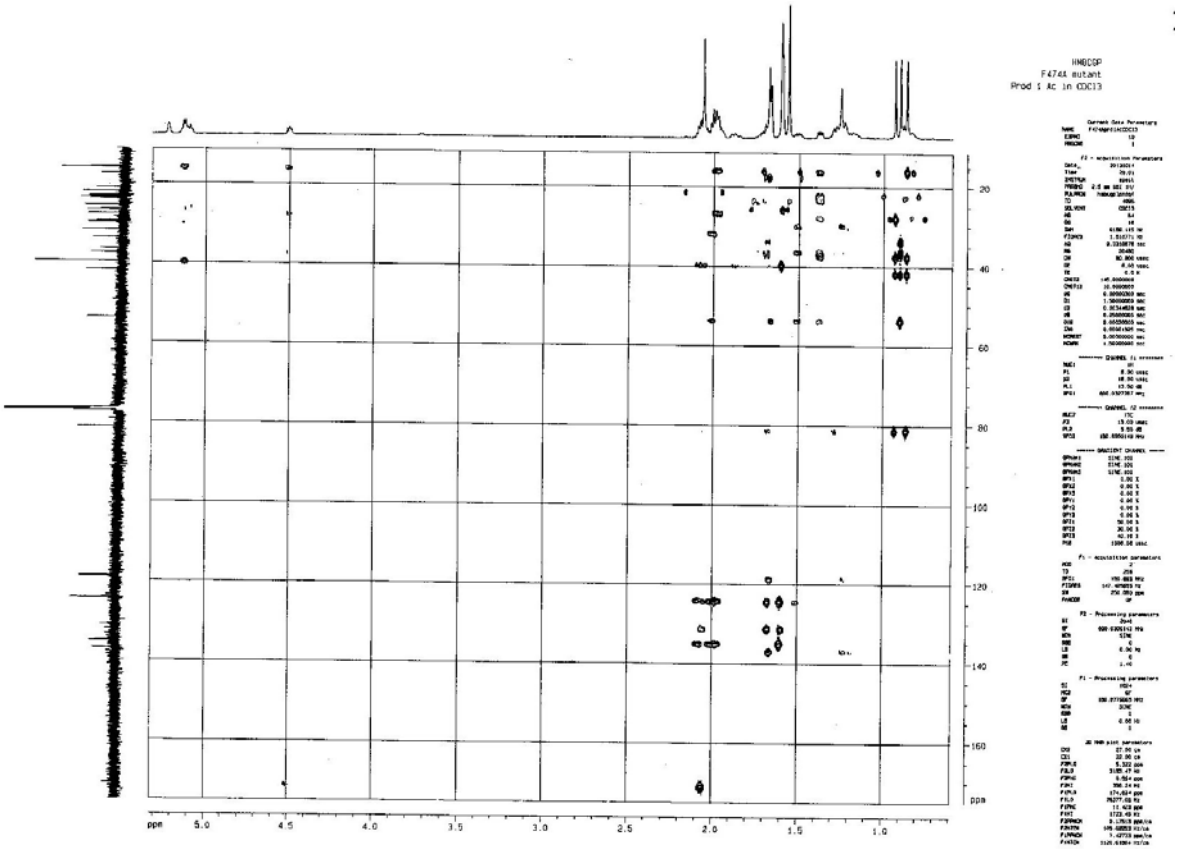
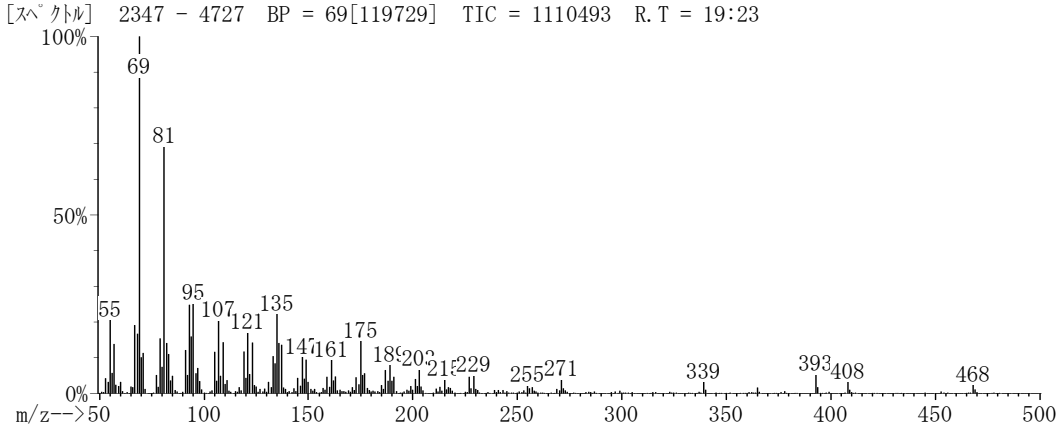


Figure S3.2. Product 10 acetate produced by F474A mutant

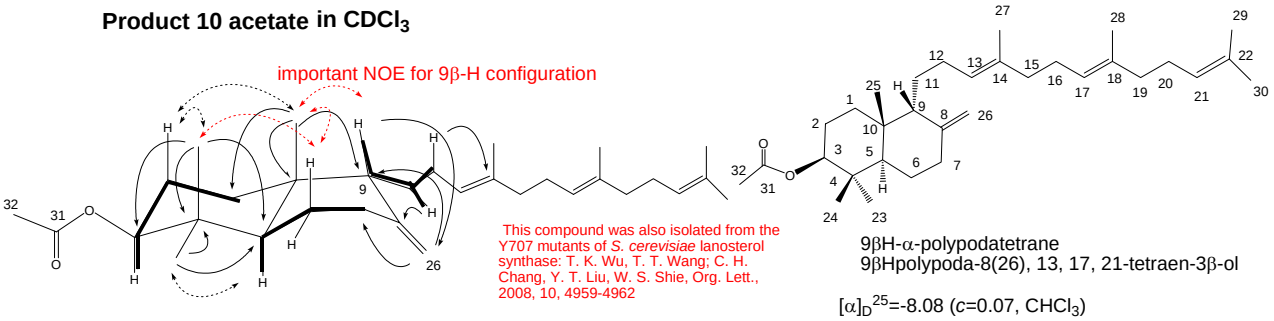
3.2.1. EIMS.



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

F474A mutant

Product 10 acetate in $CDCl_3$



This compound was also isolated from the Y707 mutants of *S. cerevisiae* lanosterol synthase: T. K. Wu, T. T. Wang; C. H. Chang, Y. T. Liu, W. S. Shie, Org. Lett., 2008, 10, 4959-4962

9βH-α-polypodatetrane
9βHpolypoda-8(26), 13, 17, 21-tetraen-3β-ol

$[\alpha]_D^{25}$=-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: ⇠⇢ HMBC: ⟶

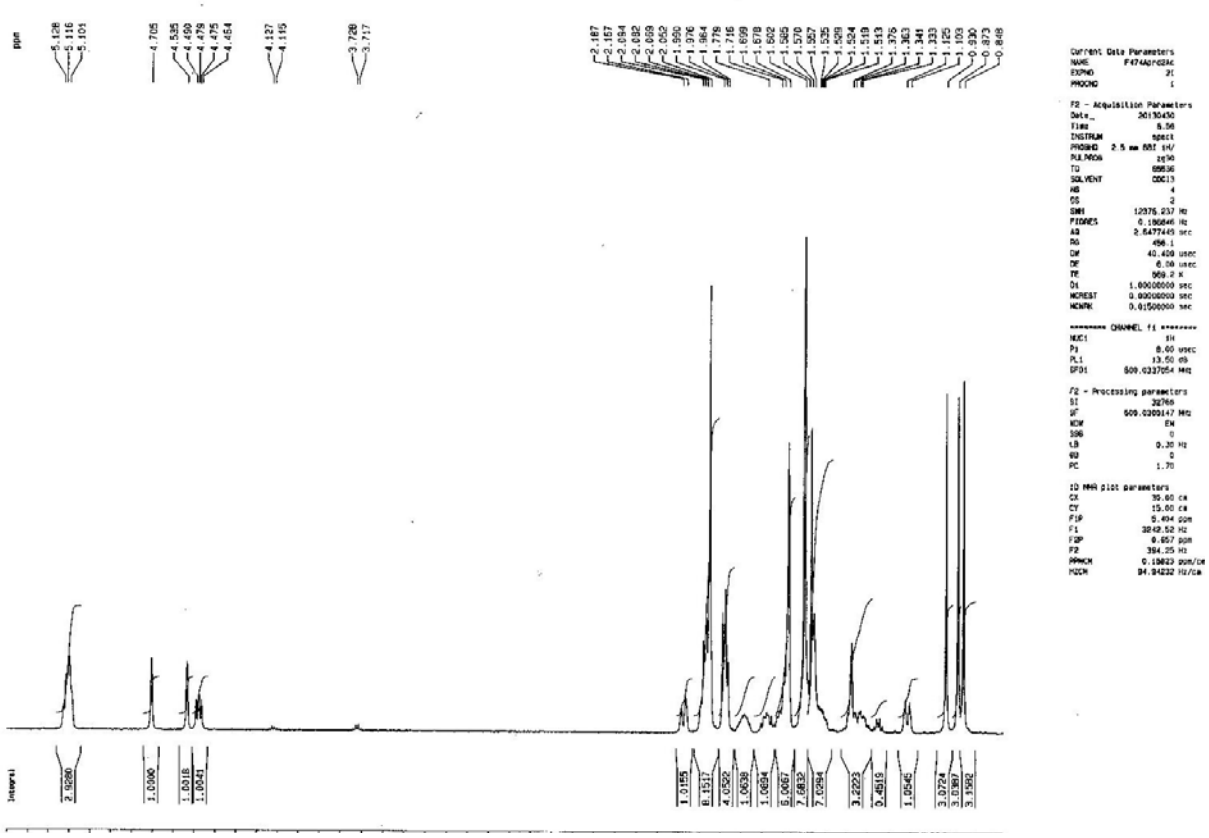
COSY & HOHAHA: ——

HREIMS
calcd. 468.39673
observed: 468.39627

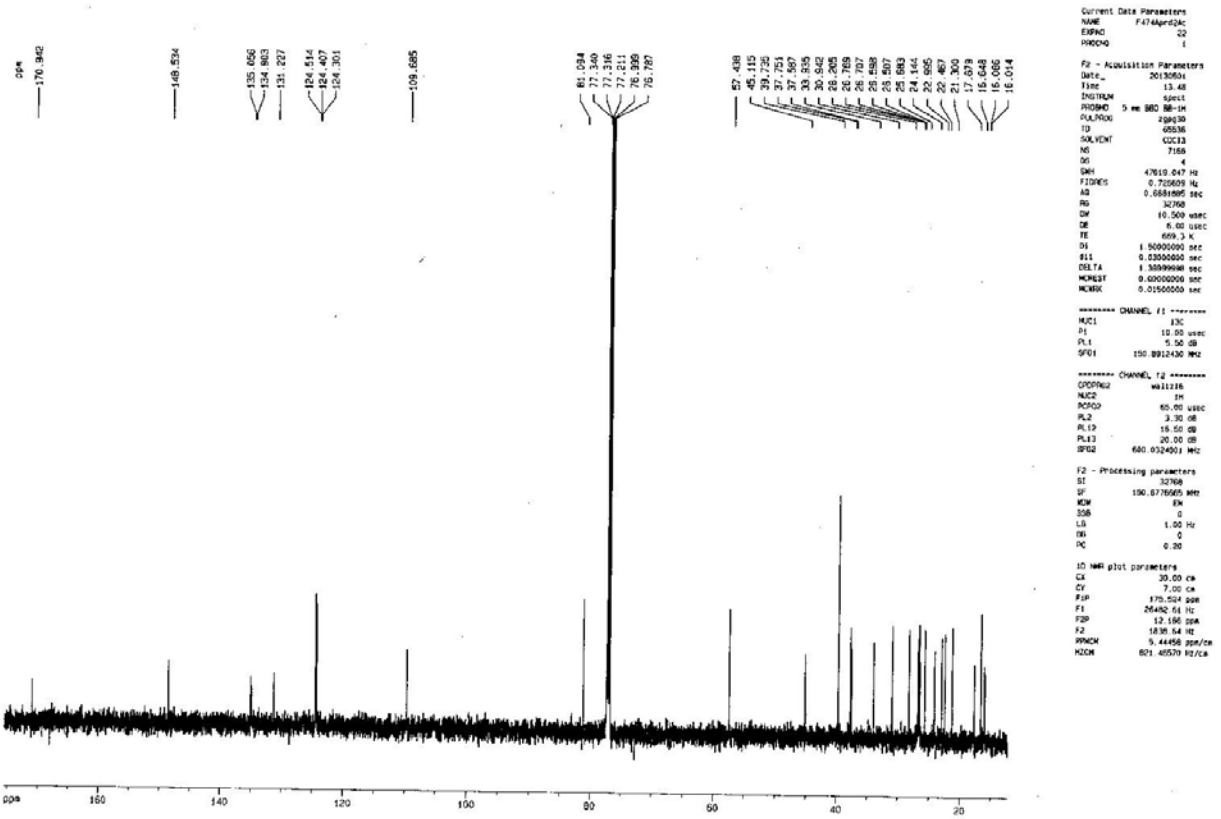
NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C
1	1.11 (bd, J=12.6 Hz);1.78 (m)	**33.94 (t)**	**9**	1.59 (m)	**57.44(d)**	**17**	5.12 (m) *e*	**124.4(d)** *f*	**25**	0.930 (3H,s)	**22.47 (q)**
2	1.68 (,2H, m)	**24.14 (t)**	**10**	——	**37.59(s)**	**18**	——	**134.9 (s)** *h*	**26**	4.54(bs); 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)** *j*	**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*	*b* **26.71(t)**	**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.

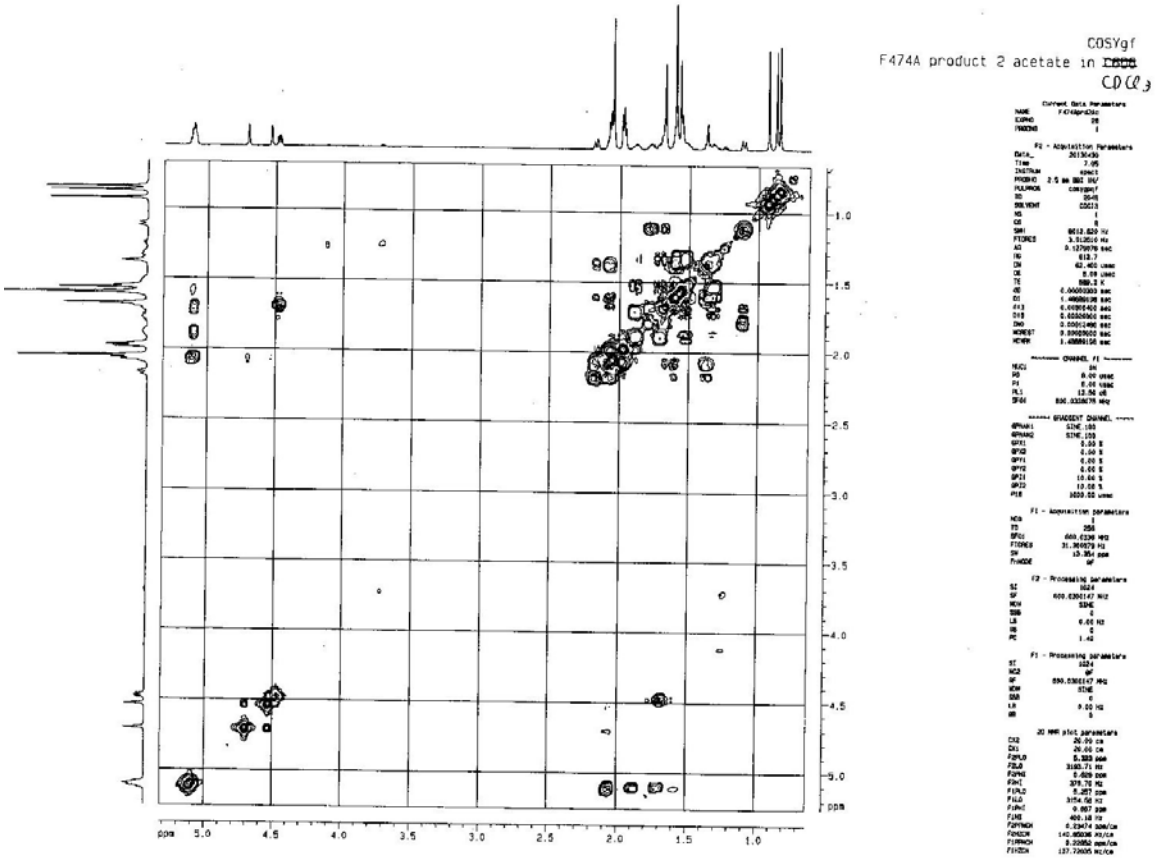
3.2.3. ^{1}H NMR in $CDCl_3$ (600 MHz)



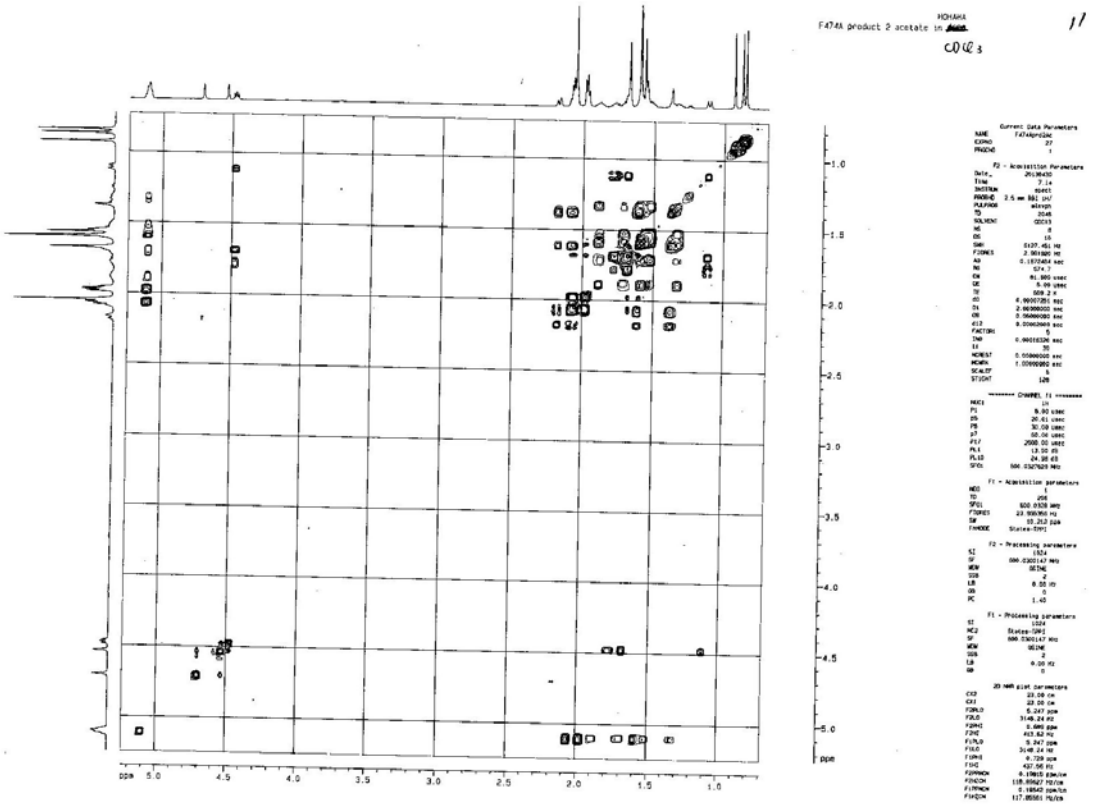
3.2.4 ^{13}C NMR in $CDCl_3$ (150 MHz)



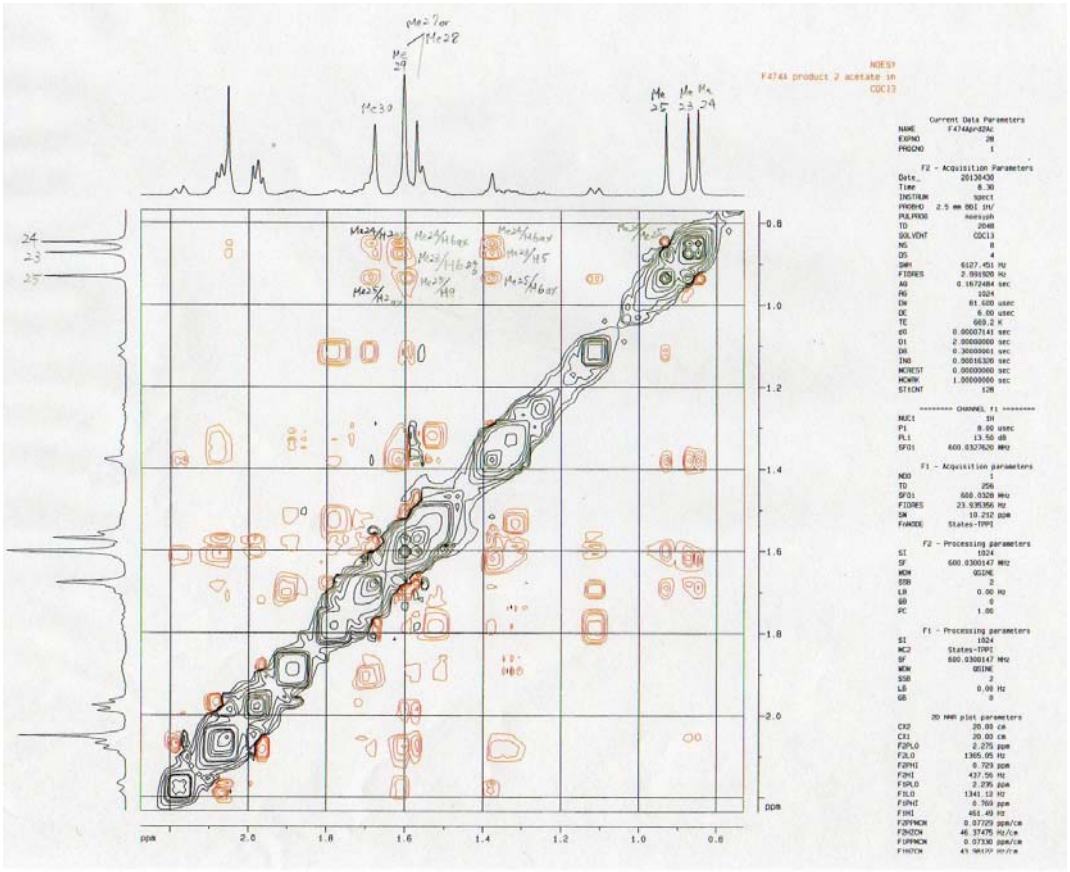
3.2.5. ^{1}H-^{1}H COSY in $CDCl_3$ (600 MHz)



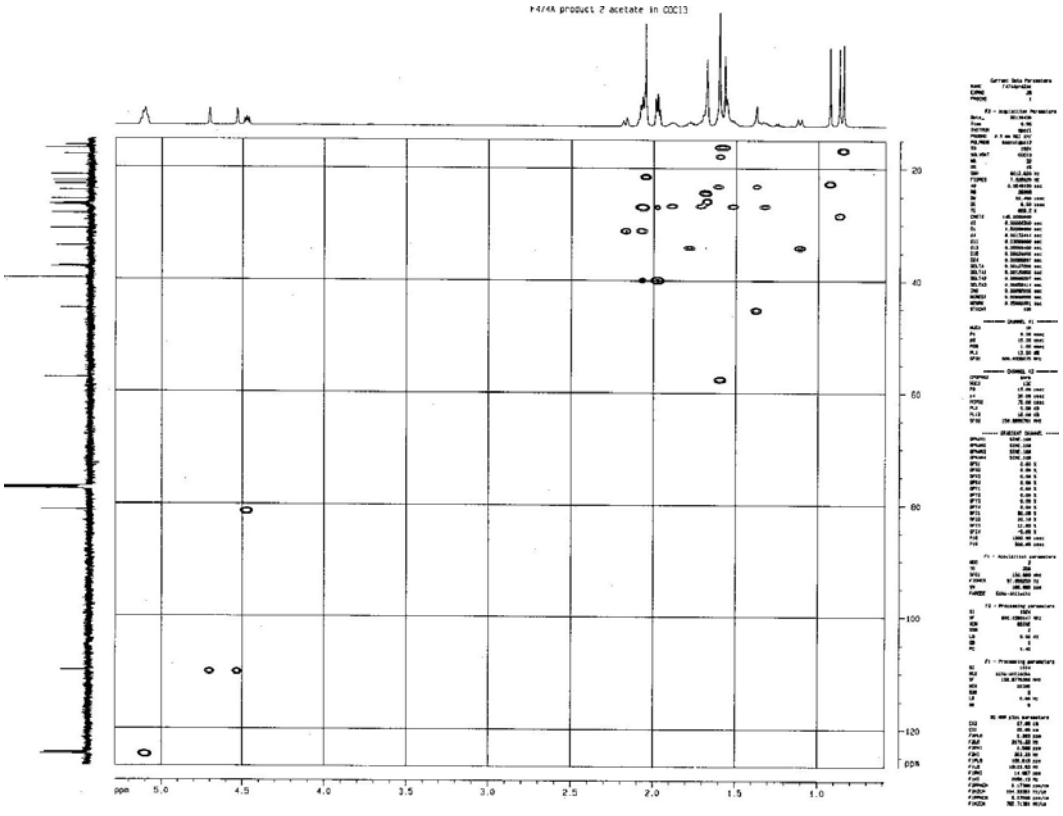
3.2.6. HOHAHA in $CDCl_3$ (600 MHz)



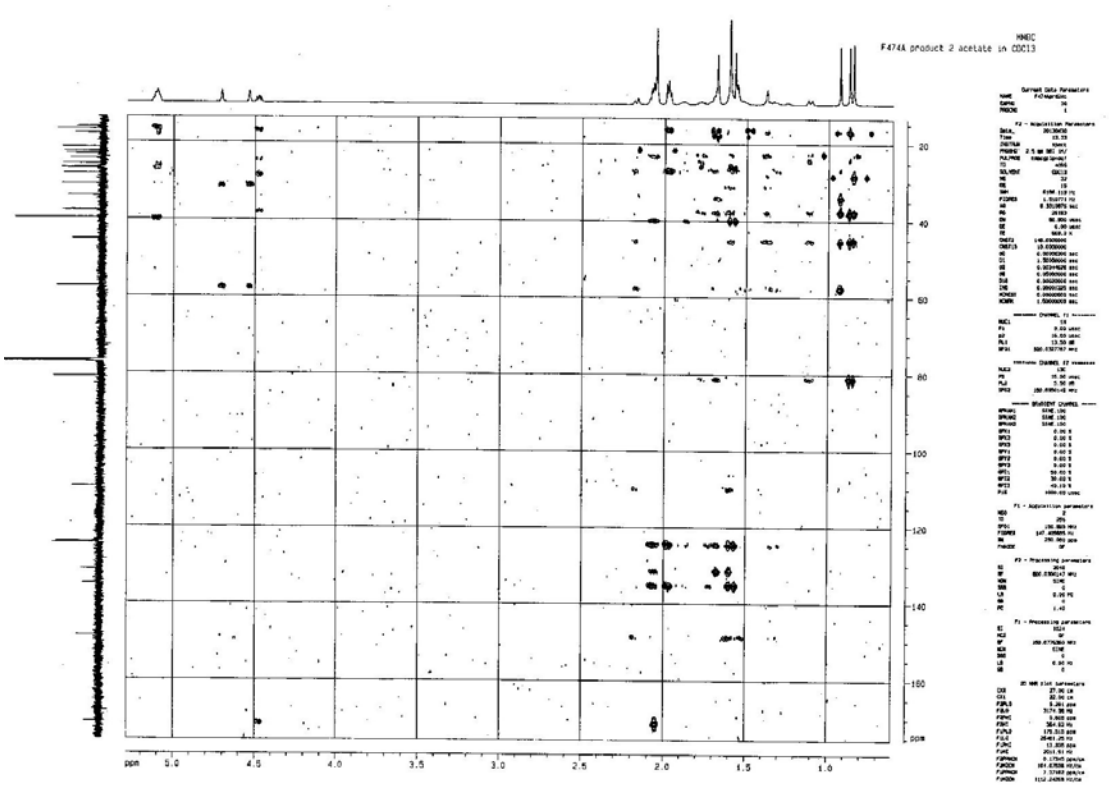
3.2.7. NOESY in CDCl3 (600 MHz)



3.2.8. HSQC in $CDCl_3$



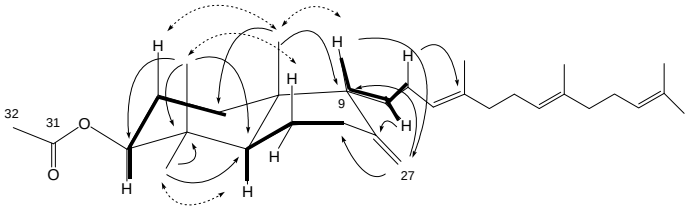
3.2.9. HMBC in $CDCl_3$

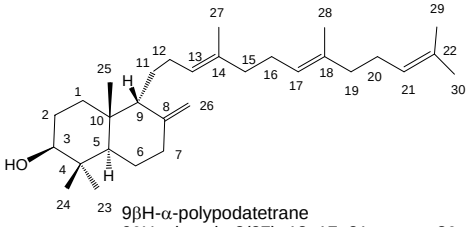


3.2.10. NMR data analyses in C_6D_6 (600 MHz).To confirm the stereochemistry of 9β-H, the NMR spectra of this compound were measured again in the different solvent C_6D_6.

F474A mutant

Product 10 acetate in (600 Hz, C_6D_6)





9βH-α-polypodatetrane
9βHpolypoda-8(27), 13, 17, 21-tetraen-3β-ol

600 MHz in C_6D_6
the solvent peak ^1H: 7.28 ppm; ^{13}C:128.0 ppm

NOE: ⇢ HMBC: ⟶
COSY & HOHAHA: ━

NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{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) *d*
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) *d*
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)	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 C_6D_6 (600 MHz)

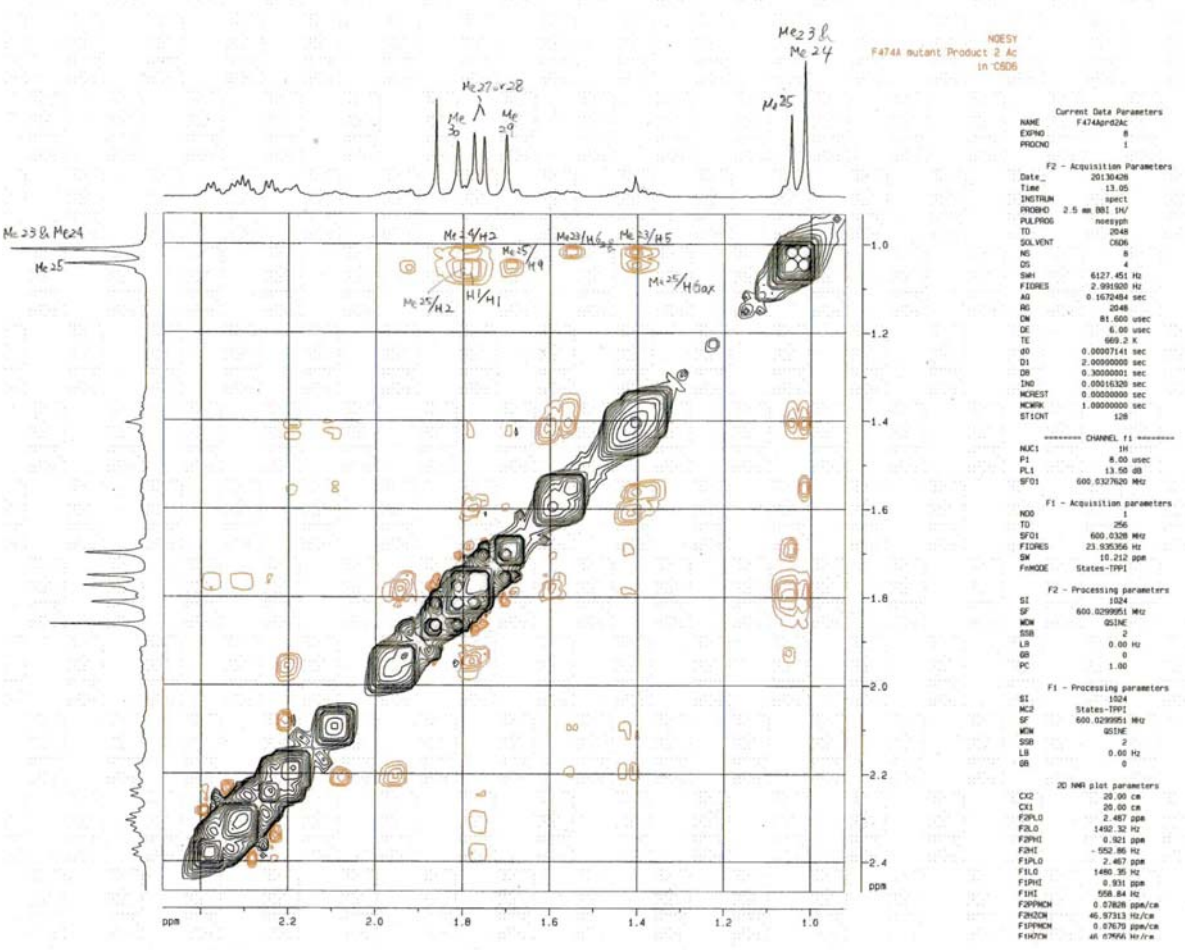
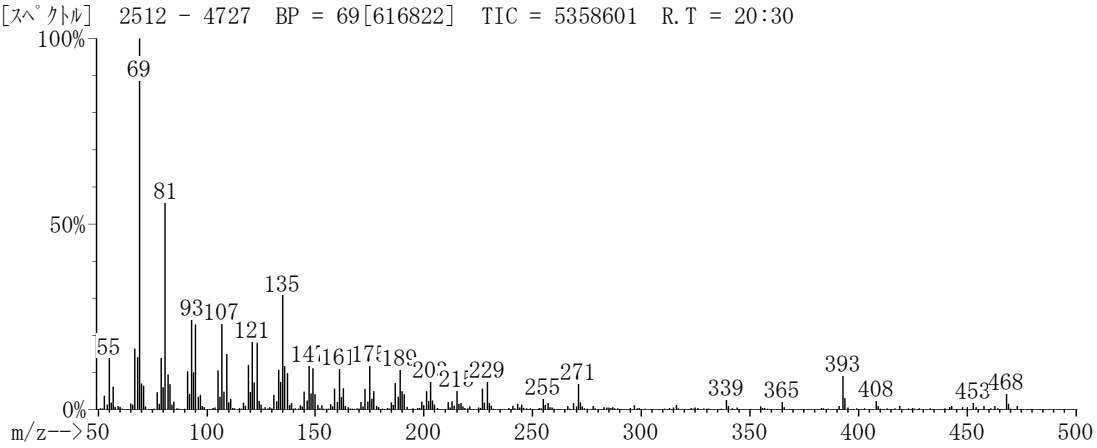


Fig. S3.3. Product 11 acetate produced by F474A mutant

3.3.1 EIMS

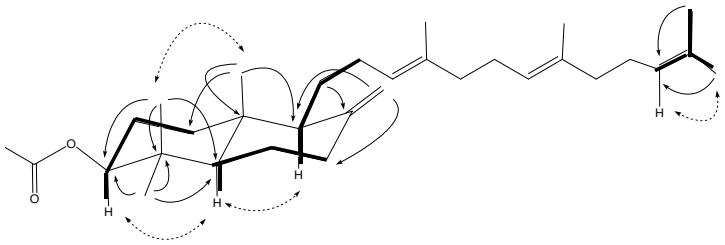


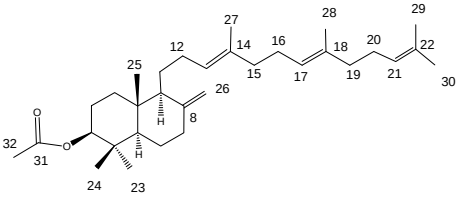
3.3.2. NMR data analyses in $CDCl_3$, $[\alpha]_D$, and HREIMS.

F474A mutant Product 11 acetate in $CDCl_3$

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

alternative name: α-polypodatetraen-3β-ol





Polypoda-8(26),13,17,21-tetraen-3β-ol
alternative name: (+)-α-polypodatetraen-3β-ol

Shiojima, Kenji; Arai, Yoko; Masuda, Kazuo; Kamada, Toshifumi; Ageta, Hiroyuki From Tetrahedron Letters (1983), 24(51), 5733-6. α-and γ-polypodatetraene wwere first isolated by Ageta et al.

$[\alpha]_D^{25}$=+ 74.4 (*c*=0.154, $CHCl_3$)

HREIMS
calcd. 468.39748
observed: 468.39627

NOE: ◄----► HMBC: ——►

COSY & HOHAHA: ▬▬

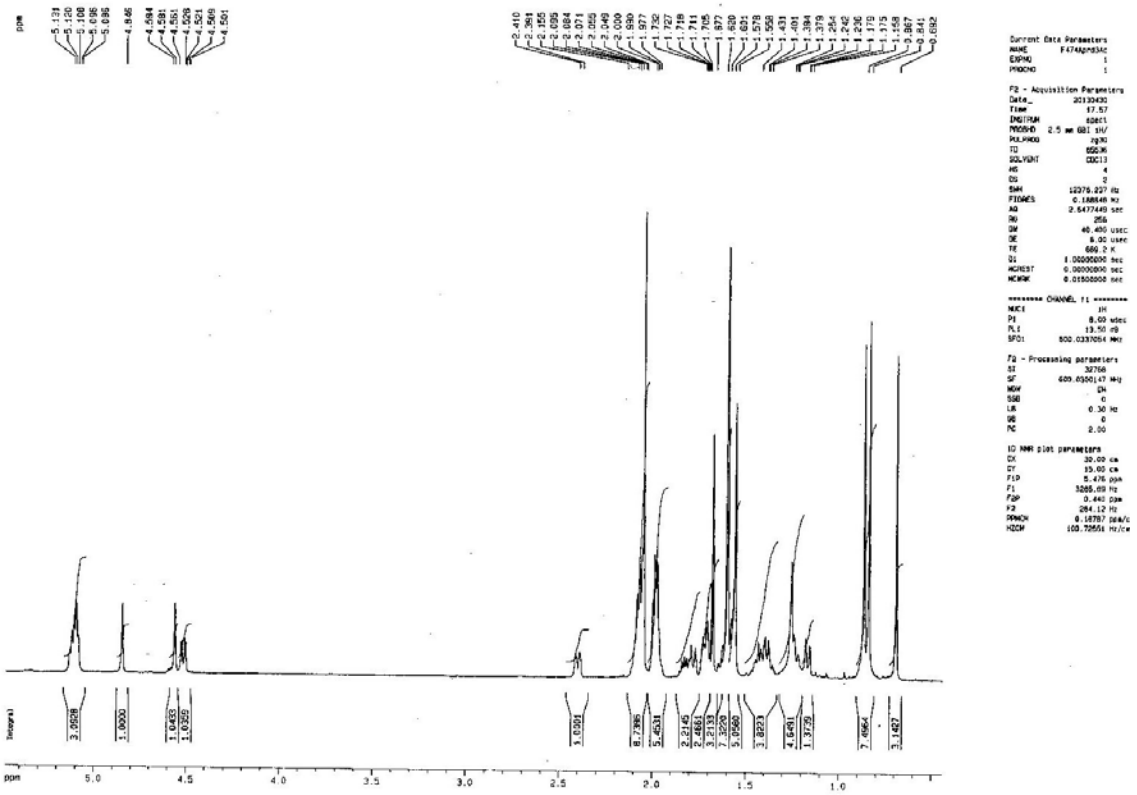
600 MHz in $CDCl_3$
the solvent peak ^1H: 7.26 ppm; ^{13}C:77.0 ppm

NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{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)**	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)** *b*	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)**

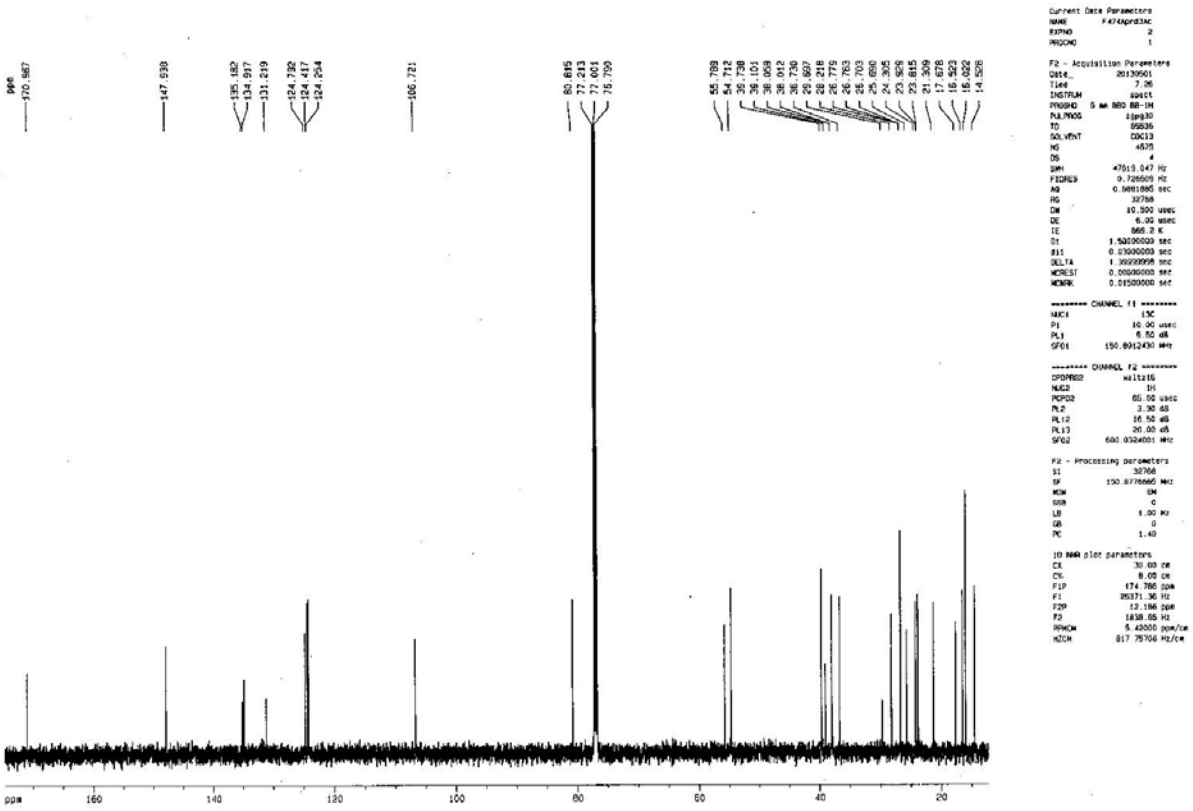
The assignments of the symbols a~h are indistinguishable between the same symbols, due to the very close or inseparable chemical shifts.

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 indistingushable.

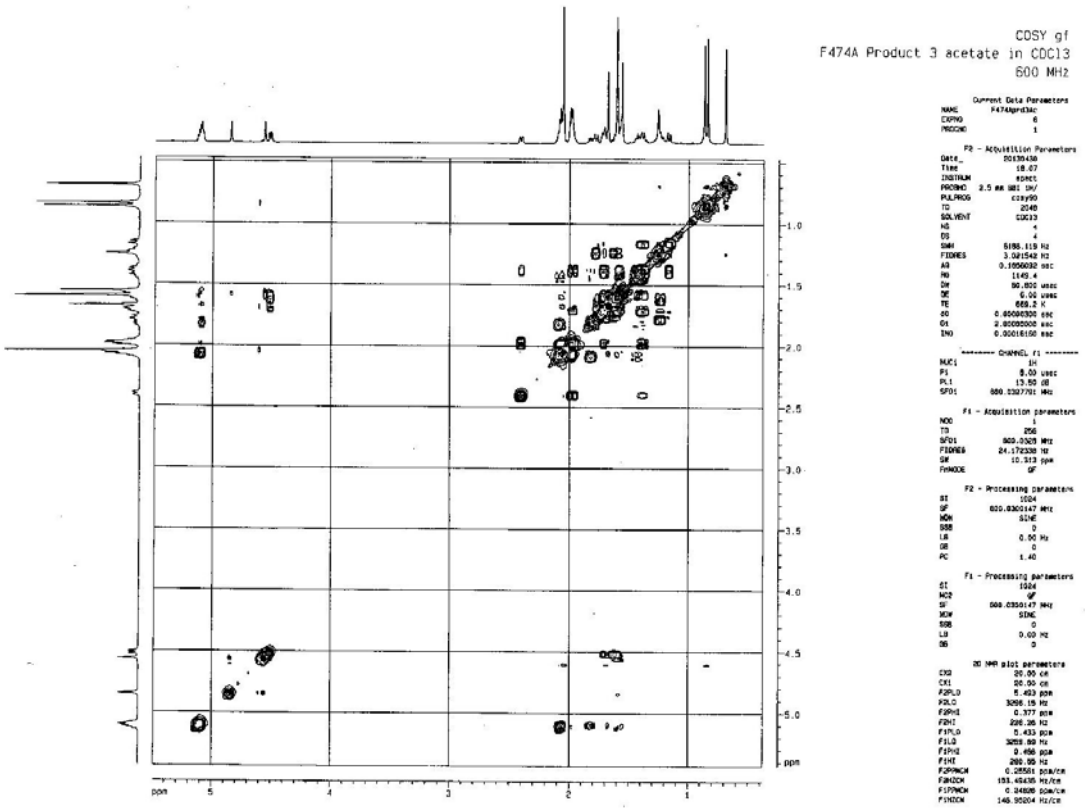
3.3.3.^{1}H NMR in C_6D_6 (600 MHz)



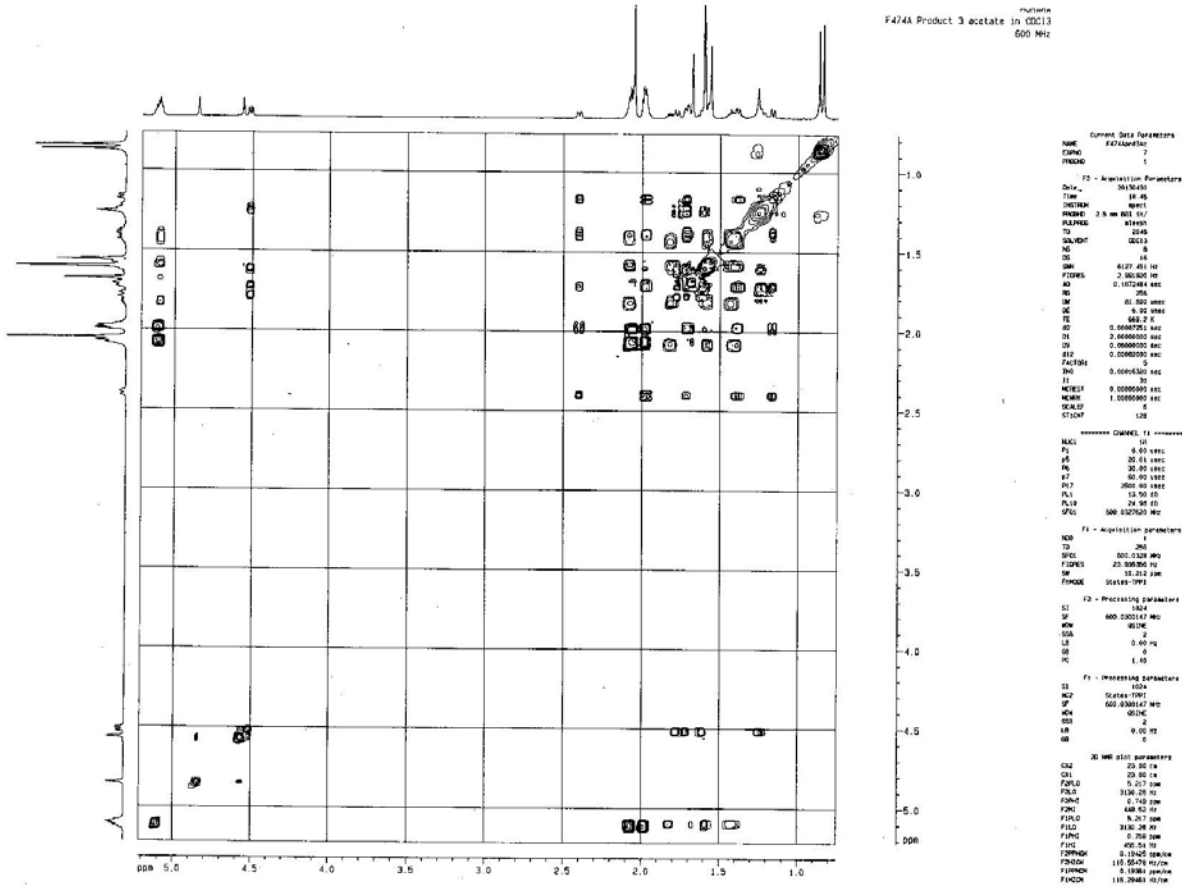
3.3.4.^{13}C NMR in C_6D_6 (150 MHz)



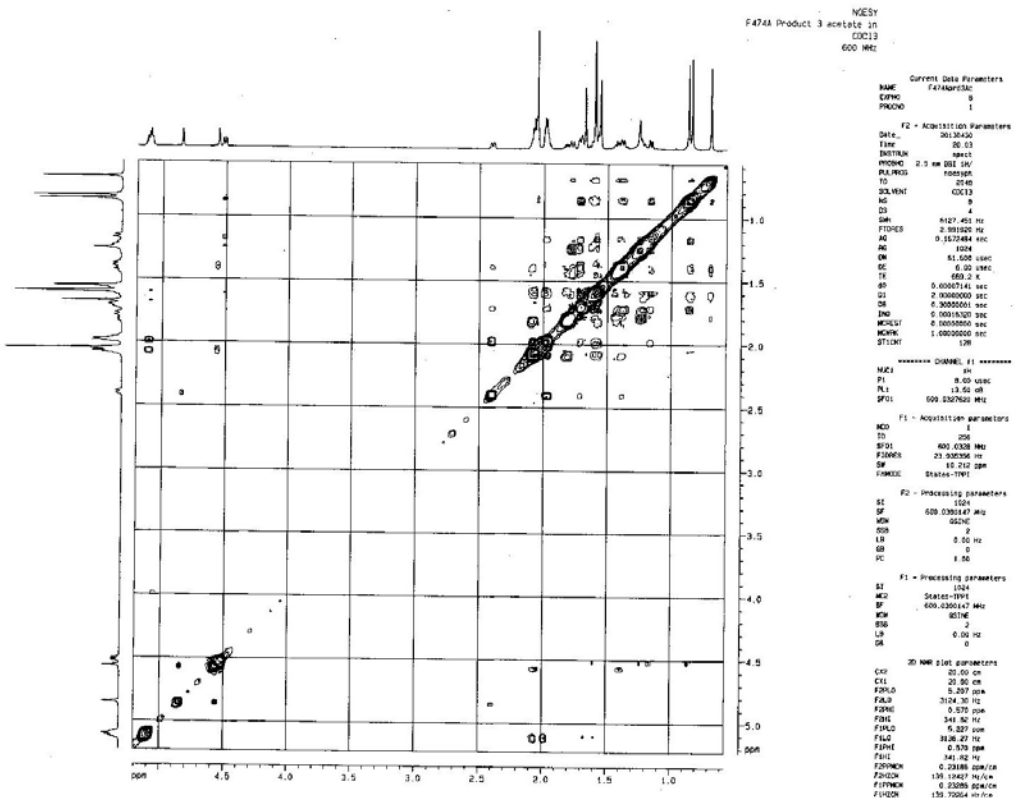
3.3.5. COSY in C_6D_6 (600 MHz)



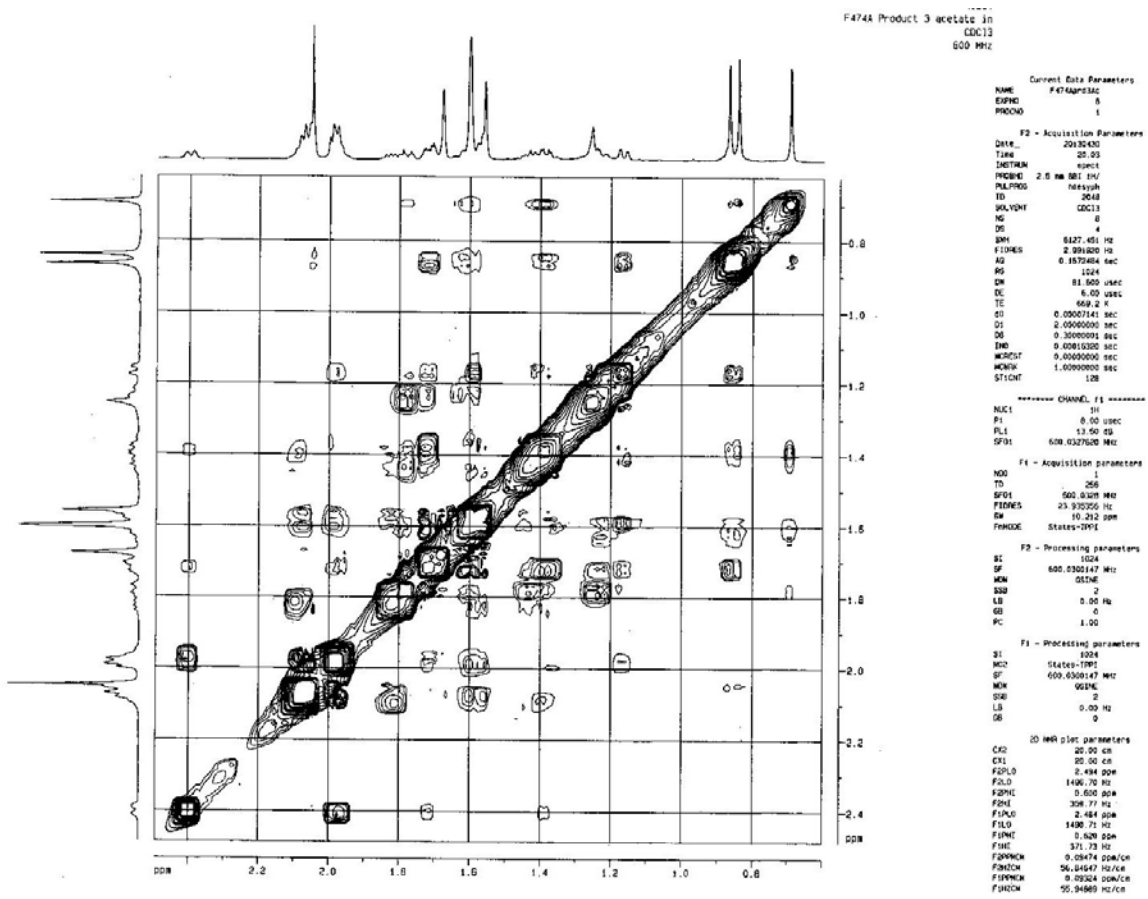
3.3.6 HOHAHA in C_6D_6 (600 MHz)



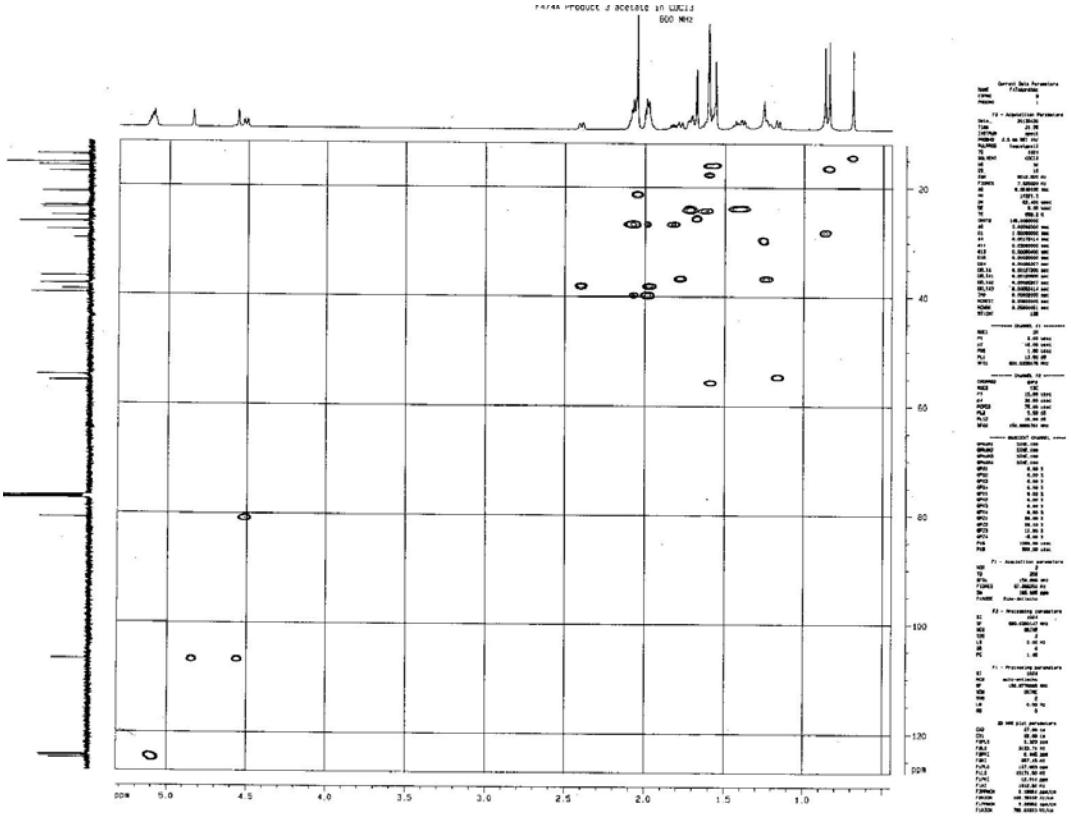
3.3.7. NOESY in C_6D_6 (600 MHz): Whole region



3.3.8. NOESY in C_6D_6 (600 MHz), expanded region



3.3.9. HSQC in C_6D_6 (600 MHz).



3.3.10. HMBC in C_6D_6 (600 MHz).

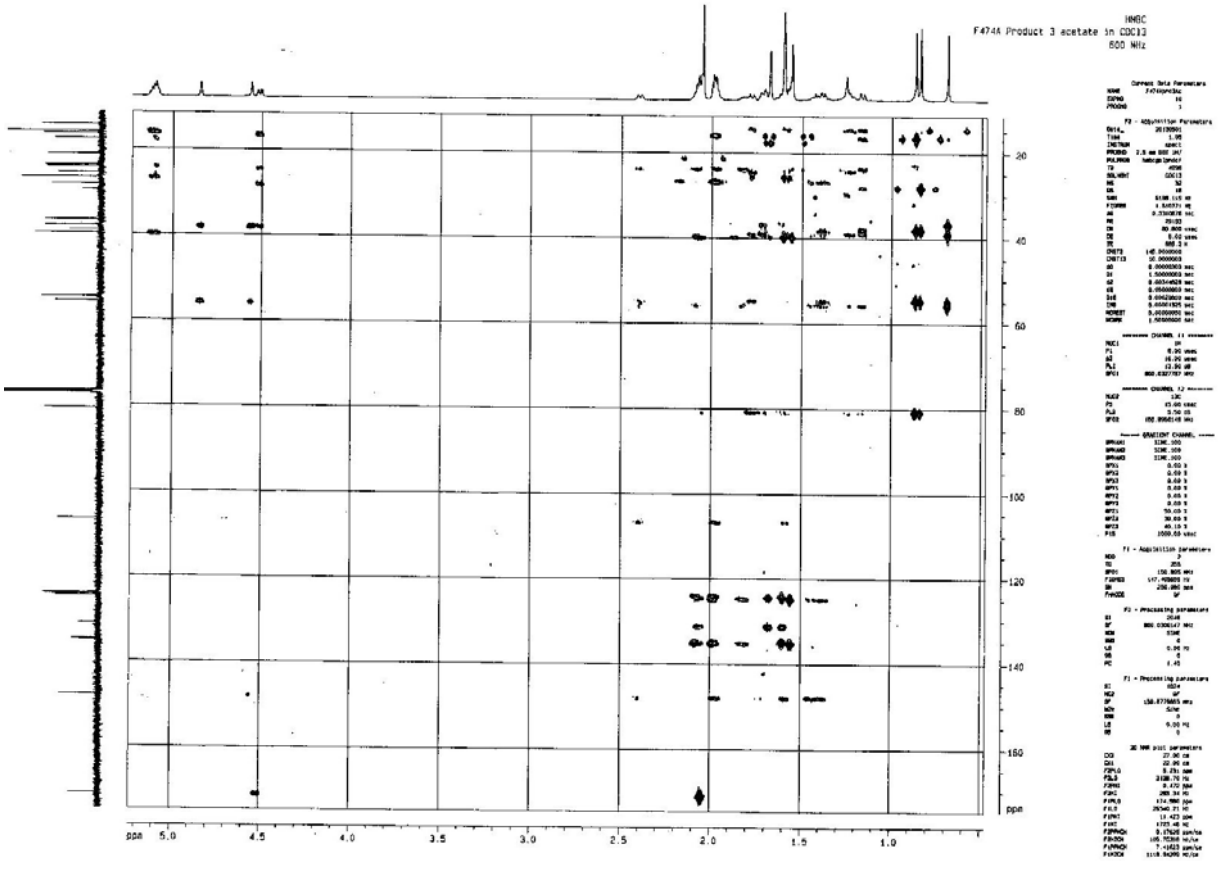
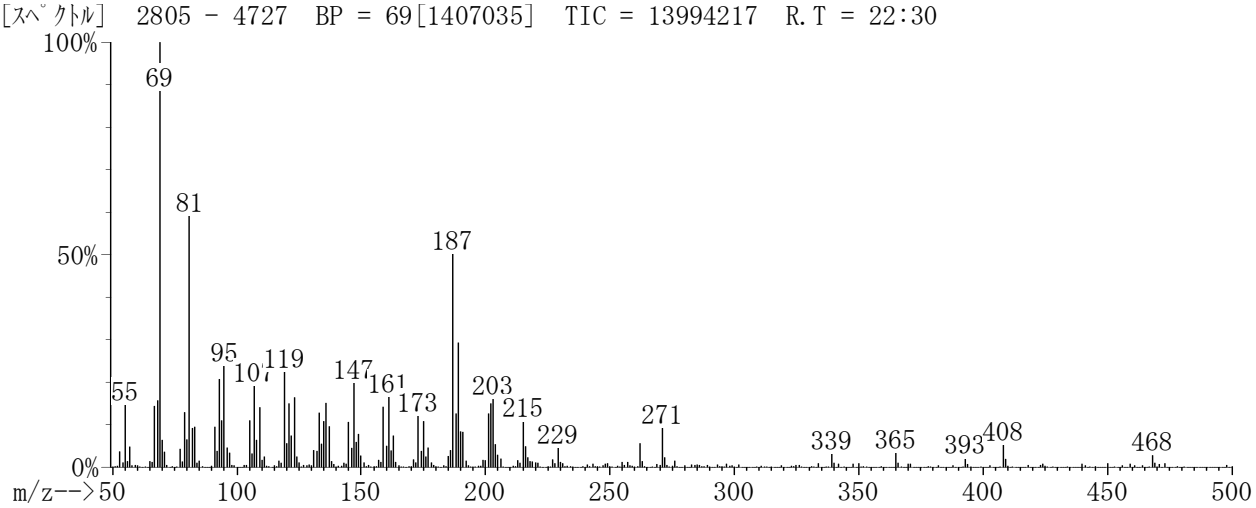


Fig. S.3. 4. Product 12 acetate produced by F474A mutant

3.4.1. EIMS

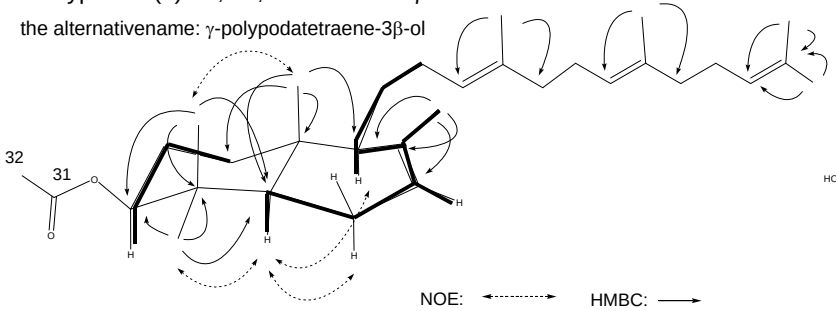


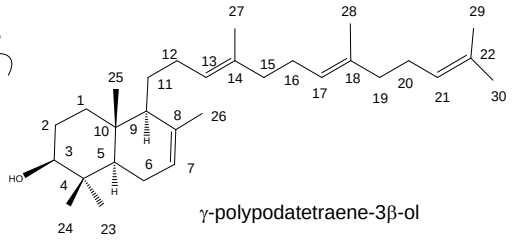
3.4.2. NMR data analyses in C_6D_6, $[\alpha]_D$, HREIMS

F474A mutant Product 12 acetate in C_6D_6

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

the alternativename: γ-polypodatetraene-3β-ol





γ-polypodatetraene-3β-ol

Shiojima, Kenji; Arai, Yoko; Masuda, Kazuo; Kamada, Toshifumi; Ageta, Hiroyuki
From Tetrahedron Letters (1983), 24(51), 5733-6. α-and γ-polypodatetraene wwere first isolated by Ageta et al.

NOE: ⟷ HMBC: ⟶

COSY & HOHAHA: ━━

$[\alpha]_D^{25}$=+ 76.9 (*c*=0.08, $CHCl_3$)

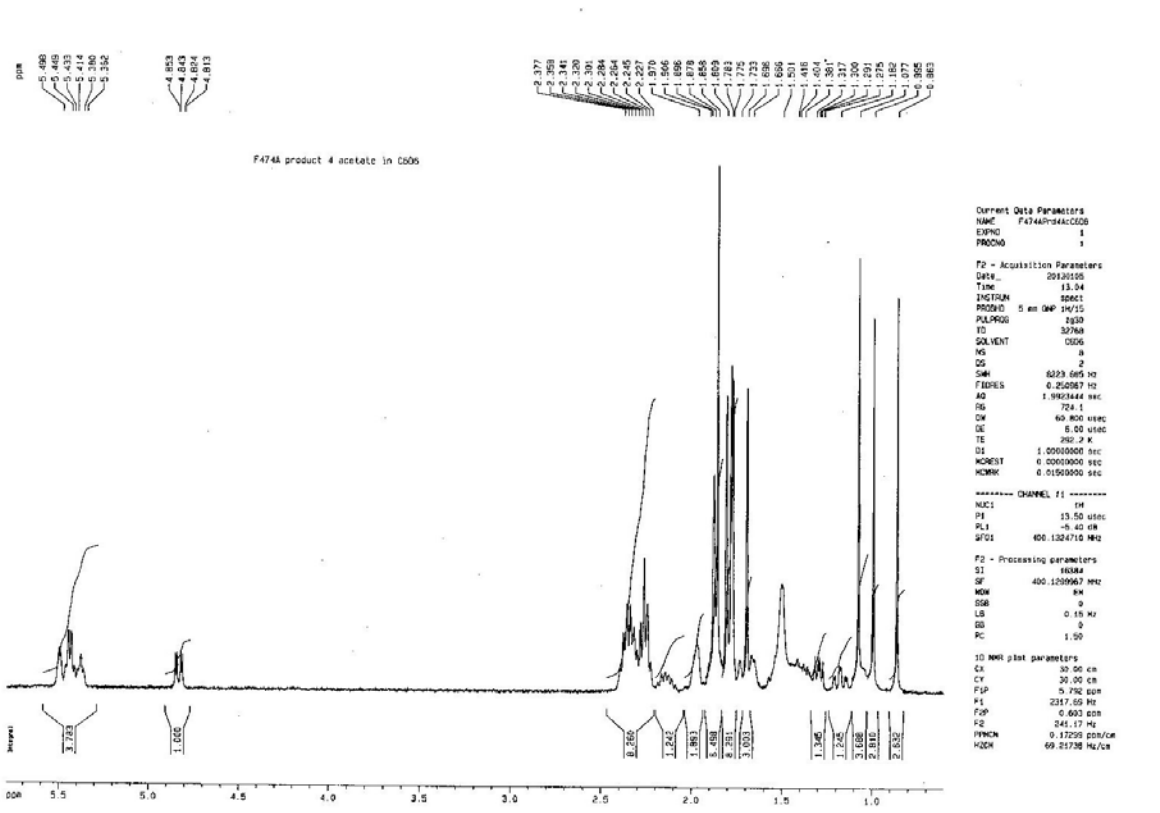
400 MHz in C_6D_6
the solvent peak ^1H: 7.28 ppm; ^{13}C:128.0 ppm

HREIMS
calcd. 468.39686
observed: 468.39627

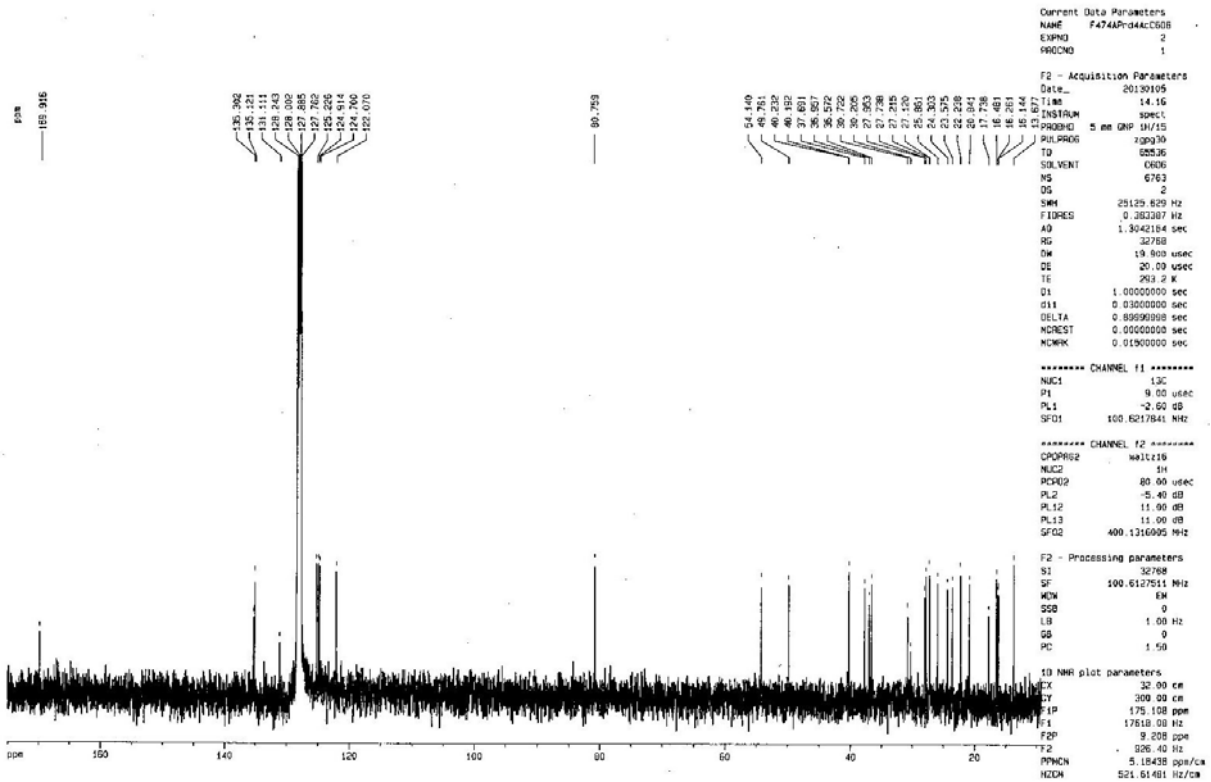
NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C
1	1.18 (ddd, J=12.8, 12.8, 3.2 Hz);1.83(m)	**36.96 (t)**	**9**	1.66 (m)	**54.14(d)**	**17**	5.43 (t, J=6.4 Hz) *e*	**124.7(d)** *f*	**25**	0.863 (3H,s)	**13.68 (q)**
2	1.70(m); 1.82 (m)	**24.30 (t)**	**10**	——	**36.57(s)**	**18**	——	**135.1 (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.

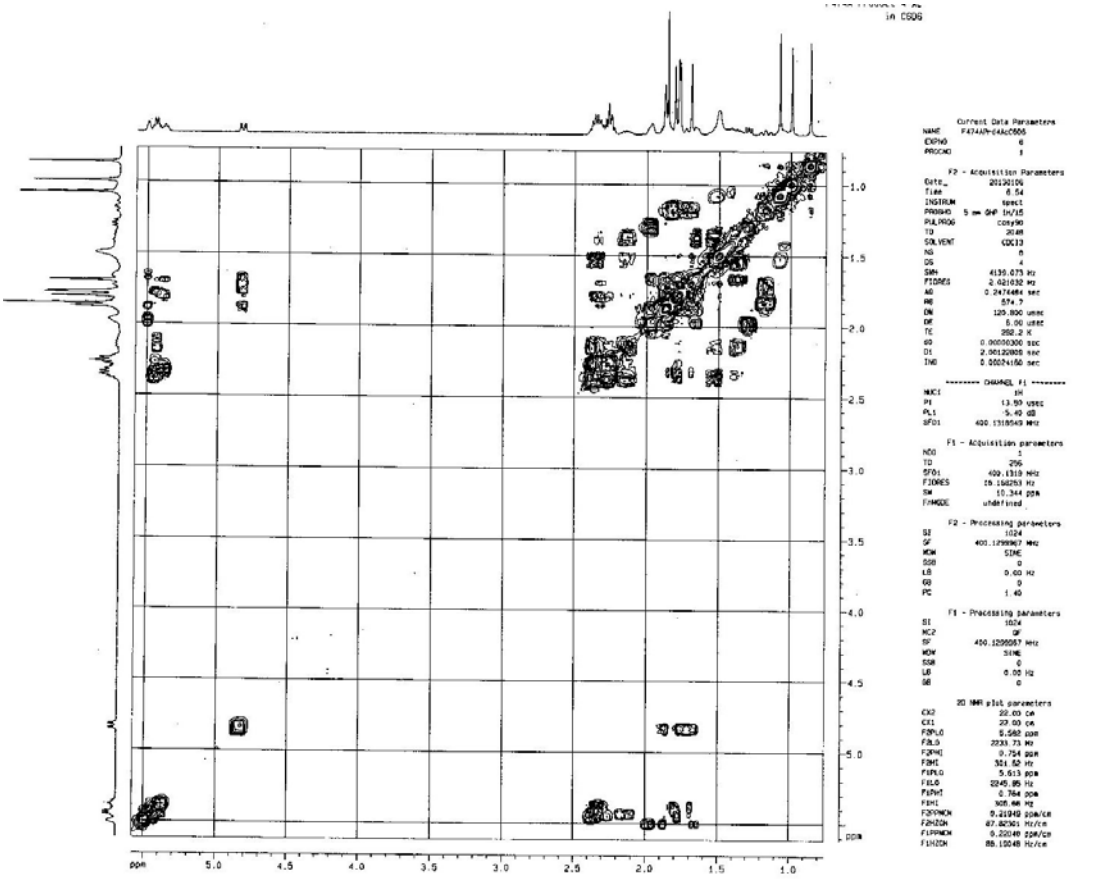
3.4.3. ^{1}H NMR in C_6D_6 (400 MHz)



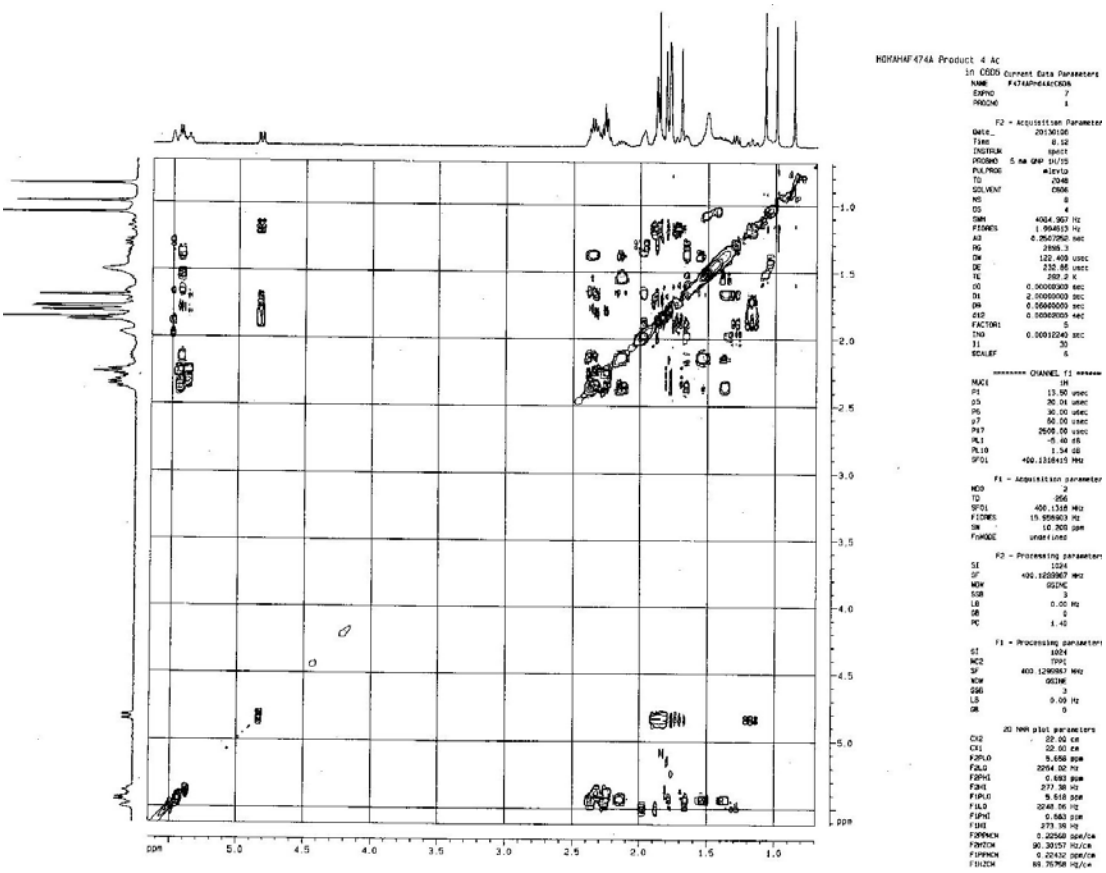
3.4.4. ^{13}C NMR in C_6D_6 (100 MHz)



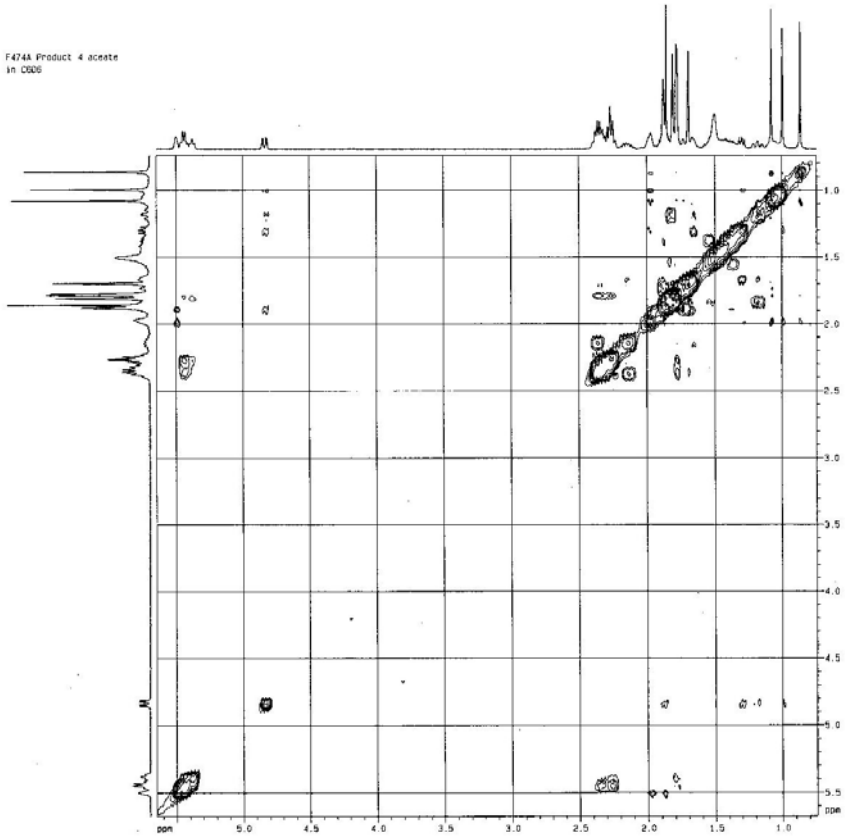
3.4.5. COSY NMR in C_6D_6 (400 MHz)



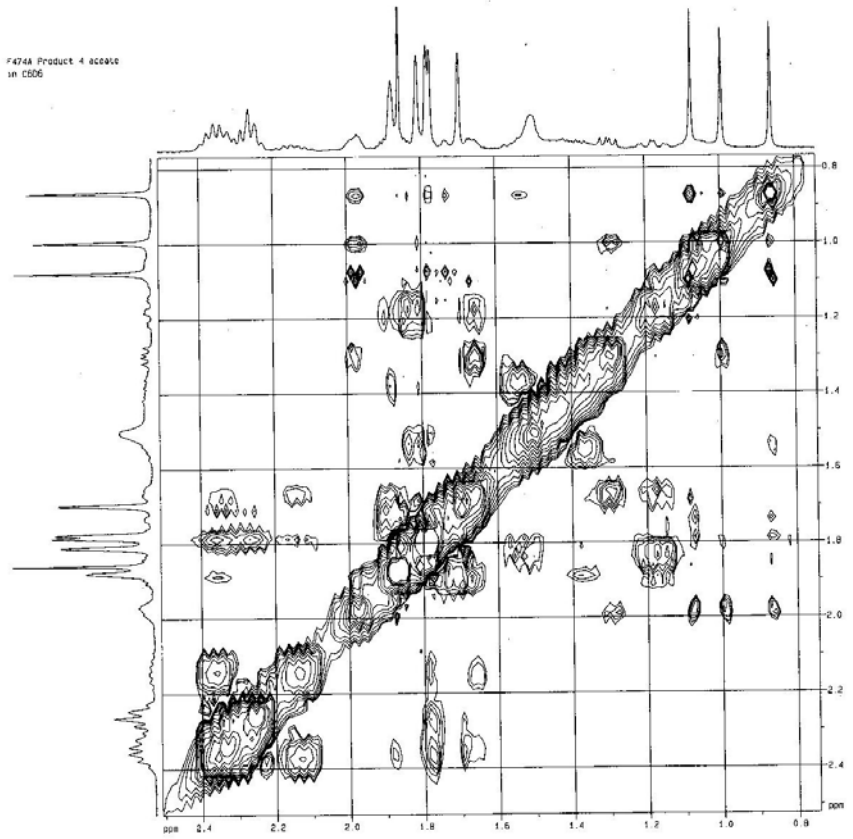
3.4.6. HOHAHA NMR in C_6D_6 (400 MHz)



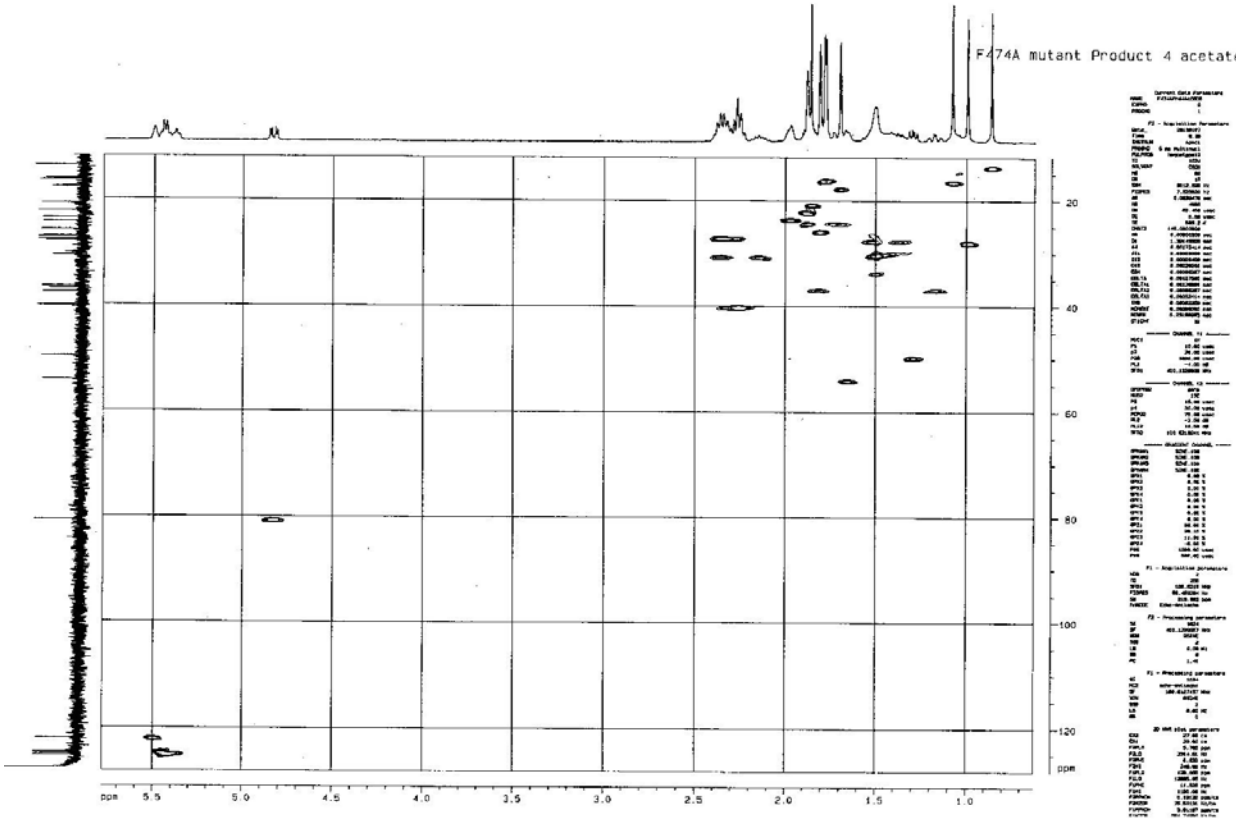
3.4.7. NOESY spectrum in C_6D_6 (400 MHz), whole region



3.4.8. NOESY spectrum in C_6D_6 (400 MHz), expanded region



3.4.9. HSQC spectrum in C_6D_6 (400 MHz).



3.4.10. HMBC spectrum in C_6D_6 (400 MHz).

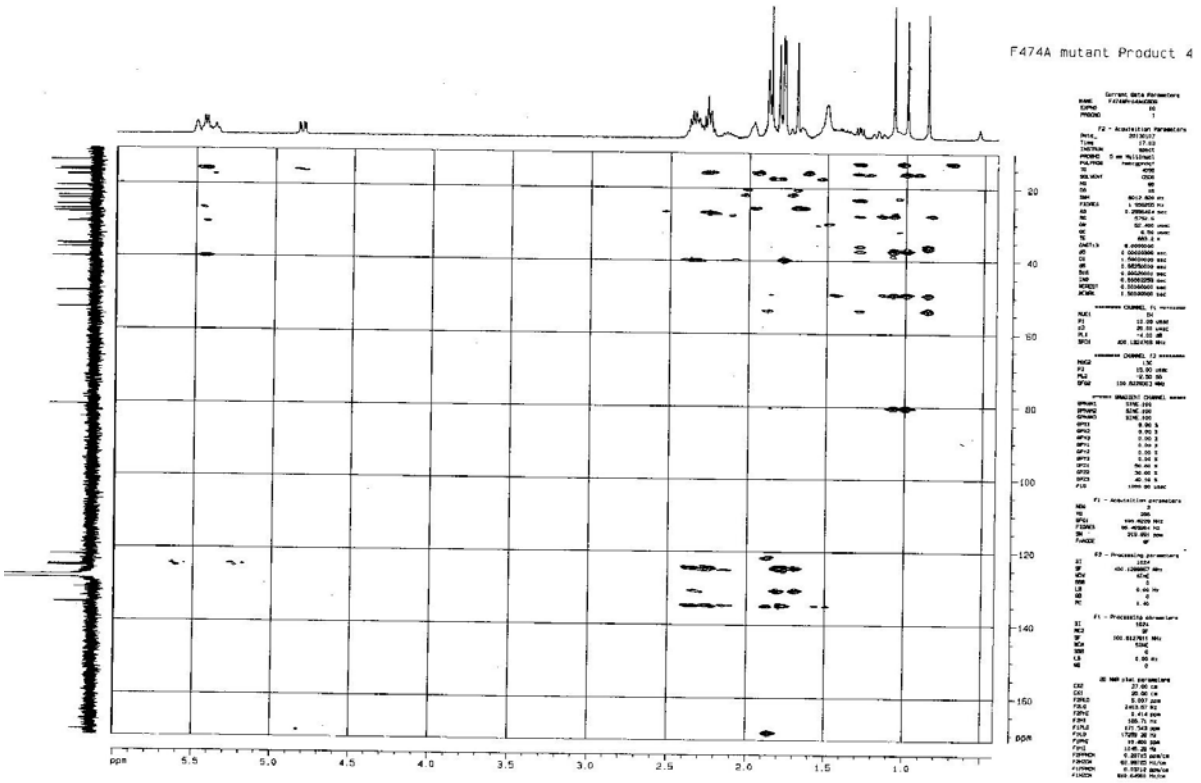
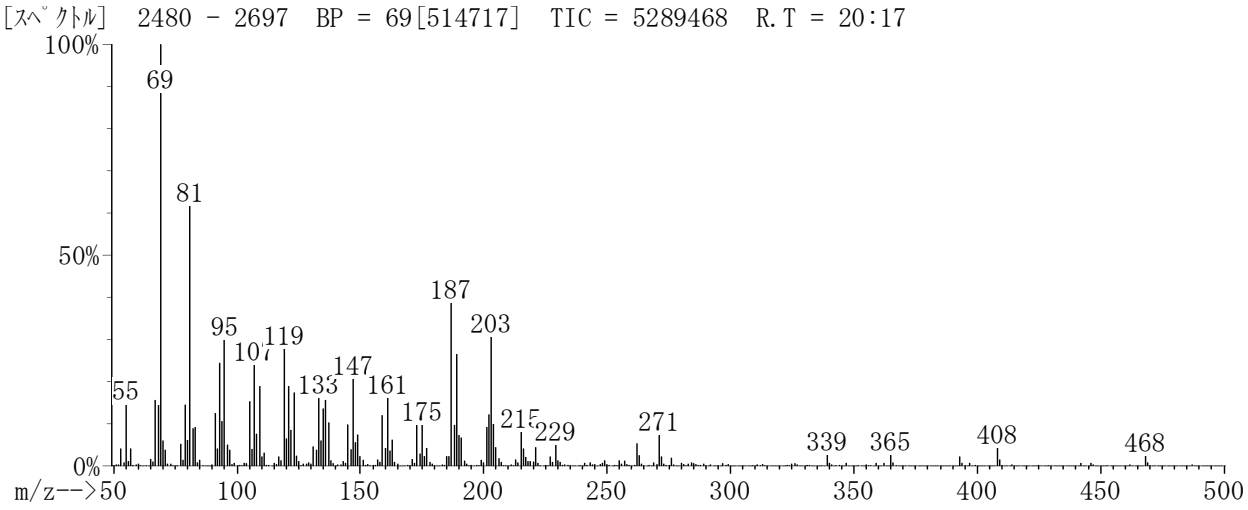


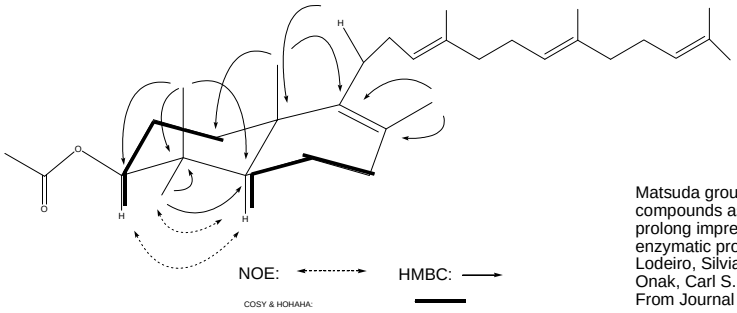
Figure S3. 5. Product 13 acetate from F474A mutant

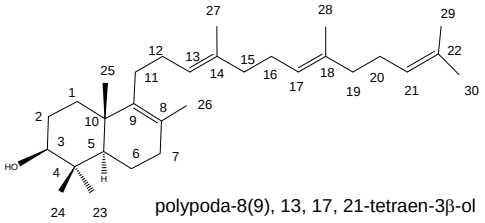
3.5.1 EIMS



3.5.2. NMR data analyses of **13** in C_6D_6, $[\alpha]_D$, HREIMS

Product 13 acetate isolated by F474A mutant





polypoda-8(9), 13, 17, 21-tetraen-3β-ol

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 SiO_2, 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, $CDCl_3$)
There is not literature for the specific rotation of this compound.

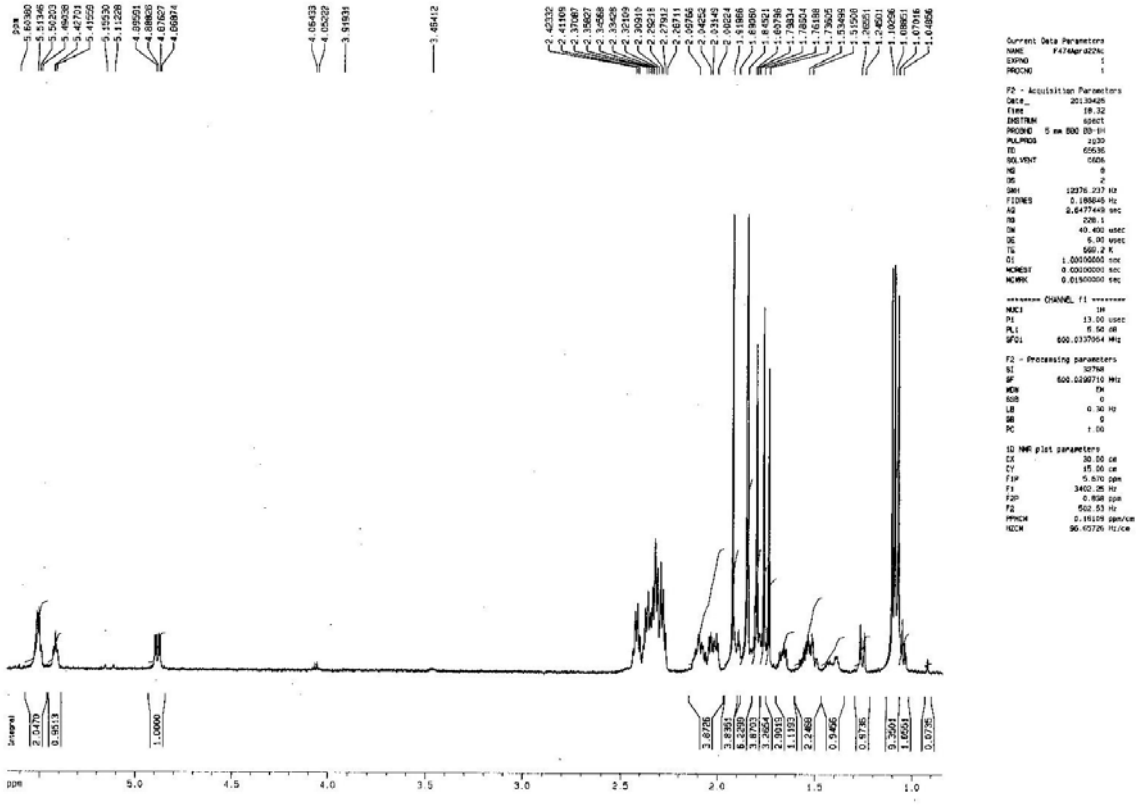
HREIMS
calcd. 468.39673; observed: 468.39657

600 MHz in C_6D_6
the solvent peak ^1H: 7.28 ppm; ^{13}C:128.0 ppm

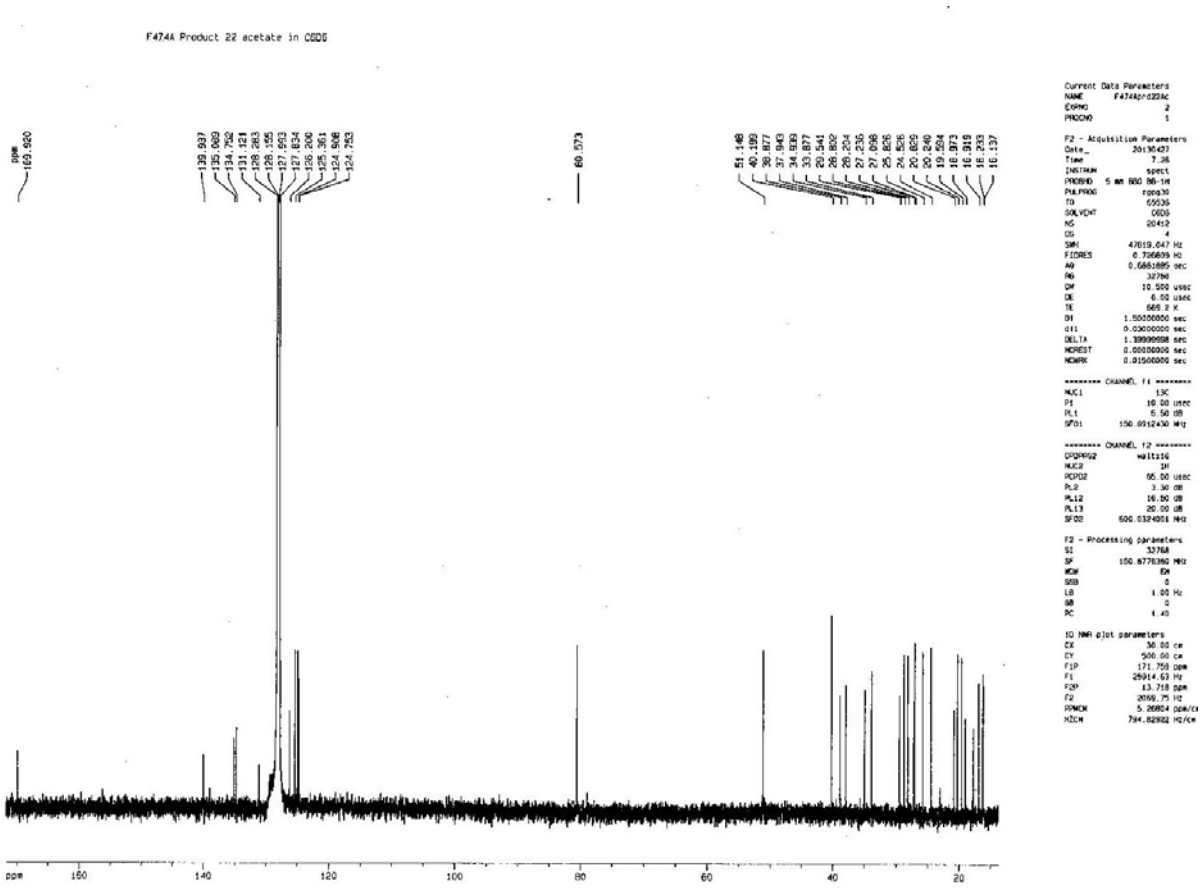
NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	^{13}C	NO.	^1H	
1	1.47 (m);1.85(m)	**34.94 (t)**	9	——	**139.9(s)**	17	5.46 (t, J=6.4 Hz) *e*	**124.7(d)** *f*	25	1.064 (3H,s)	**20.24 (q)**
2	1.76(m); 1.97 (m)	**24.53 (t)**	10	——	**38.88(s)**	18	——	**134.8 (s)**	26	1.722 (3H,s)	**19.59 (q)**
3	4.84(dd, 11.8, 4.4 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 (bd, 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.

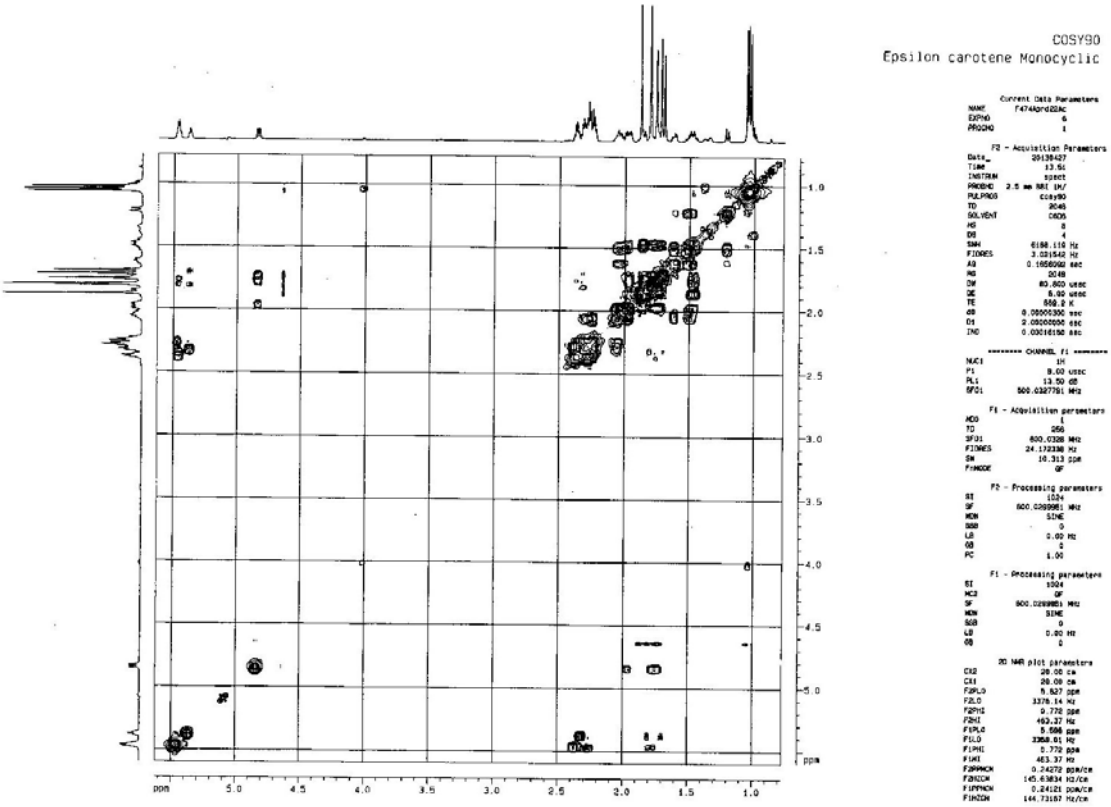
3.5.3.[1] H NMR in C_6D_6 (600 MHz)



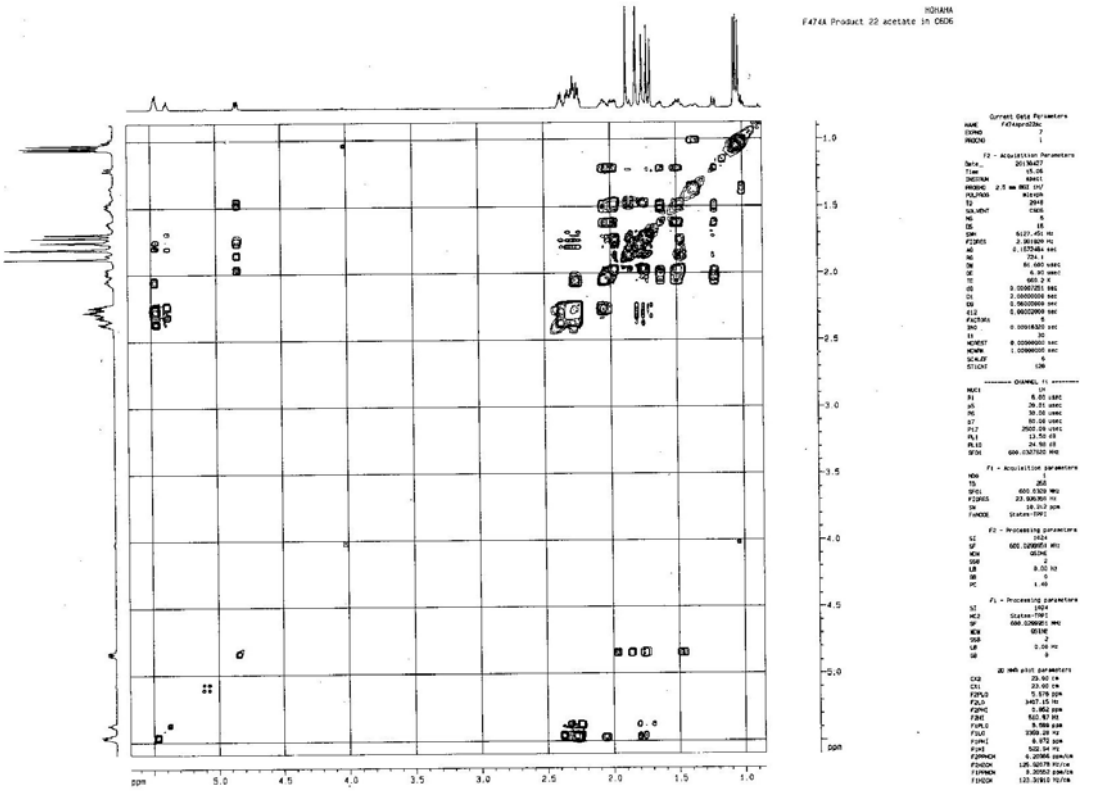
3.5.4.[13] C NMR in C_6D_6 (150 MHz)



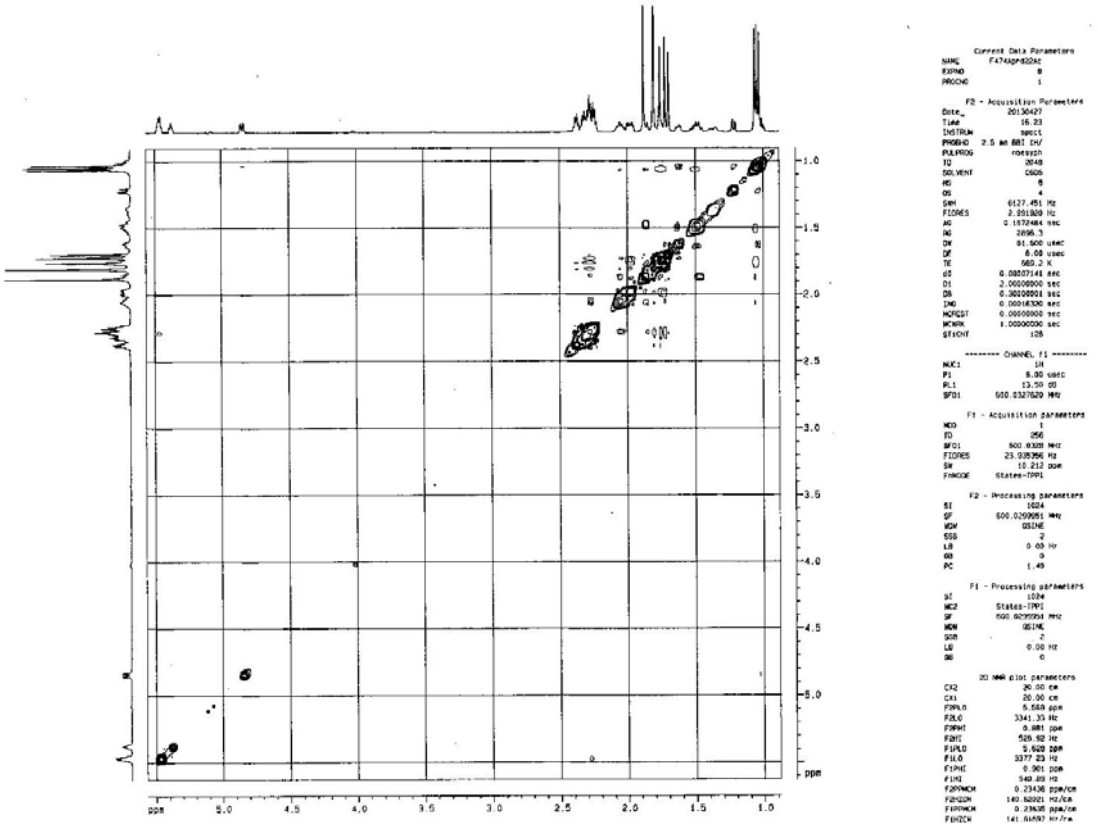
3.5.5. COSY spectrum in C_6D_6 (600 MHz)



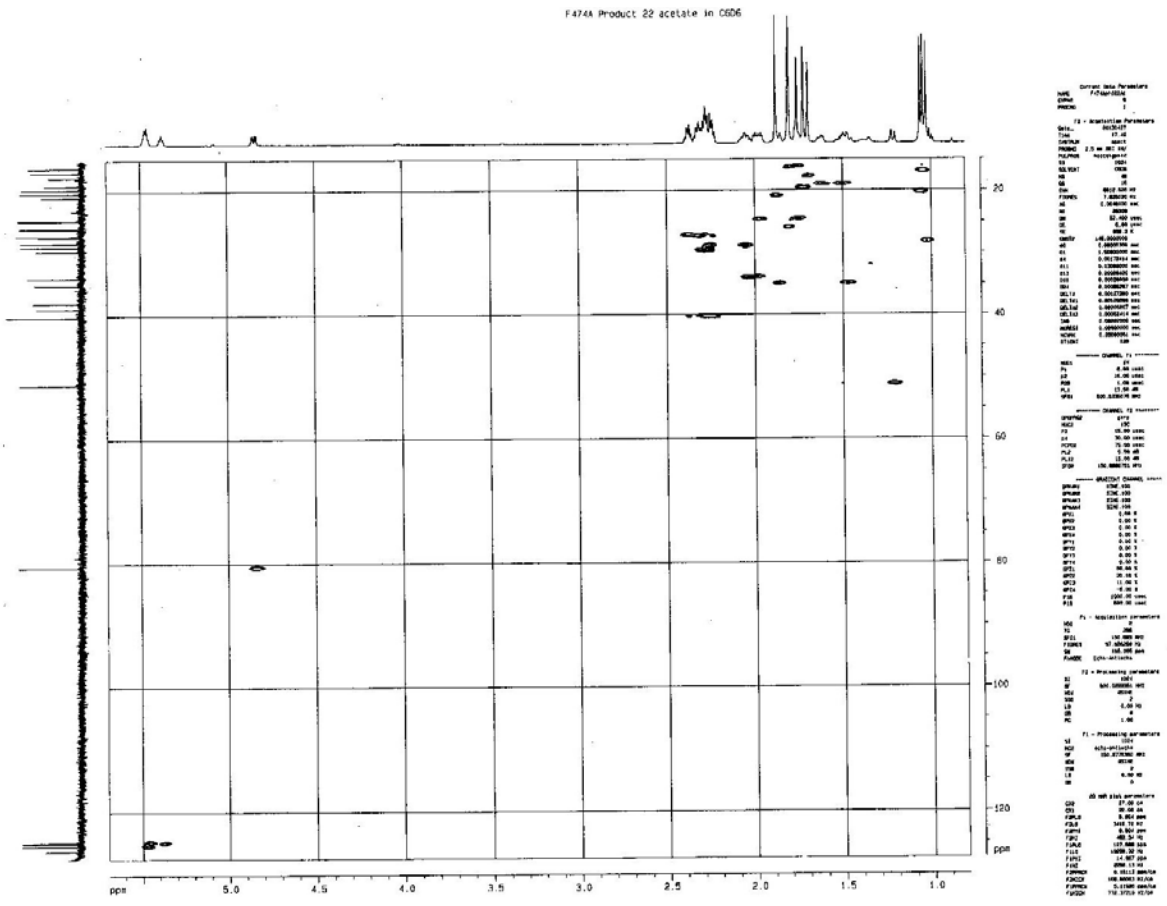
3.5.6. HOHAHA spectrum in C_6D_6 (600 MHz)



3.5.7. NOESY spectrum in C_6D_6 (600 MHz)



3.5.8.. HSQC spectrum in C_6D_6 (600 MHz)



3.5.9.. HMBC spectrum in C_6D_6 (600 MHz)

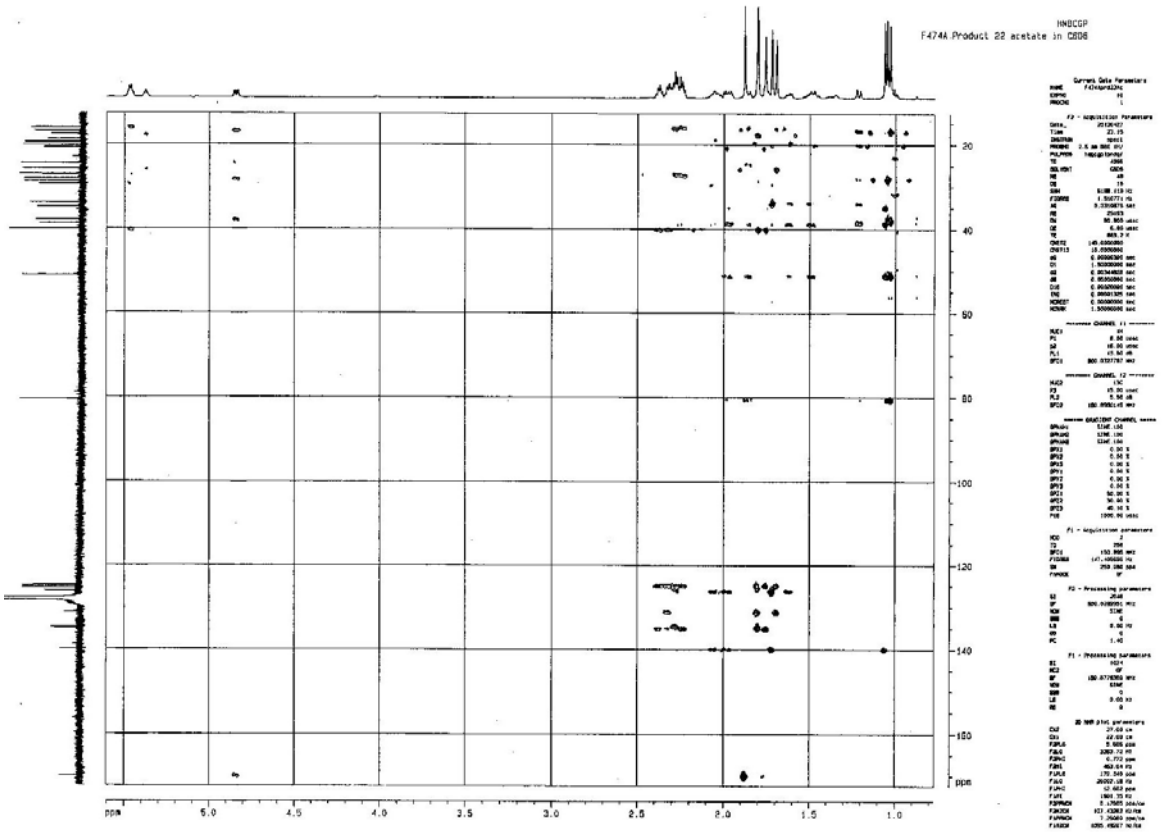


Figure. S.3.6 Product 14 acetate from F474A mutant

(butyrospermol acetate)

EIMS

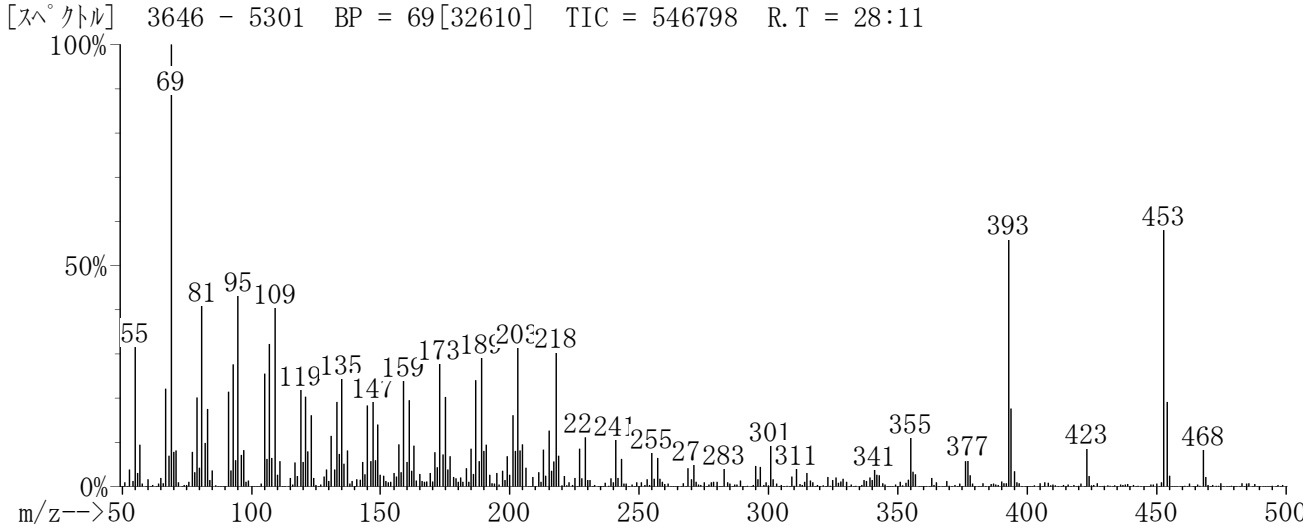


Figure . S.3. 7. Product 15 acetate from F474A mutant

(tirucalla-7,24-dien-3β-ol acetate)

EIMS

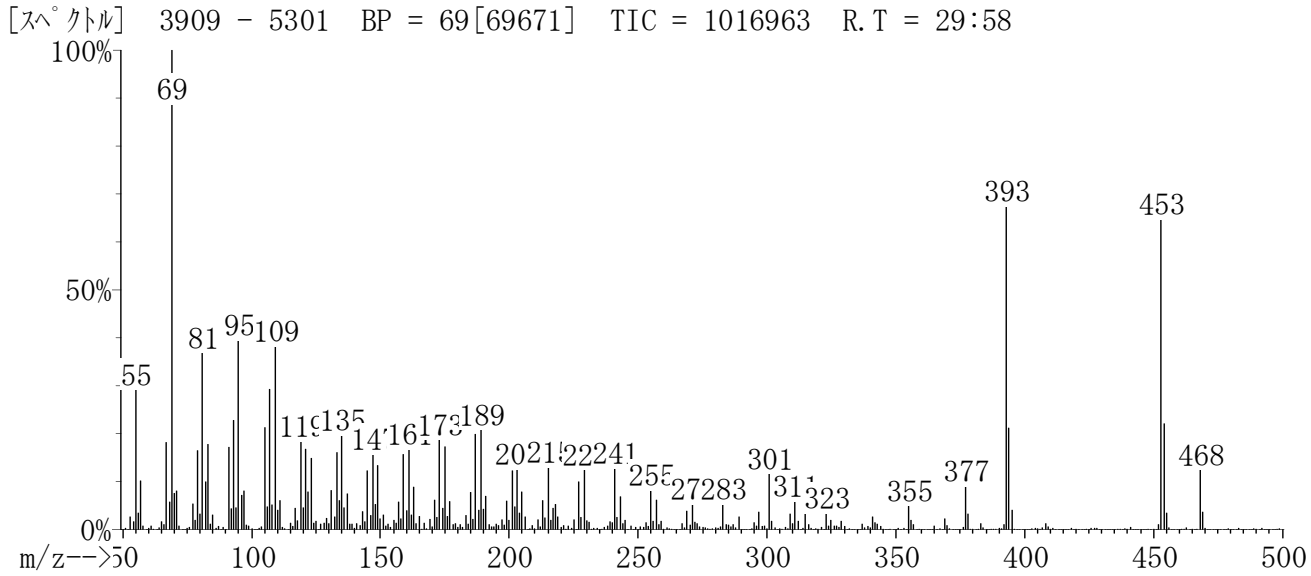
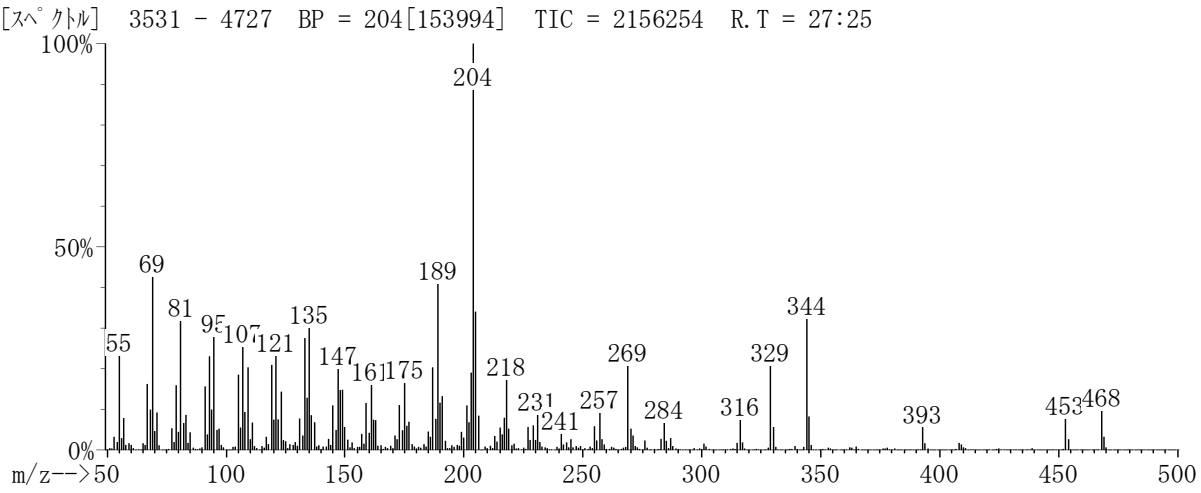


Figure S.3.8. Product 16 acetate from F474A mutant

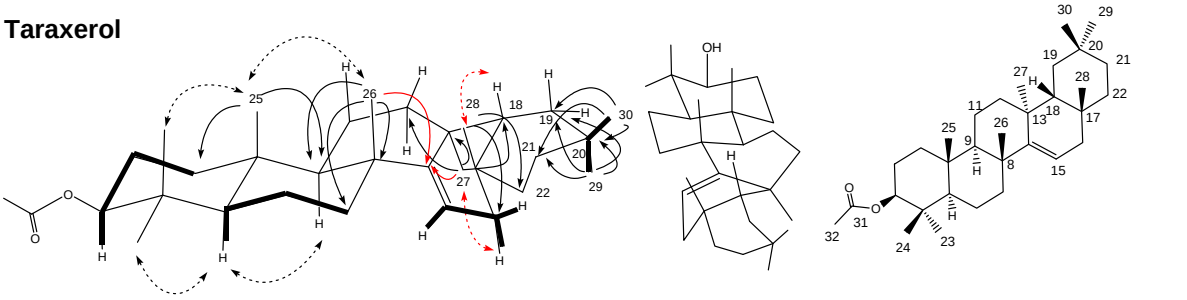
3.8.1. EIMS



3.8.2. NMR data analyses of **16** in C_6D_6 (400 MHz), $[\alpha]_D$, HREIMS

F474A Product 16 acetate

Taraxerol



400 MHz in C_6D_6
the solvent peak ^{1}H: 7.28 ppm; ^{13}C:128.0 ppm

NOE: ⇢ HMBC: →

COSY & HOHAHA: ▬

$[\alpha]_D^{25}$= + 20.1 (*c*=0.65, $CDCl_3$)

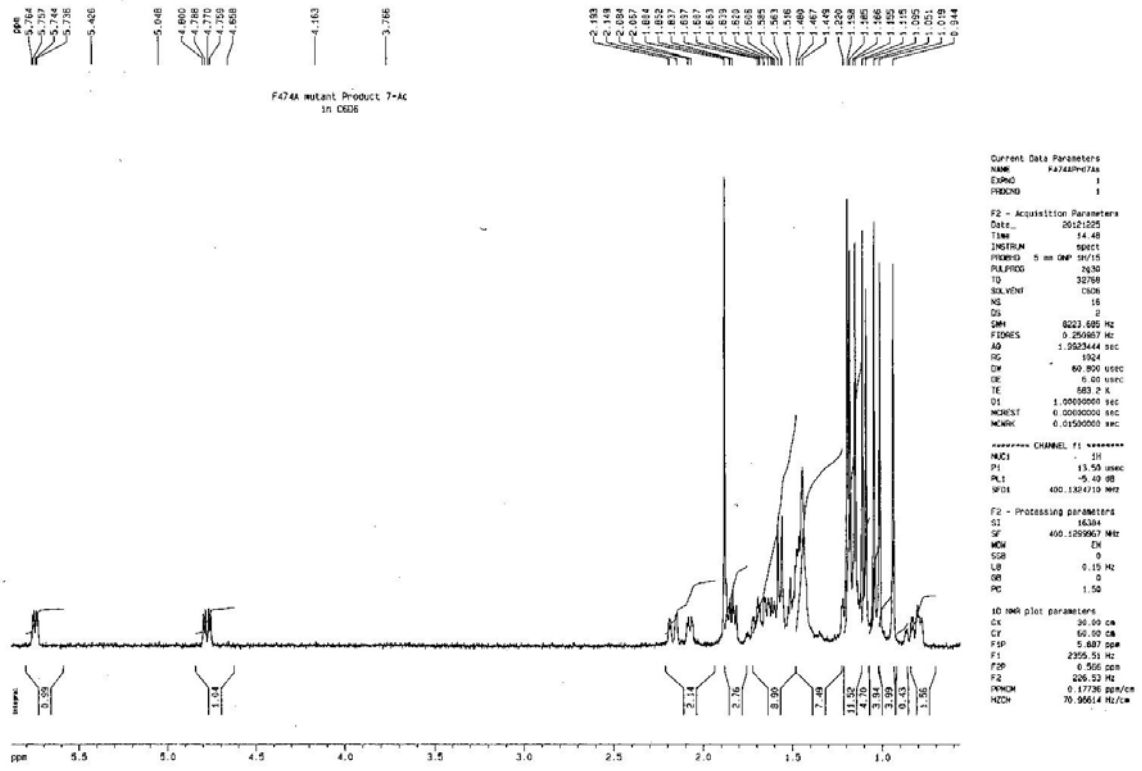
lit. value $[\alpha]_D^{23}$= 0 (*c*=0.43, $CDCl_3$) for deaetylated compound **16.**
Byung-Sun Min et al., J. Nat. Prod., 2004, 67, 1980-1984

HREIMS
calcd. 468.39673; observed: 468.39691

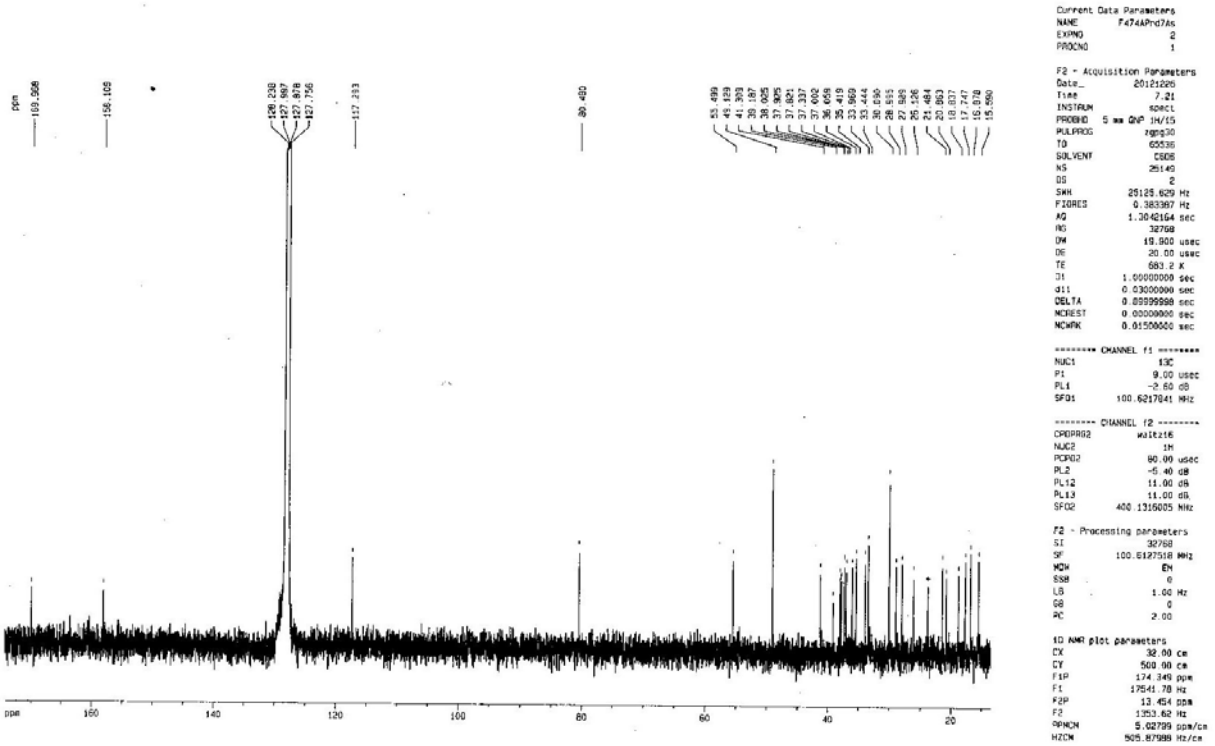
NO.	^{1}H	^{13}C	NO.	^{1}H	^{13}C	NO.	^{1}H	^{13}C	NO.	^{1}H	^{13}C
1	0.83 (ddd, J=13.2, 13.2, 3.6 Hz);1.48(m)	**37.34(t)**	9	1.44 (m)	**49.13(d)**	17	——	**36.06(s)**	25	0.944(3H,s)	**15.59(q)**
2	1.71(m); 1.84 (m)	**23.83(t)**	10	——	**37.76(s)** *a*	18	1.17 (m)	**49.13(d)**	26	1.199 (3H,s)	**26.13(q)**
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 cabon assignments were indistinguishable due to the close chemical shifts.
b: The assignments of Me-29 and Me-30 may be exchangeable.

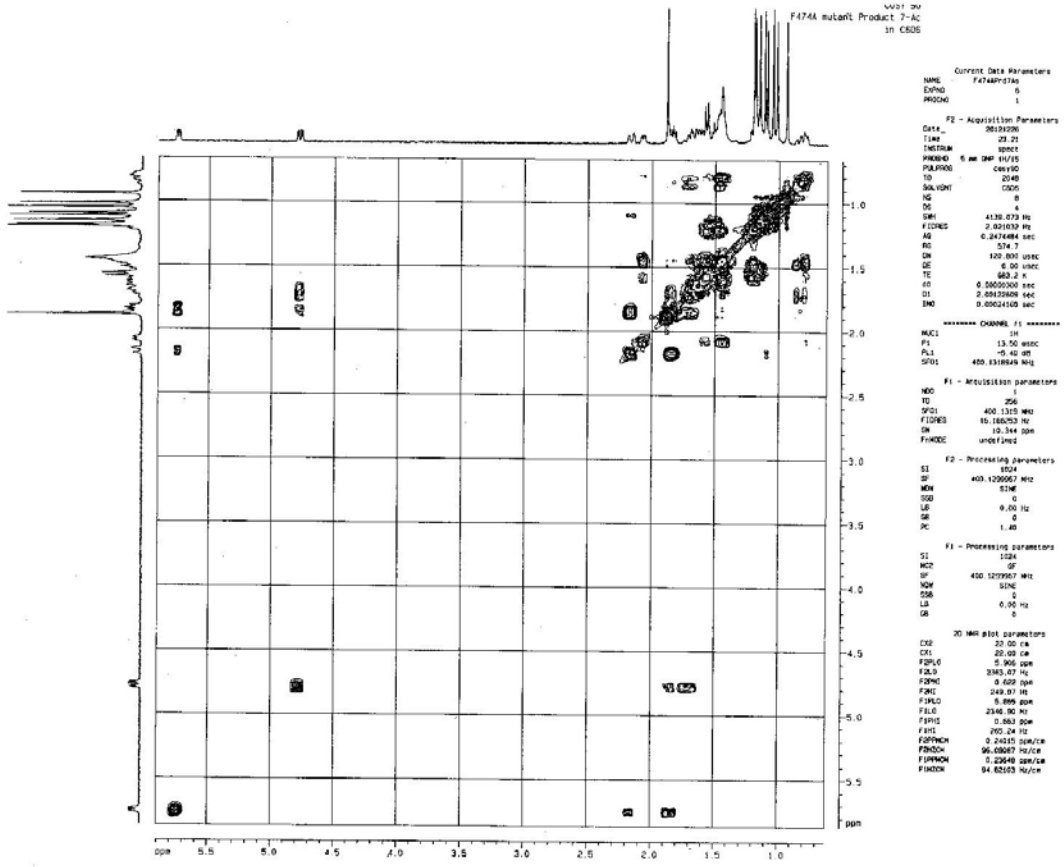
3.8.3.[1] H NMR in C_6D_6 (400 MHz)



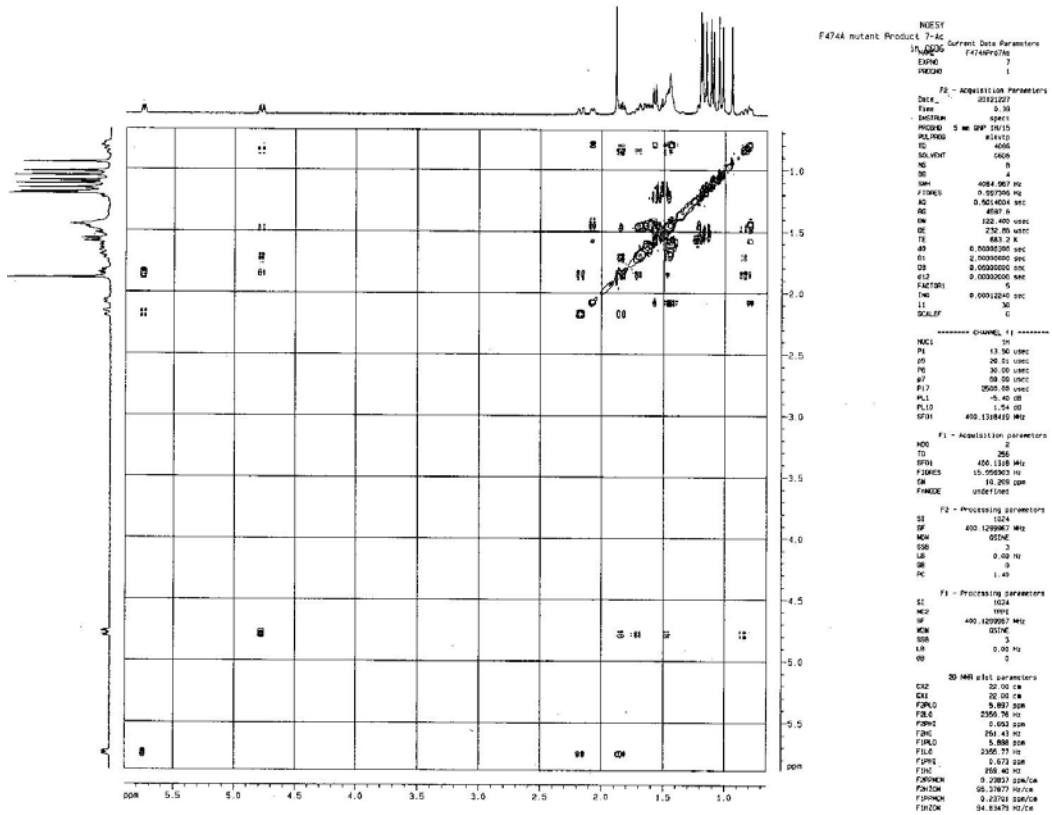
3.8.4.[13]C NMR in C_6D_6 (100 MHz)



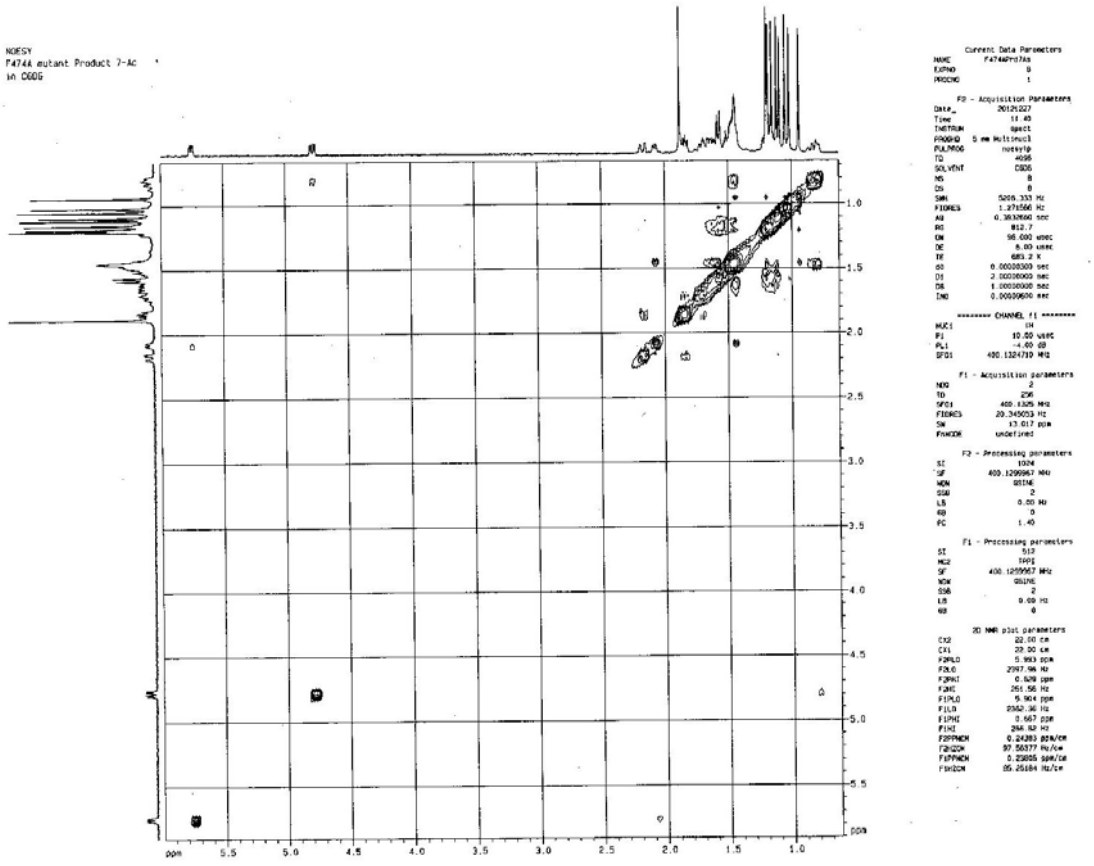
3.8.5. COSY spectrum in C_6D_6 (400 MHz)



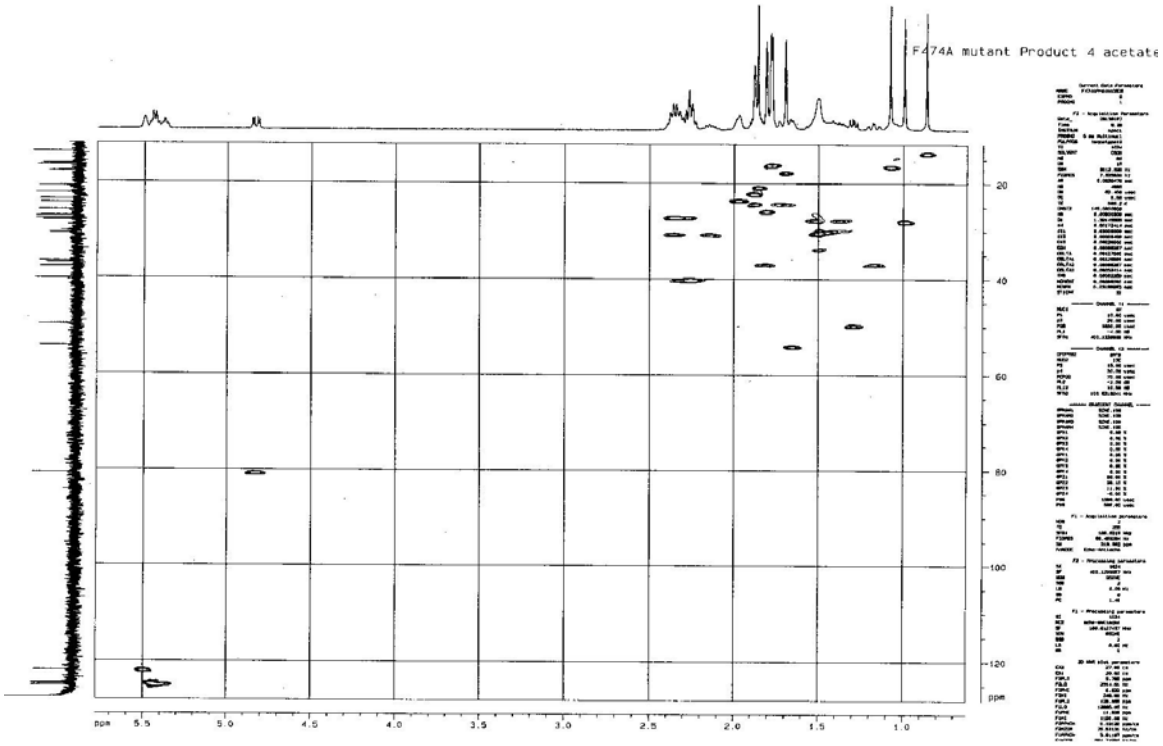
3.8.6 HOHAHA in C_6D_6 (400 MHz)



3.8.7. NOESY in C_6D_6 (400 MHz)



3.8.8. HSQC in C_6D_6 (400 MHz)



3.8.9. HMBC in C_6D_6 (400 MHz)

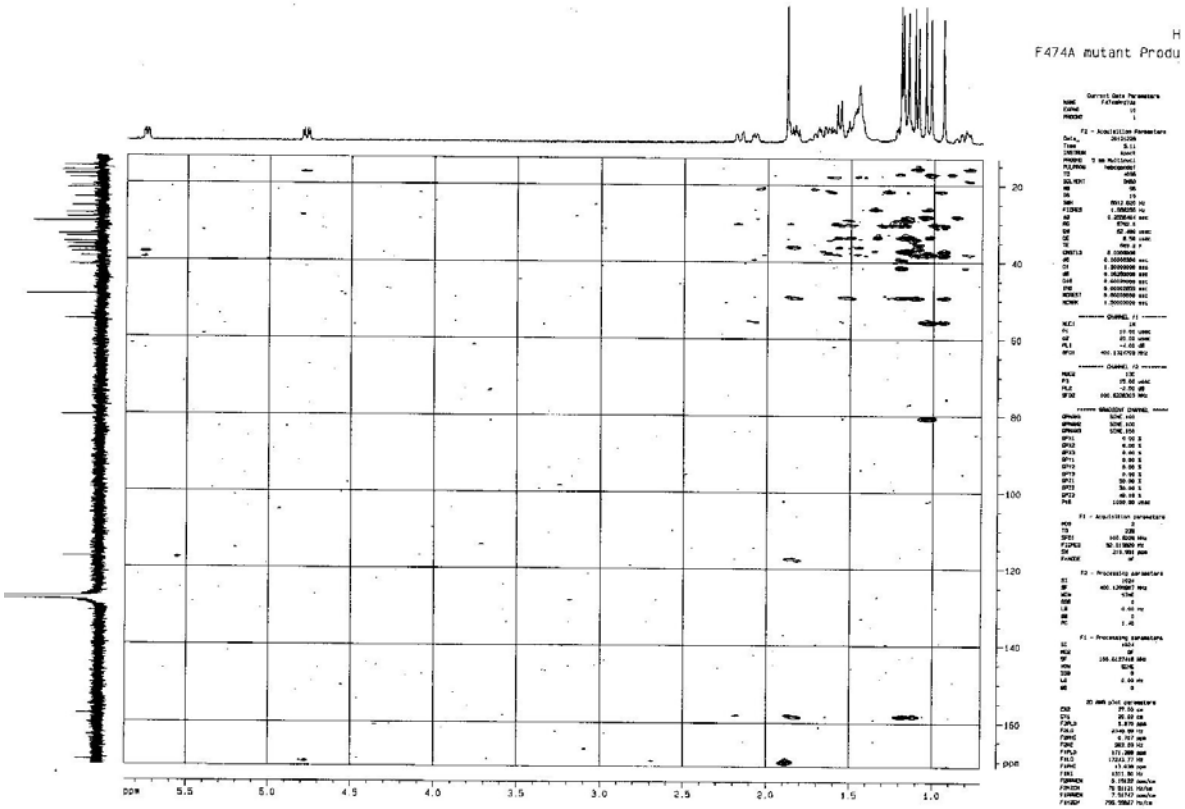
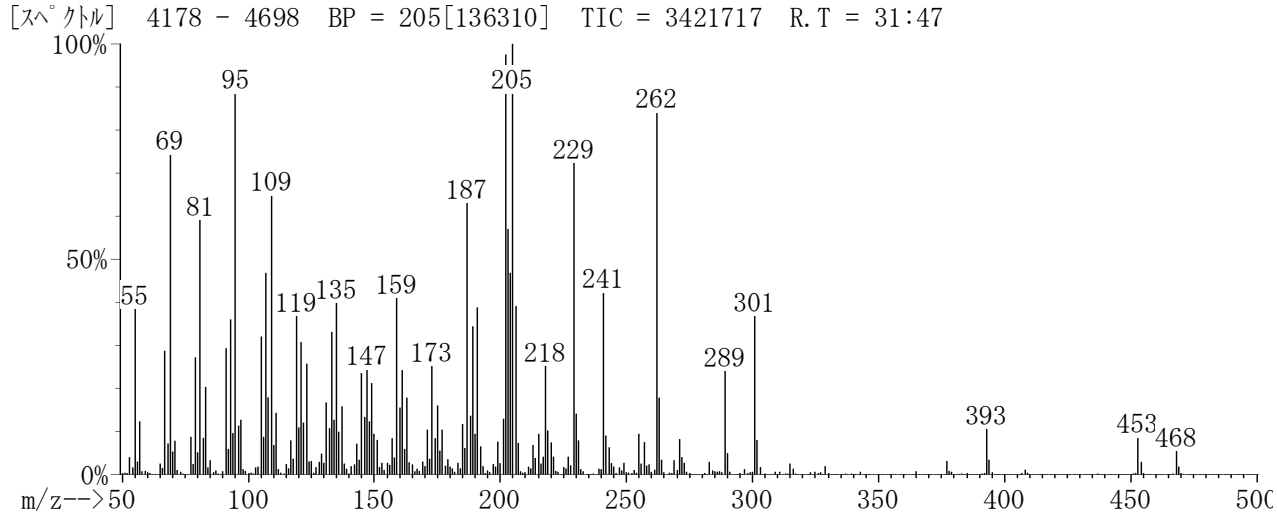


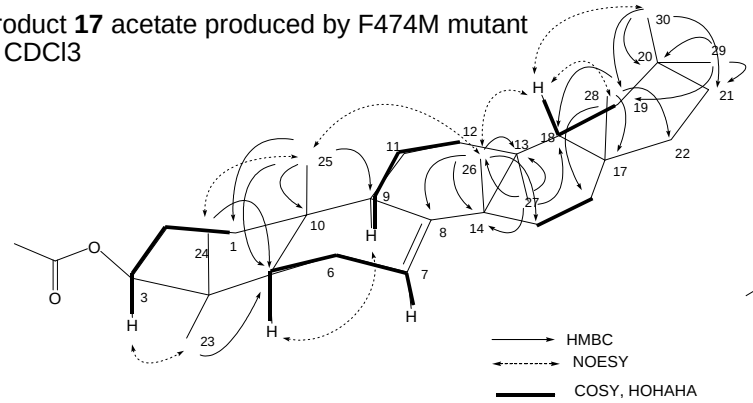
Figure S3.9. Product 17 acetate from F474A mutant

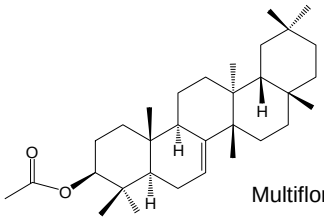
3.9.1. EIMS



3.9.2. NMR data analyses of **17** in $CDCL_3$ (600 MHz), $[\alpha]_D$, and HREIMS

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





$[\alpha]_D^{25}$= -16.9 (*c*=0.094, $CDCl_3$)
Lit. value $[\alpha]_D^{23}$= -8.7 (*c*=1.01, $CDCl_3$) for deaetylated compound **17.**
Sunil K. Talapatra et al,. Phytochemistry, 1989, 28, 1181-1185

HREIMS
calcd. 468.39673; observed: 468.39555

in $CDCl_3$

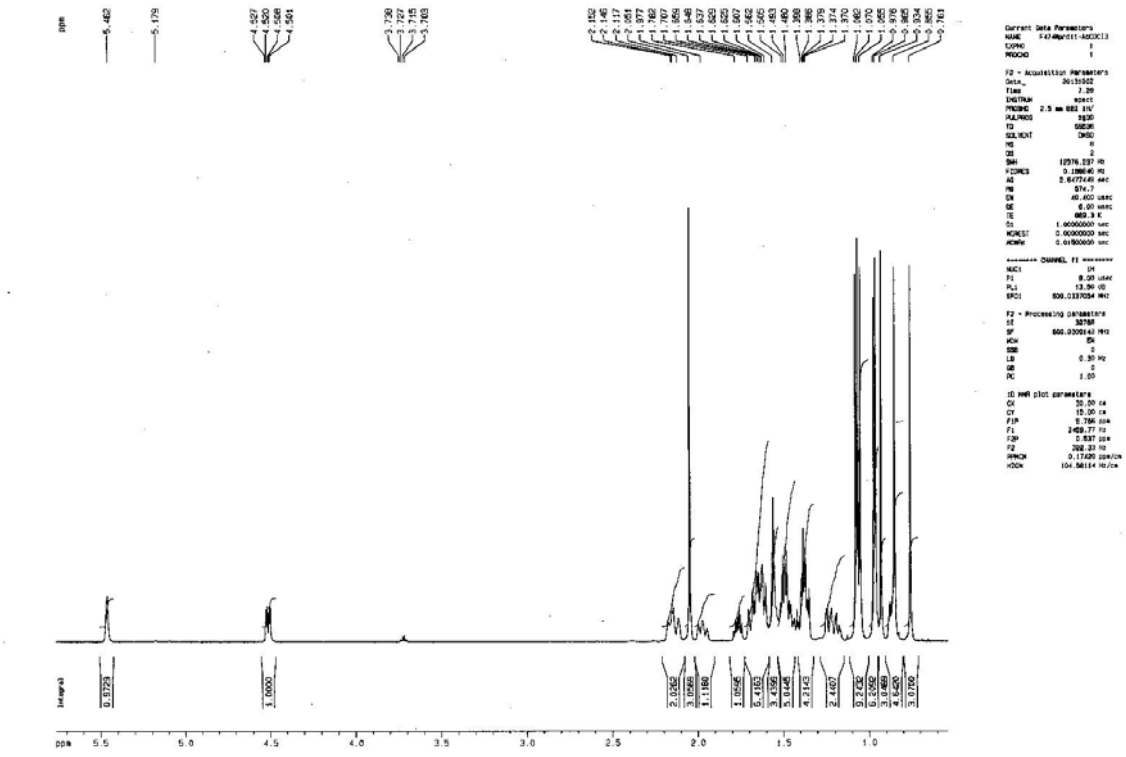
600 MHz, the solvent peak: δ_H=7.26, δ_C=77.0 ppm

The *13C signals* of Me-28 and C-17 were identical each other.

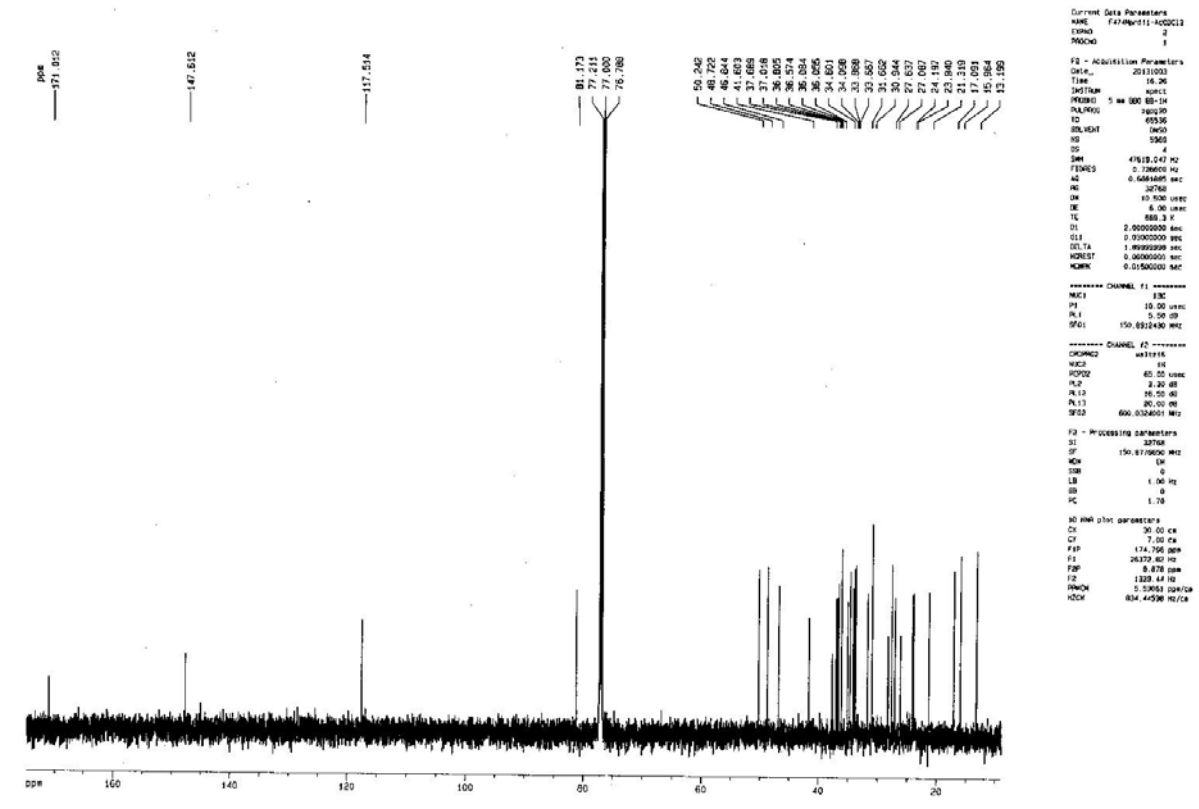
NO.	^{1}H	^{13}C	NO.	^{1}H	^{13}C	NO.	^{1}H	^{13}C	NO.	^{1}H	^{13}C
1	1.19 (m), 1.70(m)	**36.80 (t)**	**9**	2.16 (m)	**48.72(d)**	**17**	——	**30.94 (s)** *b*	**25**	0.761 (3H, s)	**13.20 (q)**
2	1.64 (2H,m)	**24.20(t)**	**10**	——	**35.06(s)**	**18**	1.50 (m)	**46.84 (d)**	**26**	1.070(3H, s)	**27.09(q)**
3	4.51(dd, J=11.4; 4.2 Hz)	**81.17(d)**	**11**	1.43 (m); 1.54(μ)	**17.09 (t)** *a*	**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..

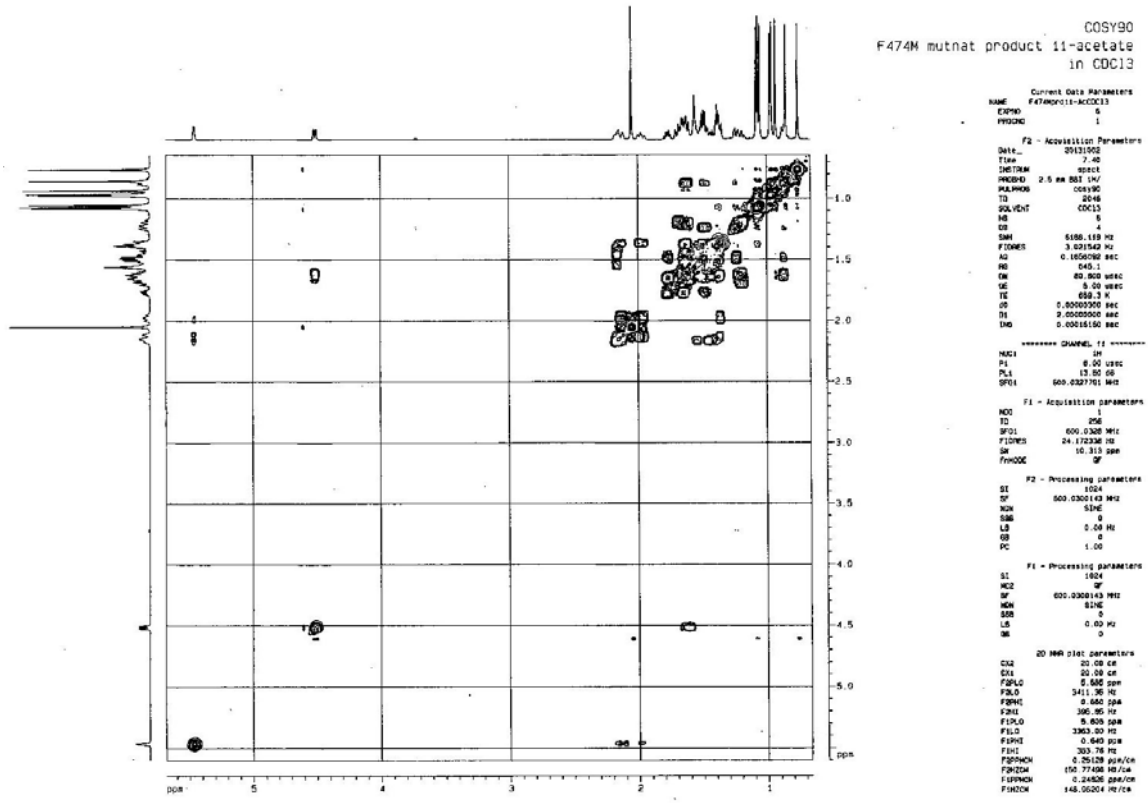
3.9.3. ^{1}H NMR in $CDCl_3$ (600 MHz)



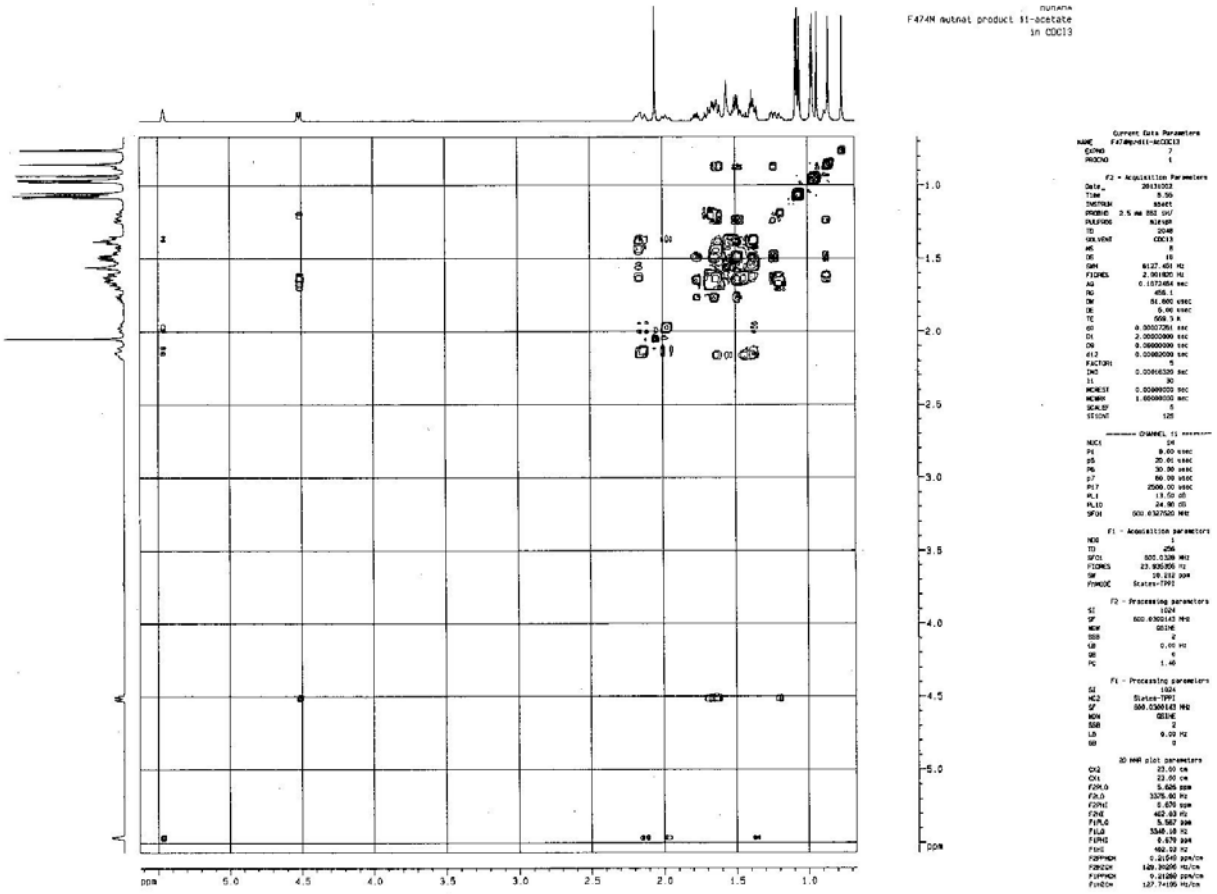
3.9.4. ^{13}C NMR in $CDCl_3$ (150 MHz)



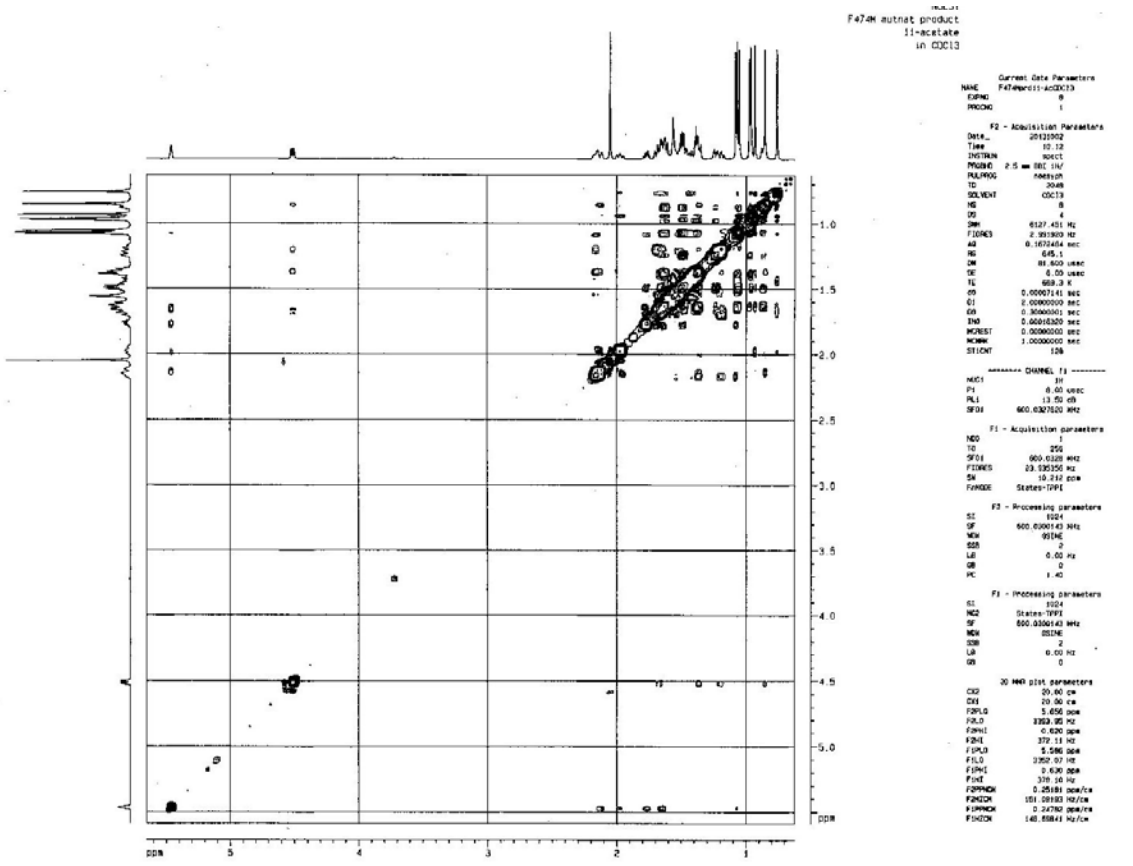
3.9.5.. COSY in $CDCl_3$ (600 MHz)



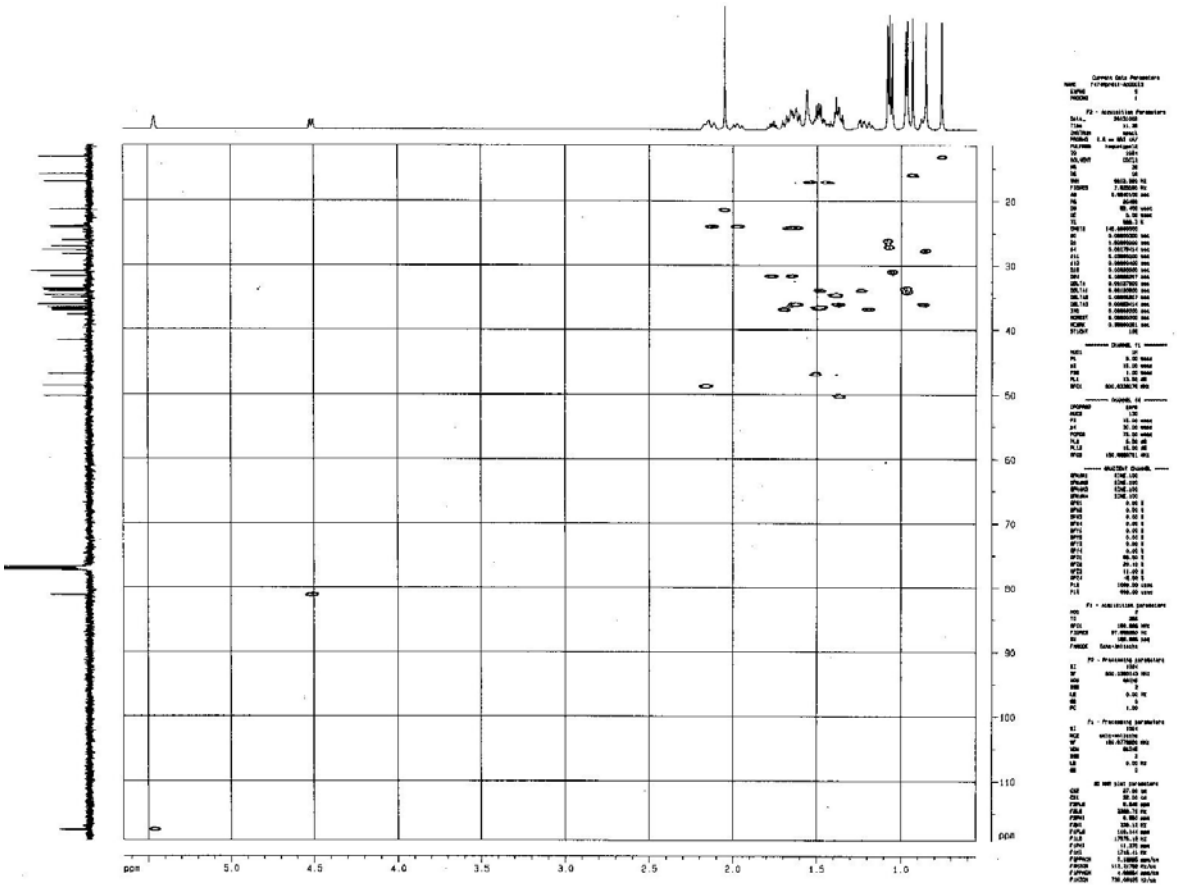
3.9.6. HOHAHA in CDCl3 (600 MHz)



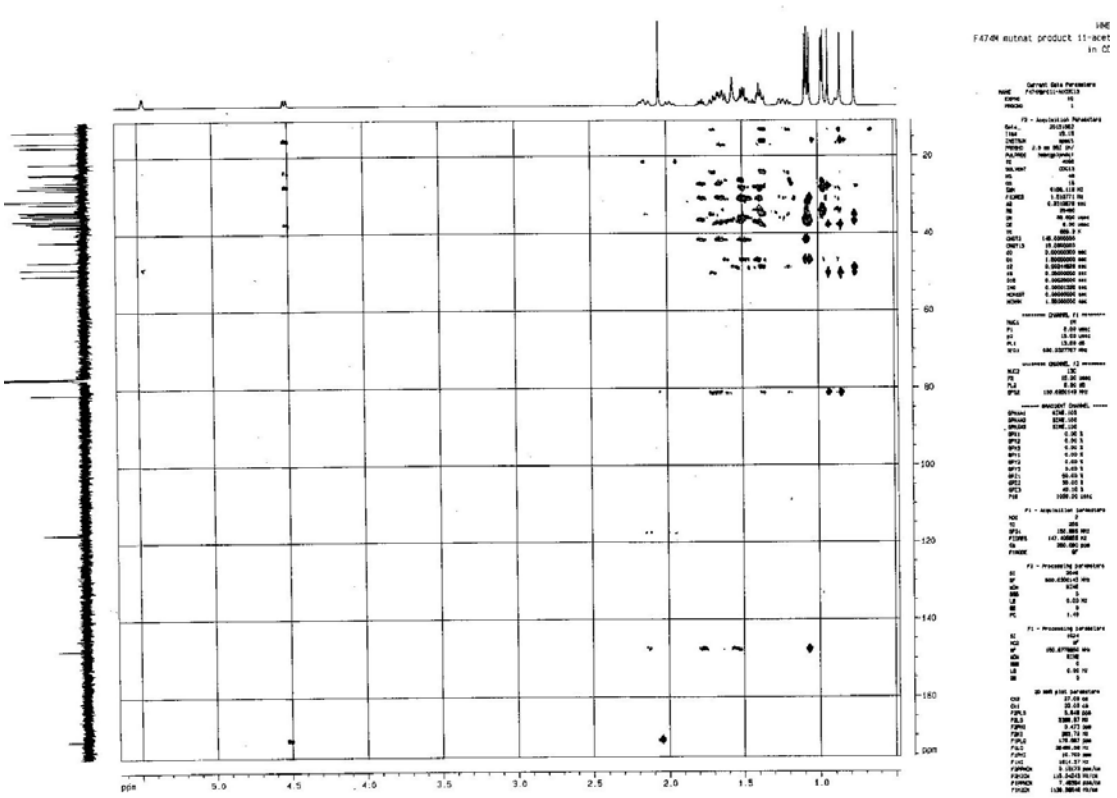
3.9.7. NOESY in CDCl3 (600 MHz)



3.9.8. HMBC .in CDCl3 (600 MHz)

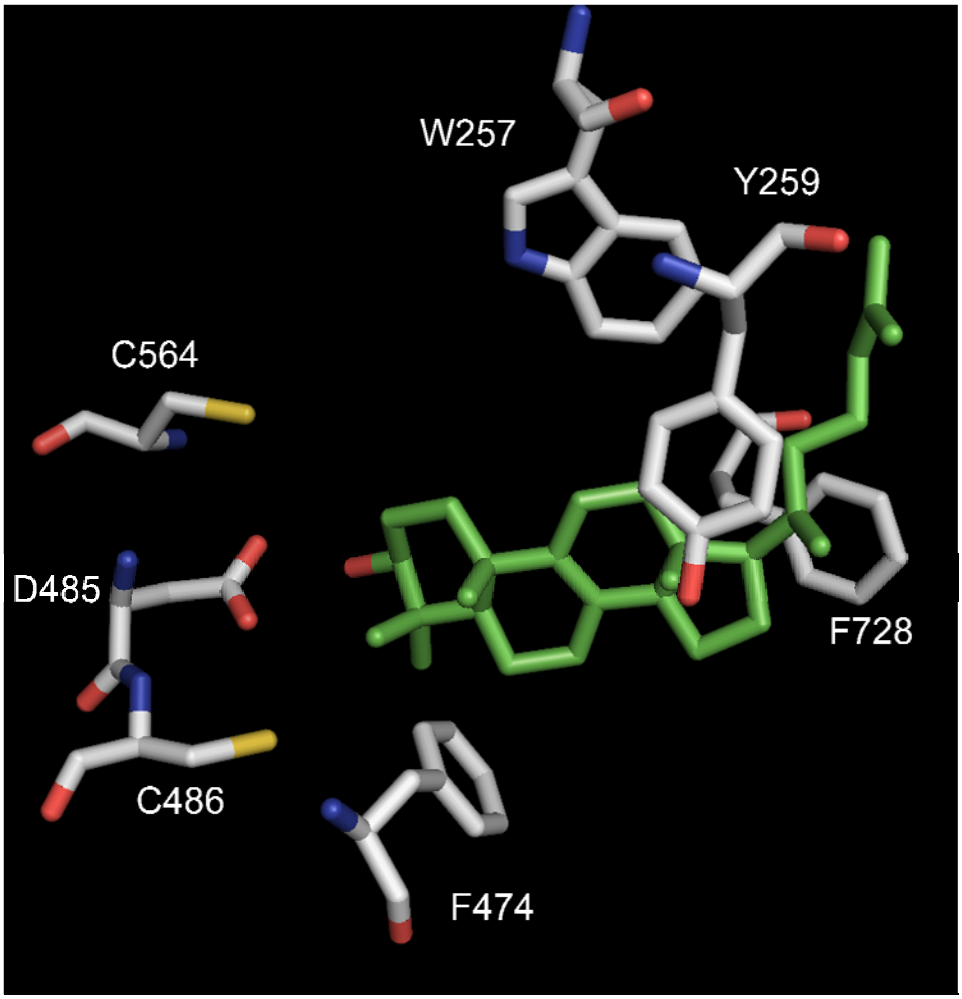


3.9.9. HMBC in $CDCl_3$ (600 MHz)



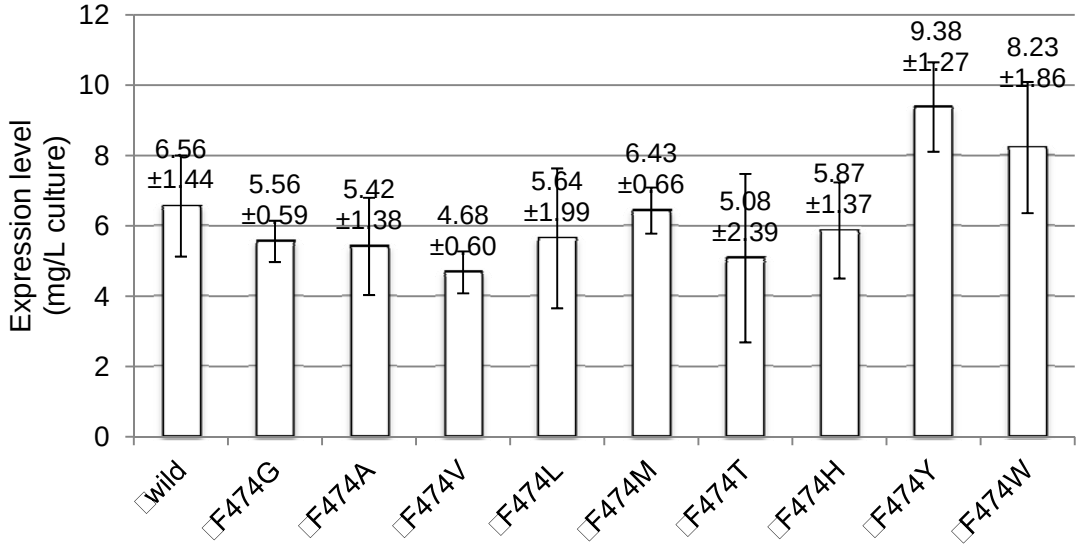
Supplementary Figure S4.

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 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.



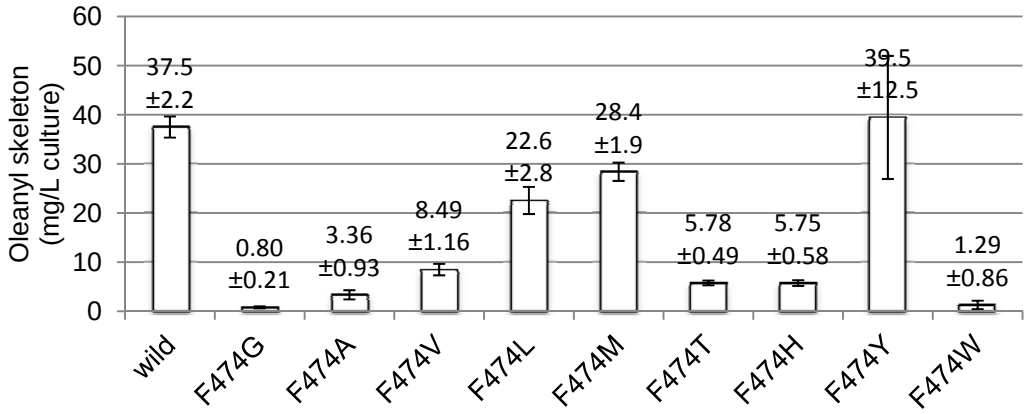
Lanosterol molecule is shown with green color. F728 residue is located in approximate to the D/E ring. We have reported the detailed functional analysis of F728 in the previous paper (R. Ito, I. Hashimoto, Y. Masukawa and T. Hoshino, *Chem. Eur. J*. **2013**, *19*, 17150-17158). By the mutagenesis experiments, we found that $D^{485}C^{486}$TA 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. Kushiro, 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.



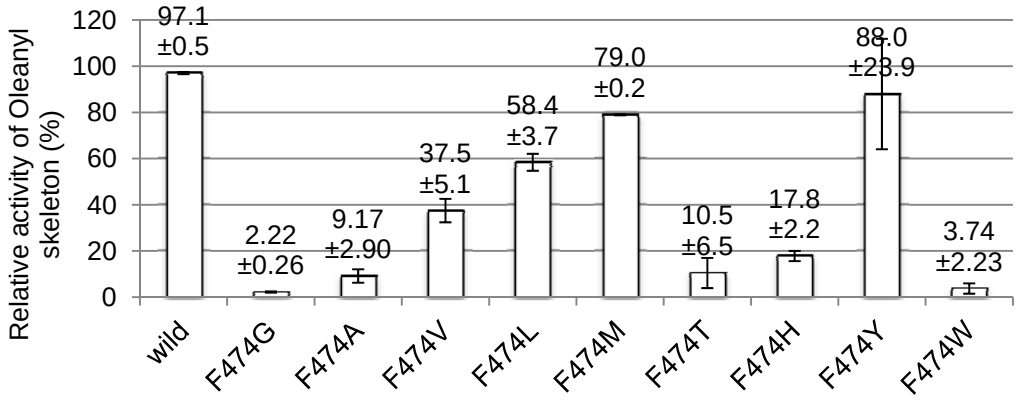
<Expression level of β-amyrin synthase (EtAS)>

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.



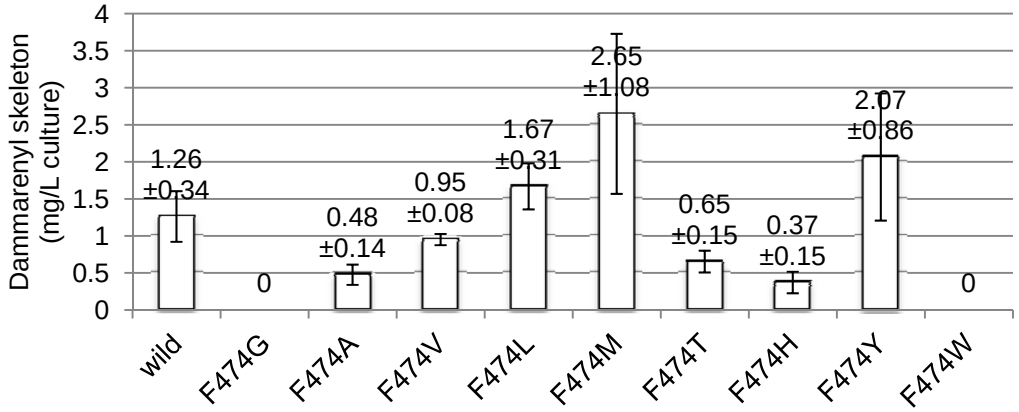
＜GC analyses for the quantities of oleanane-type triterpenes＞

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.



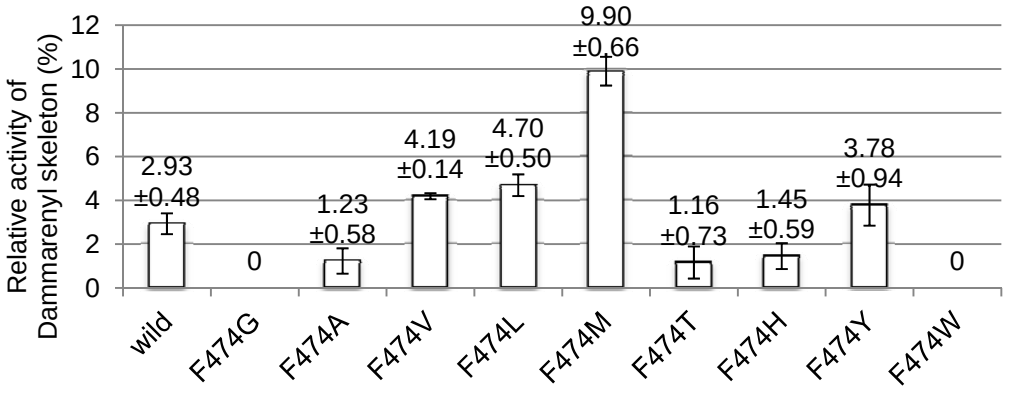
＜Enzyme activities for the production of oleanane-type triterpenes＞

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.



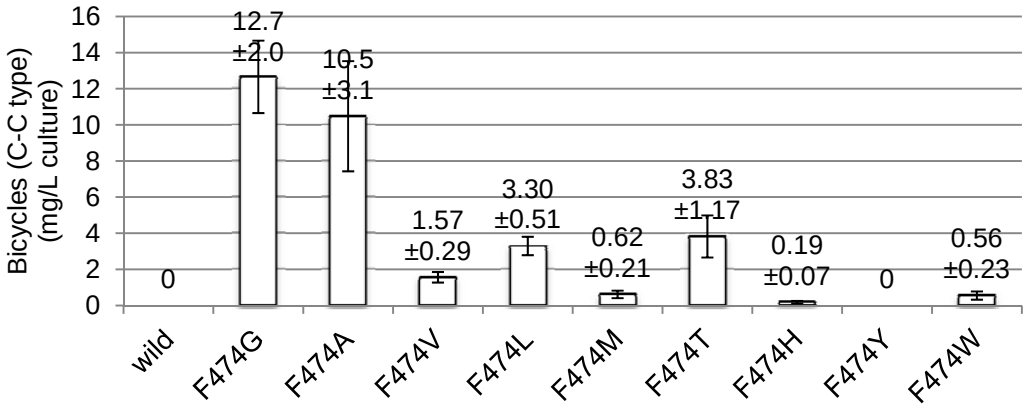
< GC analyses for the quantities of Dammarane-types>

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.



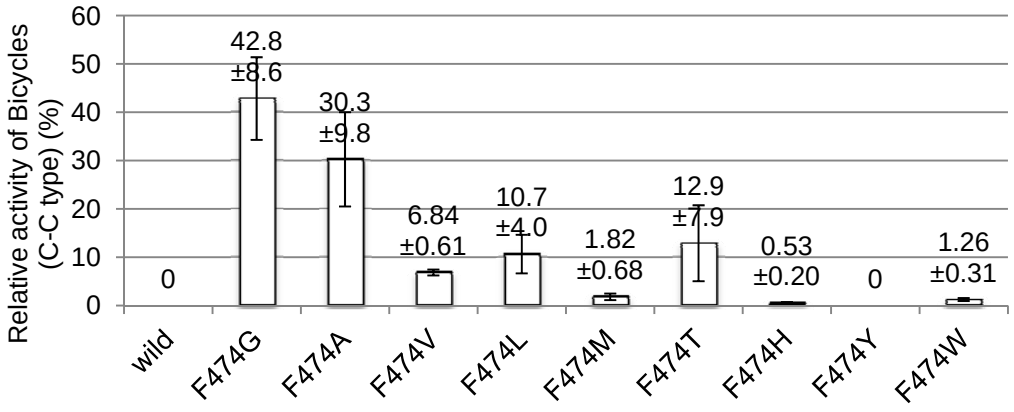
< Enzyme activities for the production of Dammarane-types>

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±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%.



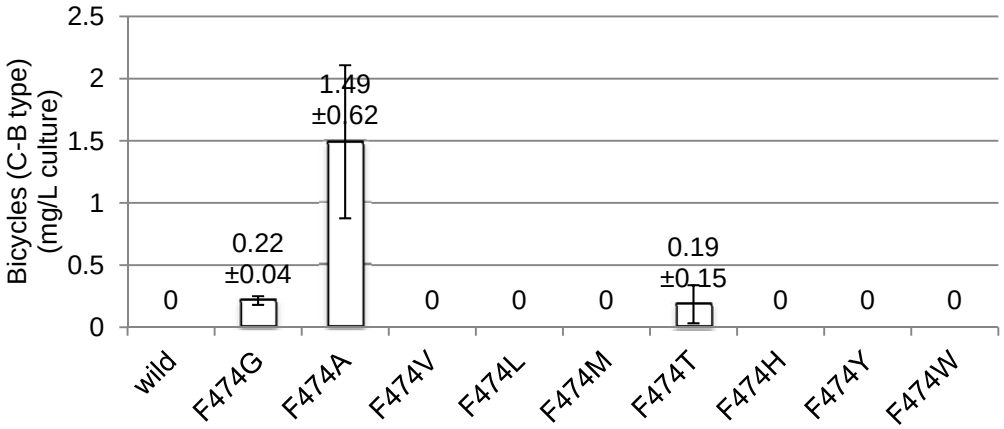
＜GC analyses for the quantities of Bicycles (C-C type)＞

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**.



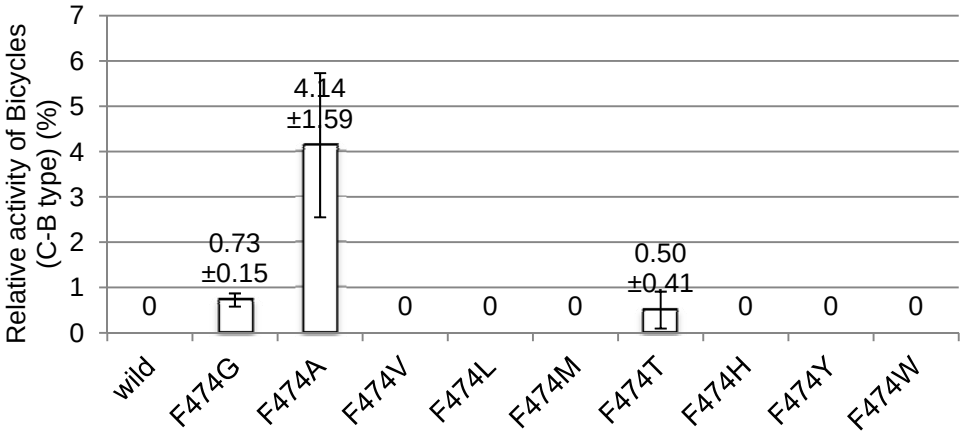
＜Enzyme activities for the production of Bicycles (C-C type)＞

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**.

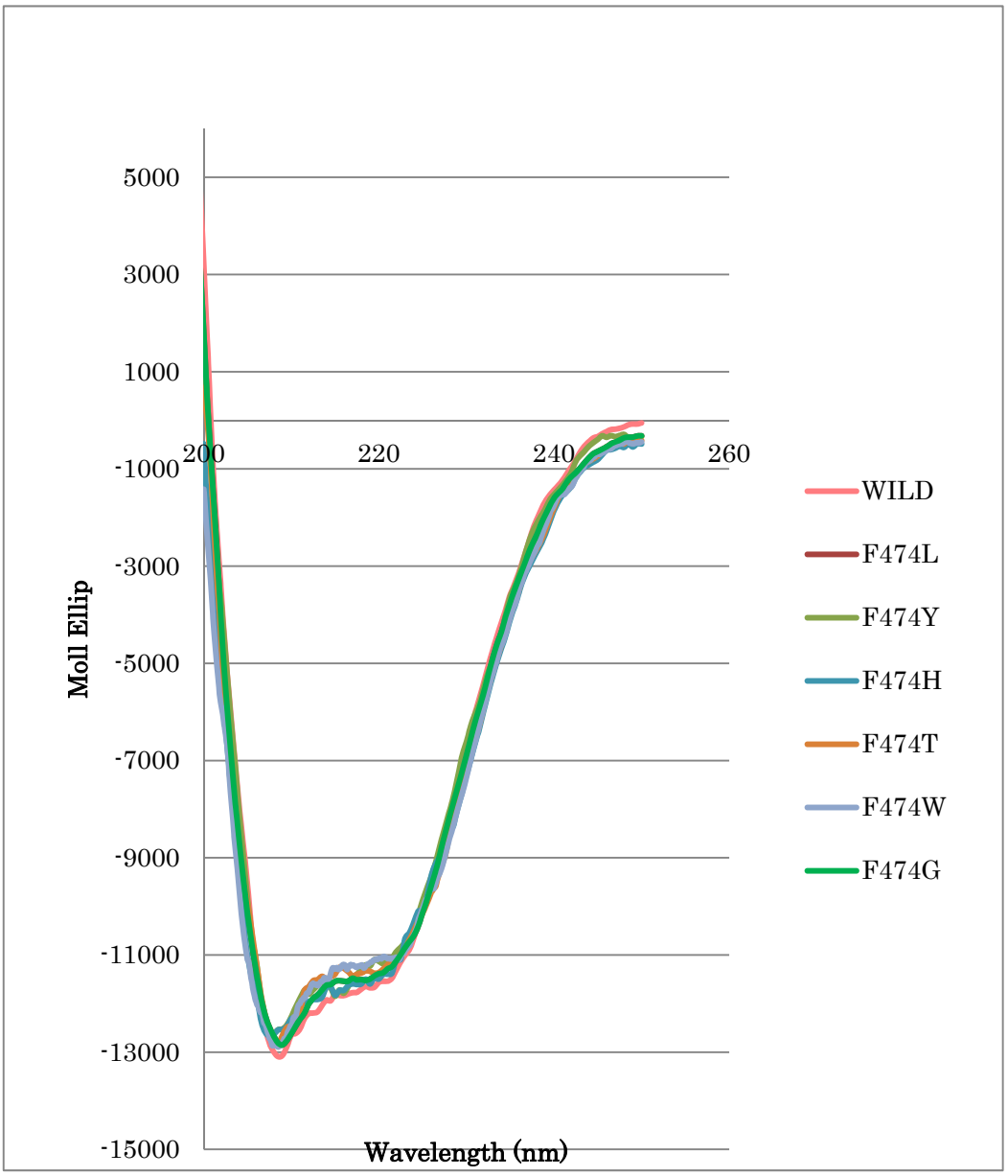


<Enzyme activities for the production of Bicycles (C-B)>

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.ttced.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	*Gemmata obscuriglobus*	
OSC_8	50896403	764	cucurbitadienol synthase[1]	*Cucurbita pepo*	dicot
	64310763	415	cycloartenol synthase	*Dioscorea zigiberensis*	monocot
OSC_9	108743267	761	oxidosqulane cyclase [2]	*Lotus japonicus*	dicot
	218198199	410	hypothetical	*Oryza sativa*	monocot
	47834395	757	β−amyrin synthase (AY698696)[3]	*Avena longiglumis*	monocot
	15866696	757	β−amyrin synthase (AY618699)[3,4]	*Avena strigosa*	monocot
	47834397	757	β−amyrin synthase (AY618698)[3]	*Avena prostrata*	monocot
	47834401	757	β−amyrin synthase (AY618700) [3]	*Avena ventricosa*	monocot
	47834391	757	β−amyrin synthase (AY618695) [3]	*Avena clauda*	monocot
	242096958	649	hypothetical	*Sorghum bicolor*	monocot
	195614202	760	cycloartenol synthase	*Zea mays*	monocot
	115485121	756	hypothetical (OsOSC5)[5]	*Oryza sativa*	monocot
	108864253	617	putative cycloartenol synthase	*Oryza sativa*	monocot
	218185593	551	hypothetical	*Oryza sativa*	monocot
	218185587	406	hypothetical	*Oryza sativa*	monocot
	6734726	548	similar to β−amyrin synthase	*Oryza sativa*	monocot
	115485113	760	OsOSC6[5]	*Oryza sativa*	monocot
	242084188	733	hypothetical	*Sorghum bicolor*	monocot
	218190027	656	hypothetical	*Oryza sativa*	monocot
	115485919	762	hypothetical (OsOSC4) [5]	*Oryza sativa*	monocot
	115444143	495	hypothetical	*Oryza sativa*	monocot

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