Electronic Supplementary Information (ESI)

Substrate specificity of Rv3378c, an enzyme from *Mycobacterium tuberculosis*, and the inhibitory activity of the bicyclic diterpenoids against macrophage phagocytosis

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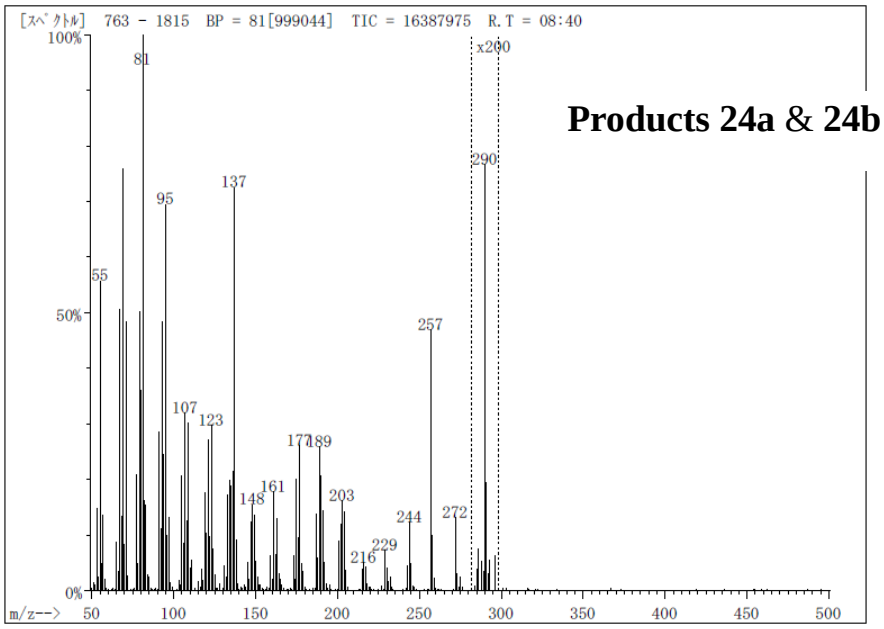
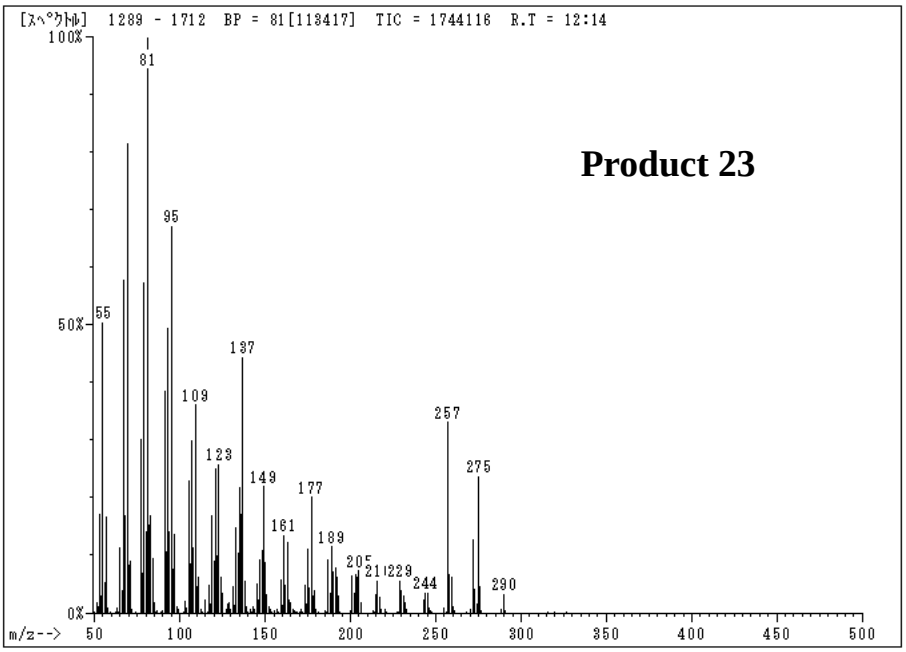
Fig. S1. EIMS spectra of enzymatic products 23-28

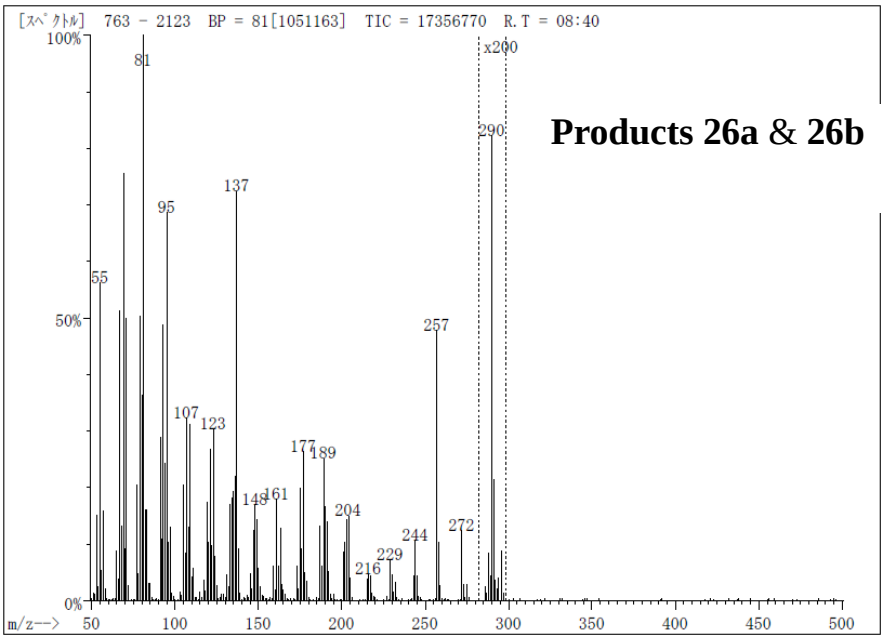
The GC and GC-MS traces were inseparable between **24a** and **24b**, between **26a** and **26b**, and between **28a** and **28b** in a ZB-5ms capillary column (30m, Zebron); see Fig. 3 in the text. However, a chiral GC column (CYCLOSILB GC capillary column) gave the complete separation between their diastereomers (see Fig. 5 in the text).

Products 23, 24a & 24b

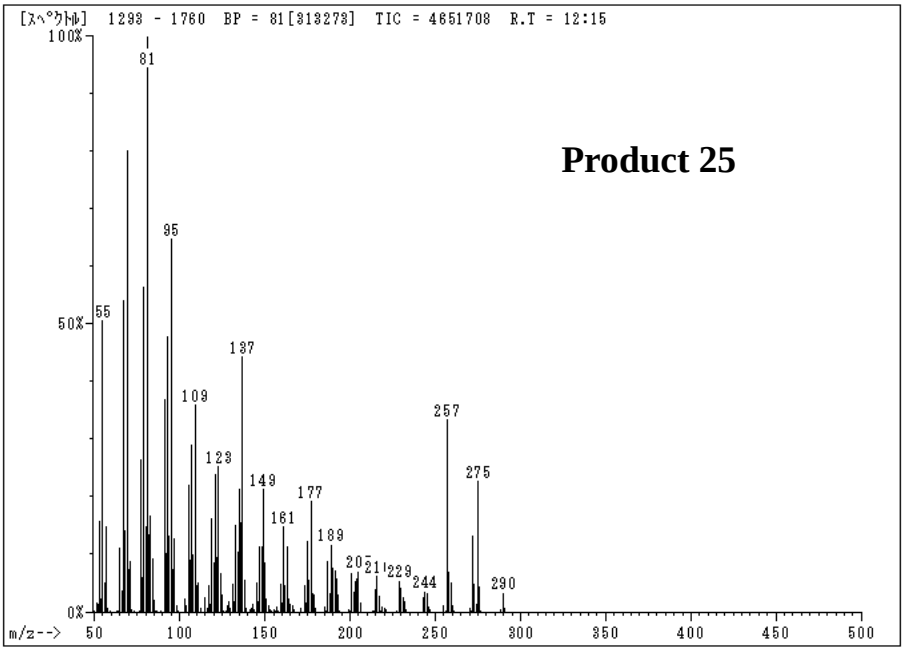




Products 25, 26a & 26b

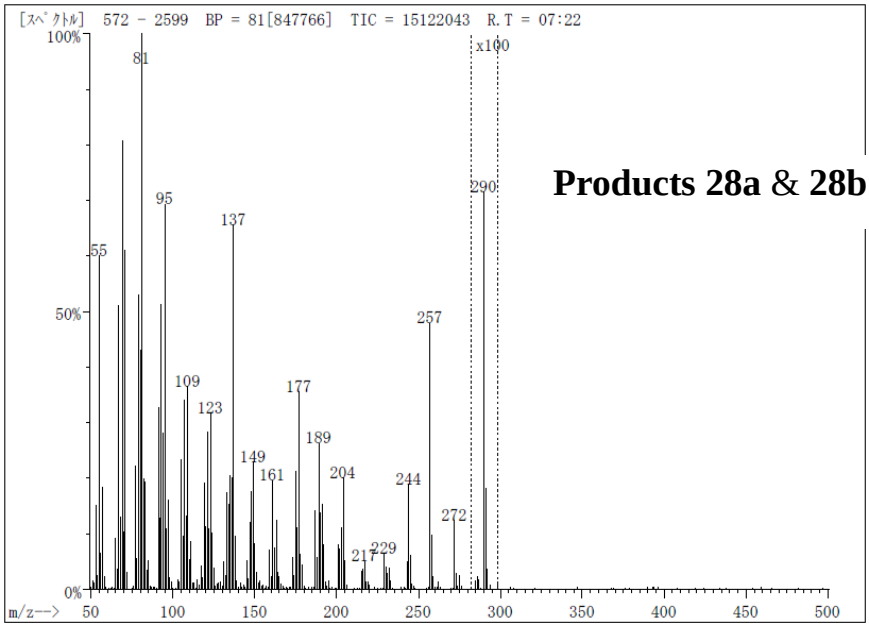


Products 26a & 26b



Product 25

Products 27, 28a & 28b



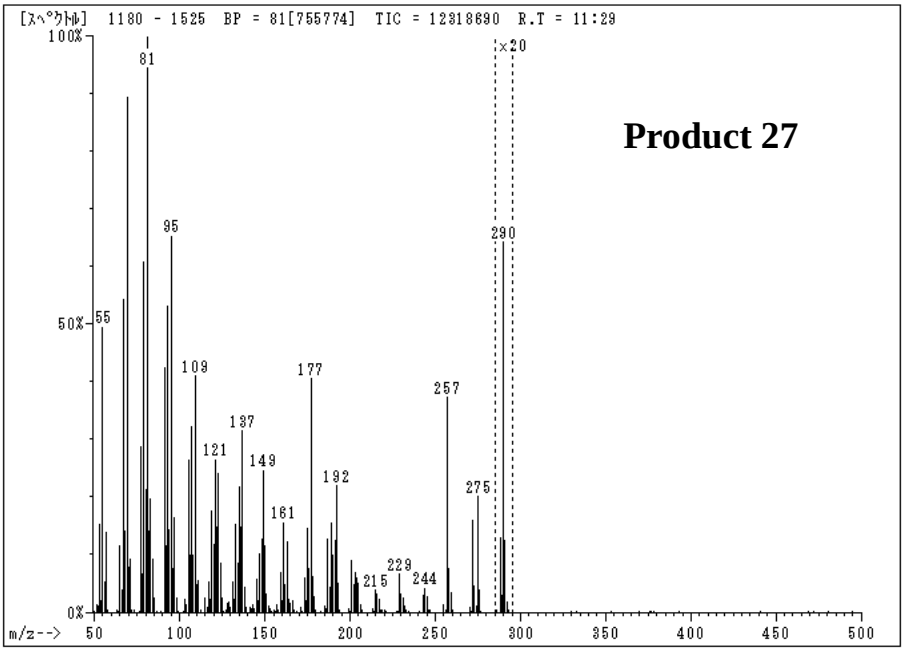
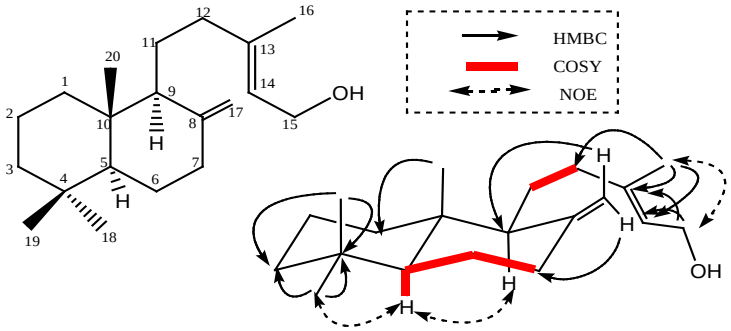


Fig. S2. NMR data of Product 23

copalol



Observed : 290.2601
Calculated : 290.2610

$C_{20}H_{34}O$

$[\alpha]_D^{24}$ + 30.6
(*c* 0.12, $CHCl_3$)

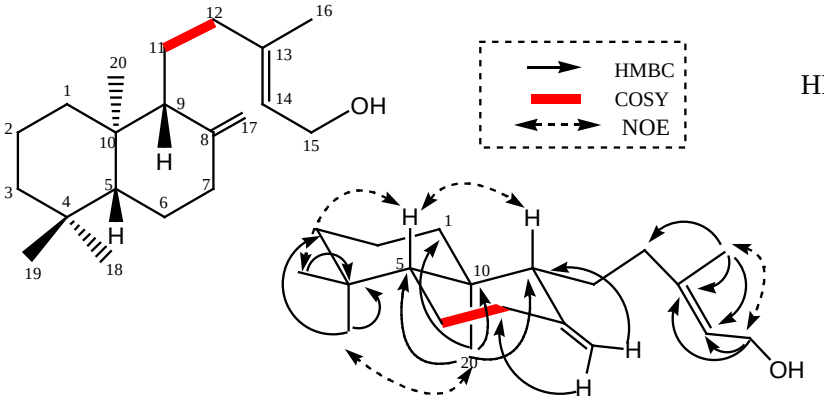
400 MHz in C_6D_6 referred to the solvent of C_6D_6 : ^1H ; 7.28ppm , ^{13}C ; 128.0ppm

	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.03(m);1.77(m)	**39.20**	**6**	1.38(m);1.73(m)	**24.74**	**11**	1.60(m);1.72(m)	**22.19**	**16**	1.65 (3H, s)	**16.30**
2	1.53 (2H, m);1.66(m)	**19.74**	**7**	2.07(m);2.50(m)	**38.68**[a]	**12**	1.97(m);2.35(m)	**38.79**[a]	**17**	5.07 (bs); 4.78 (bs)	**106.7**
3	1.24(m);1.48(m)	**42.39**	**8**	—	**148.7**	**13**	—	**139.0**	**18**	0.963 (3H, s)	**33.71**
4	—	**33.64**	**9**	1.69 (m)	**56.53**	**14**	5.57 (bt, *J*=6.8Hz)	**124.5**	**19**	0.922 (3H, s)	**21.89**
5	1.10 (dd, *J*=12.8, 2.8Hz)	**55.60**	**10**	—	**39.87**	**15**	4.13 (2H, d, *J*=6.4Hz)	**59.37**	**20**	0.855 (3H, s)	**14.74**

a: The assignments of C-7 and C-12 may be interchangeable due to the close chemical shifts.

Fig. S3. NMR data of Product 25

***ent*-copalol**



HREIMS M^+

Observed : 290.2614
Calculated : 290.2610

$C_{20}H_{34}O$

$[\alpha]_D^{22}$ - 30.0
(*c* 0.11, $CHCl_3$)

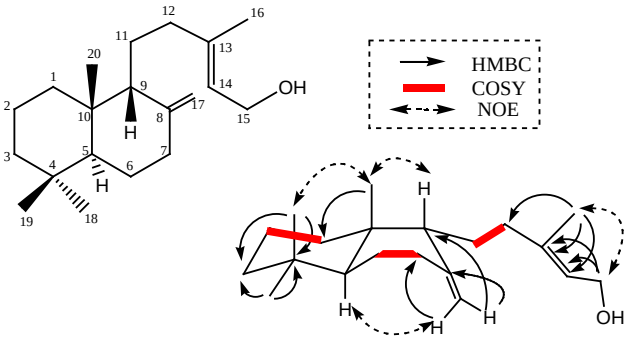
400 MHz in C_6D_6 relative to C_6D_6 : ^1H ; 7.28ppm , ^{13}C ; 128.0ppm

	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.02 (m);1.76 (m)	**39.19**	**6**	1.37 (m); 1.74 (m)	**24.73**	**11**	1.62 (m); 1.73 (m)	**22.18**	**16**	1.65 (3H, s)	**16.29**
2	1.52 (m);1.66(m)	**19.74**	**7**	2.07 (m); 2.51 (m)	**38.67**[a]	**12**	1.98 (m); 2.33 (m)	**38.79**[a]	**17**	5.07 (bs); 4.78 (bs)	**106.7**
3	1.25 (m); 1.48 (m)	**42.38**	**8**	—	**148.7**	**13**	—	**139.0**	**18**	0.962 (3H, s)	**33.71**
4	—	**33.64**	**9**	1.68 (m)	**56.53**	**14**	5.57 (bt, *J*=6.7 Hz)	**124.5**	**19**	0.921 (3H, s)	**21.89**
5	1.10 (dd, *J*=12.4, 2.8 Hz)	**55.59**	**10**	—	**39.86**	**15**	4.12 (2H, d, *J*=6.7 Hz)	**59.36**	**20**	0.854 (3H, s)	**14.73**

a: The assignments of C-7 and C-12 may be interchangeable due to the close chemical shifts.

Fig. S4. NMR data of Product 27

***syn*-copalol**



HREIMS M^+

Observed : 290.2617
Calculated : 290.2610

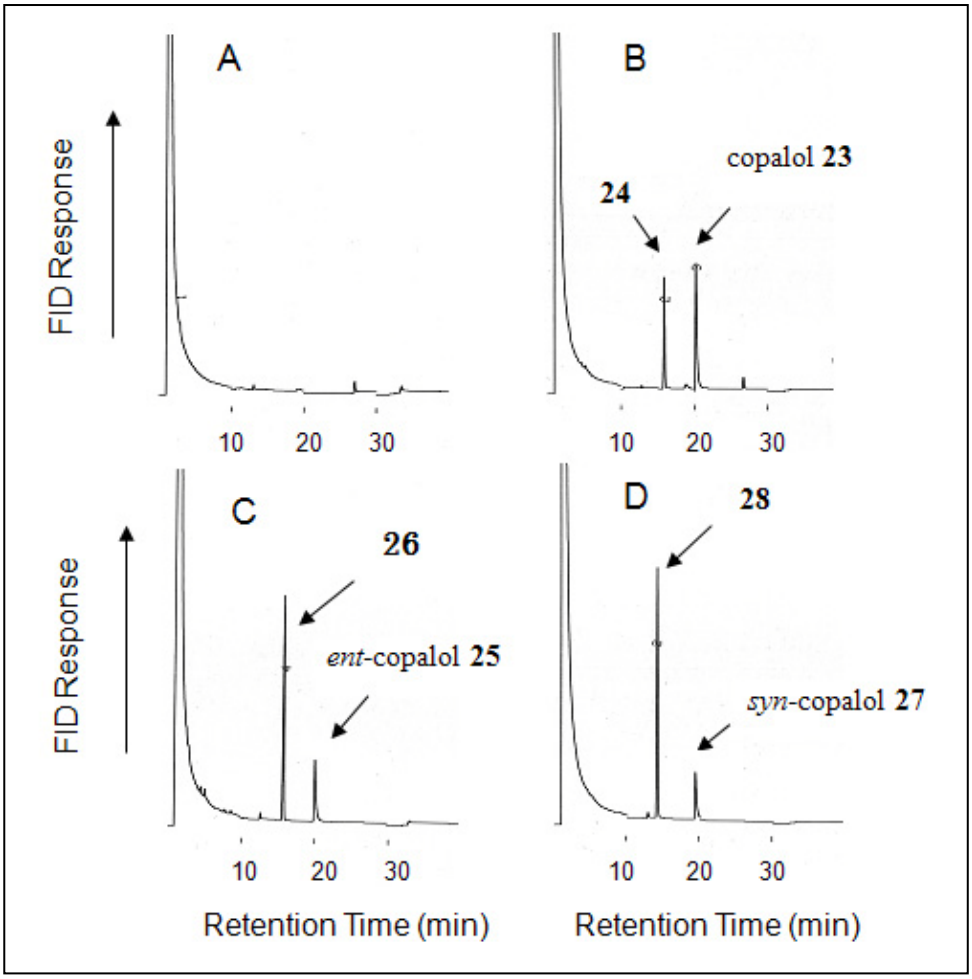
$C_{20}H_{34}O$

$[\alpha]_D^{24}$ + 16.2
(*c* 0.17, $CHCl_3$)

400 MHz in C_6D_6 referred to C_6D_6 : ^1H, 7.28ppm; ^{13}C, 128.0ppm

	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.68 (m);1.14 (m)	**37.06**	**6**	1.65 (m); 1.38 (m)	**23.98**	**11**	1.81 (m); 1.58 (m)	**24.79**	**16**	1.631 (3H, s)	**16.39**
2	1.72 (m); 1.55 (m)	**19.52**	**7**	2.24 (m); 2.14 (m)	**31.86**	**12**	2.09 (m); 1.91 (m)	**38.52**	**17**	4.94 (bs); 4.76 (bs)	**110.0**
3	1.50 (m); 1.26 (m)	**42.94**	**8**	—	**149.3**	**13**	—	**138.9**	**18**	0.935 (3H, s)	**22.33**
4	—	**33.32**	**9**	1.66 (m)	**58.11**	**14**	5.57 (bs)	**124.6**	**19**	0.990 (3H, s)	**33.67**
5	1.41 (m)	**46.14**	**10**	—	**38.27**	**15**	4.15 (2H, d, *J*=5.6)	**59.38**	**20**	1.112 (3H, s)	**22.63**

Fig. S5. GC trace of the enzymatic products for testing the substrate specificity of the Rv 3378c enzyme. The incubation mixtures were extracted with hexane. An excess of Triton X-100 included in the hexane-extracts was removed by passing a short SiO_2 column (hexane:EtOAc=100:30). The incubation mixtures were not subjected to phosphatase treatment. Thus, **23**, **25** and **27** were the true enzymatic products, but not the chemical artifacts (see Text).



A: Incubation mixture of GGPP **1** with a mixture of terpentedienyl-PP (**6**) synthase (CYC1) and the Rv3378c enzyme: no reaction

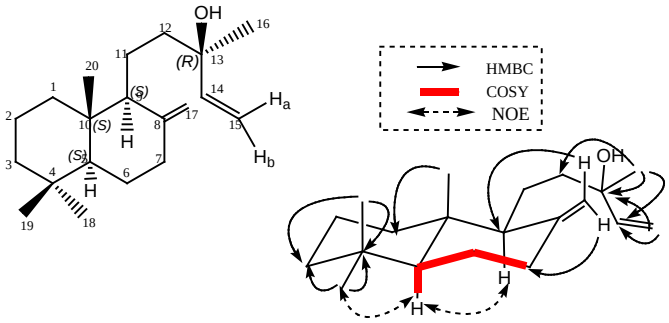
B: Incubation mixture of **1** with a mixture of (+)-copalyl-PP (**7**) synthase and the Rv3378c enzyme

C: Incubation mixture of **1** with a mixture of (-)-*ent*-copalyl-PP (**8**) synthase and the Rv3378c enzyme

D: Incubation mixture of **1** with a mixture of *syn*-copalyl-PP (**9**) synthase with the Rv3378c enzyme.

Fig, S6. NMR data of Product 24a (manool, 13*R*).

manool (13*R*) from CDP+Rv3378c



[a] $_D^{24}$=+26.9
(c=0.05,$CHCl_3$)

400 MHz in C_6D_6 relative to C_6D_6 : ^1H ; 7.28ppm , ^{13}C ; 128.0ppm

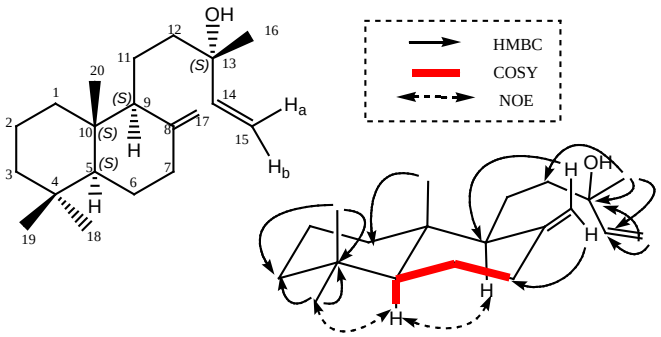
	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.84 (m);1.05 (m)	**39.22***	**6**	1.75 (m); 1.40 (m)	**24.71***	**11**	1.74(m);1.59(m)	**18.01***	**16**	1.257 (3H, s)*	**28.53***
2	1.65(m); 1.54 (m)	**19.73***	**7**	2.51 (bd, *J*=12.8Hz); 2.08 (ddd, *J*=12.8, 12.8, 4.8 Hz)	**38.70**	**12**	1.88 (m); 1.40 (m)	**41.74***	**17**	5.08 (bs);4.83 (bs)*	**106.9***
3	1.48 (m); 1.26 (m)	**42.45**	**8**	—	**148.8**	**13**	—	**73.24***	**18**	0.973 (3H, s)	**33.74**
4	—	**33.64**	**9**	1.62 (m)	**57.48**	**14**	5.94 (dd, *J*=17.2, 10.8)*	**145.7***	**19**	0.916 (3H, s)	**21.88**
5	1.10 (bd, *J*=12.4 Hz)	**55.65***	**10**	—	**40.08**	**15**	Ha 5.34 (dd, *J*=17.2, 1.6)* Hb 5.10 (dd, *J*=10.8, 1.6)*	**111.4***	**20**	0.857 (3H, s)*	**14.68**

The symbol * indicates that the chemical shifts are separable between 13*R* and 13*S*-manool

The ^1H and ^{13}C chemical shifts listed here are derived from authentic **24a**, purchased from Industrial Research Limited (New Zealand).

Fig. S7. NMR data of Product 24b (13-*epi*-manool, 13*S*)

13-epi-manool (13*S*) from CDP+Rv3378c



HREIMS M^+

Observed : 290.2605
Calculated : 290.2610

$C_{20}H_{32}O$

$[\alpha]_D^{24}$ + 31.5
(*c* 0.08, $CHCl_3$)

400 MHz in C_6D_6 referred to C_6D_6: ^1H, 7.28ppm; ^{13}C, 128.0ppm

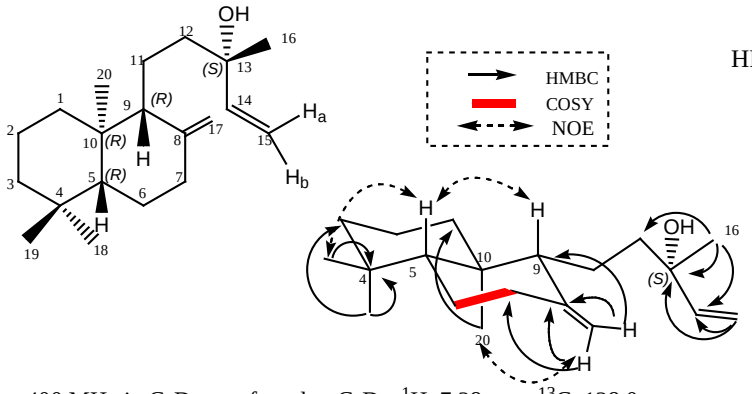
	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.84 (m);1.05 (m)	**39.23***	**6**	1.75 (m); 1.40 (m)	**24.71***	**11**	1.74(m);1.59(m)	**18.07***	**16**	1.264 (3H, s) *	**28.22***
2	1.65(m); 1.54 (m)	**19.75***	**7**	2.51 (bd, *J*=12.8Hz); 2.08 (ddd, *J*=12.8, 12.8, 4.8 Hz)	**38.70**	**12**	1.90 (m); 1.38 (m)	**41.80***	**17**	5.08 (s); 4.88 (s)*	**107.1***
3	1.48 (m); 1.26 (m)	**42.45**	**8**	—	**148.7**	**13**	—	**73.14***	**18**	0.973 (3H, s)	**33.74**
4	—	**33.64**	**9**	1.62 (m)	**57.53***	**14**	5.93 (dd, *J*=17.2, 10.8)*	**145.9***	**19**	0.916 (3H, s)	**21.88**
5	1.10 (bd, *J*=12.4 Hz)	**55.68***	**10**	—	**40.09**	**15**	Ha 5.32 (dd, *J*=17.2, 1.6) * Hb 5.09 (dd, *J*=10.8, 1.6) *	**111.2***	**20**	0.857 (3H, s)	**14.68**

The symbol * indicates that the chemical shifts are separable between 13*R* and 13*S*-manool.

Fig. S8. NMR data of Product 26b

Product 25b (major product from *ent*-CDP + Rv3378c

13(*S*)-*ent*-manool



HREIMS M^+

Observed : 290.2605
Calculated : 290.2610

$C_{20}H_{34}O$

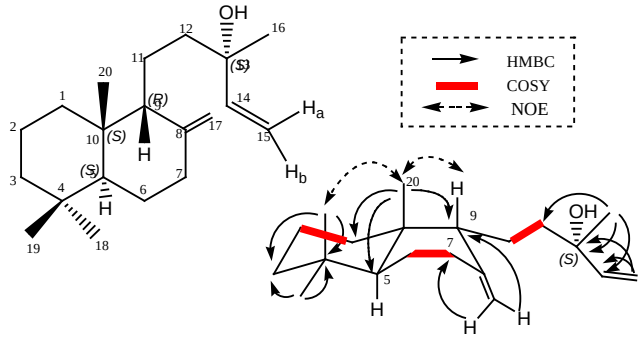
$[\alpha]_D^{20}$ - 32.1
(*c* 0.1, $CHCl_3$)

400 MHz in C_6D_6 referred to C_6D_6: ^1H, 7.28ppm; ^{13}C, 128.0ppm

	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.84 (m);1.03 (m)	**39.22**	**6**	1.75 (m); 1.39 (m)	**24.74**	**11**	1.73(m); 1.55(m)	**18.02**	**16**	1.256 (3H, s)	**28.54**
2	1.65(m); 1.56 (m)	**19.74**	**7**	2.51 (bd, *J*=12.8Hz); 2.08 (ddd, *J*=12.8, 12.8, 4.8 Hz)	**38.70**	**12**	1.85 (m); 1.40 (m)	**41.74**	**17**	5.07 (bs); 4.83 (bs)	**106.9**
3	1.47 (m); 1.25 (m)	**42.45**	**8**	—	**148.8**	**13**	—	**73.25**	**18**	0.971 (3H, s)	**33.75**
4	—	**33.65**	**9**	1.62 (m)	**57.49**	**14**	5.94 (dd, *J*=17.2, 10.8)	**145.8**	**19**	0.915 (3H, s)	**21.89**
5	1.10 (bd, *J*=12.4 Hz)	**55.66**	**10**	—	**40.08**	**15**	Ha 5.34 (dd, *J*=17.2, 1.6) Hb 5.10 (dd, *J*=10.8, 1.6)	**111.4**	**20**	0.857 (3H, s)	**14.68**

Fig. S9. NMR data of Product 28b (major product; 13*S*-vitexifolin A), which was produced from *syn*-CDP and the Rv3378c enzyme

13*S*-Vitexifolin A



HREIMS M^+

Observed : 290.2619
Calculated : 290.2610

$C_{20}H_{34}O$

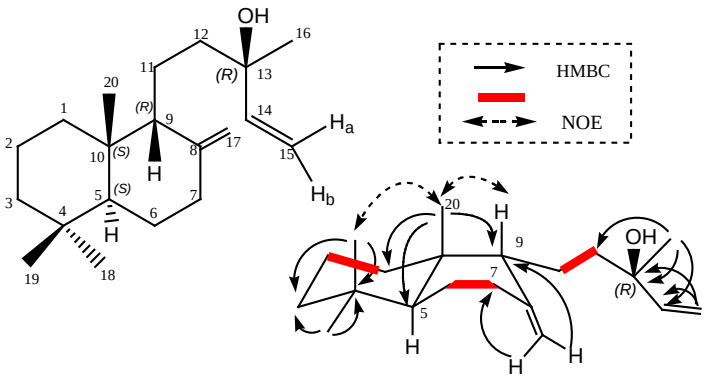
$[\alpha]_D^{20}$ + 15.1
(*c* 0.13, $CHCl_3$)

400 MHz in C_6D_6 relative to C_6D_6 : ^1H ; 7.28ppm , ^{13}C ; 128.0ppm

	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.75 (m);1.18 (m)	**37.08**	**6**	1.63 (m); 1.35 (m)	**24.02**	**11**	1.87(m); 1.61(m)	**20.41**	**16**	1.246 (3H, s)	**28.73**
2	1.75 (m); 1.52(m)	**19.54**	**7**	2.26 (2H, m)	**31.92**	**12**	1.60 (m); 1.37 (m)	**41.35**	**17**	4.93 (t, *J=2.2*); 4.75 (m)	**109.7**
3	1.46 (m); 1.23 (m)	**42.82**	**8**	—	**149.6**	**13**	—	**72.91**	**18**	0.927 (3H, s)	**22.36**
4	—	**33.30**	**9**	1.62 (m)	**58.76**	**14**	5.89 (dd, *J*=17.2, 10.8)	**145.8**	**19**	0.948 (3H, s)	**33.60**
5	1.45 (m)	**46.10**	**10**	—	**38.39**	**15**	Ha 5.31(dd, *J*=17.2, 1.6) Hb 5.06 (dd, *J*=10.8, 1.6)	**111.3**	**20**	1.124 (3H, s)	**22.70**

Fig. S10. NMR data of 13*R*-vitexifolin A 28a, gifted by Prof. Ono

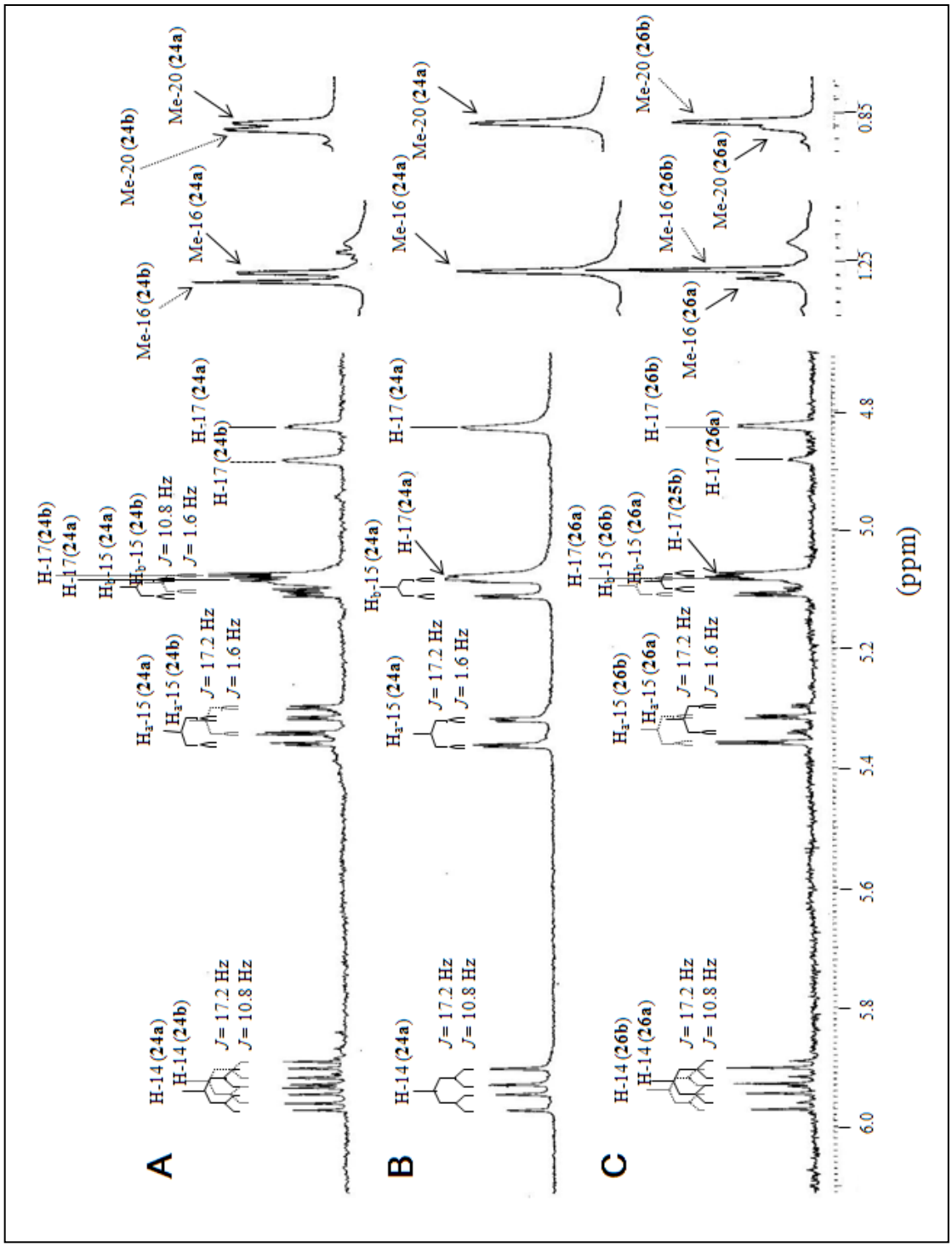
13*R*-vitexifolin A



400 MHz in C_6D_6 relative to C_6D_6 : ^1H ; 7.28ppm , ^{13}C ; 128.0ppm

	^1H	^{13}C		^1H	^{13}C		^1H	^{13}C		^1H	^{13}C
1	1.75 (m);1.17 (m)	**37.09**	**6**	1.65 (m); 1.42 (m)	**24.02**	**11**	1.83(m); 1.63(m)	**20.40**	**16**	1.241 (3H, s)	**28.36**
2	1.75 (m); 1.54(m)	**19.54**	**7**	2.25 (2H, m)	**31.91**	**12**	1.63 (m); 1.34 (m)	**41.34**	**17**	4.93 (t, *J*=2.2); 4.74 (m)	**109.7**
3	1.47 (m); 1.24 (m)	**42.84**	**8**	—	**149.6**	**13**	—	**72.82**	**18**	0.954 (3H, s)	**33.60**
4	—	**33.30**	**9**	1.63 (m)	**58.83**	**14**	5.91 (dd, *J*=17.2, 10.8)	**146.0**	**19**	0.928 (3H, s)	**22.36**
5	1.46 (m)	**46.08**	**10**	—	**38.41**	**15**	Ha 5.31(dd, *J*=17.2, 1.6) Hb 5.07 (dd, *J*=10.8, 1.6)	**111.2**	**20**	1.124 (3H, s)	**22.70**

Fig. S11. ^{1}H NMR spectra of the three manools measured in C_6D_6 (400 MHz). Solid lines: 13*R*-configurartion; Dotted lines: 13*S*-configuration.



A) manool (a mixture of 13*R*-**24a** and 13*S*-configuration **24b**, ca 1:1.2), prepared in this experiment**.**

B) authentic 13*R*-manool (**24a**) purchased from Industrial Research Limited (New Zealand).

C) *ent*-manool (a mixture of 13*R*- **26a** and 13*S*-configuration **26b**, ca 1:5), obtained by this incubation experiment. The structures of **24a** and **26b** are in an enantiomeric relationship; ^{1}H-NMR spectrum of **26b** was be identical to that of authentic **24a** (B). Thus, the major product is assigned to be *ent*-13*S*-manool **26b**.

Fig. S12. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of *ent*-manool (a mixture of **26a** and **26b**) obtained by incubating *ent*-CDP **8** with the Rv3378c enzyme.

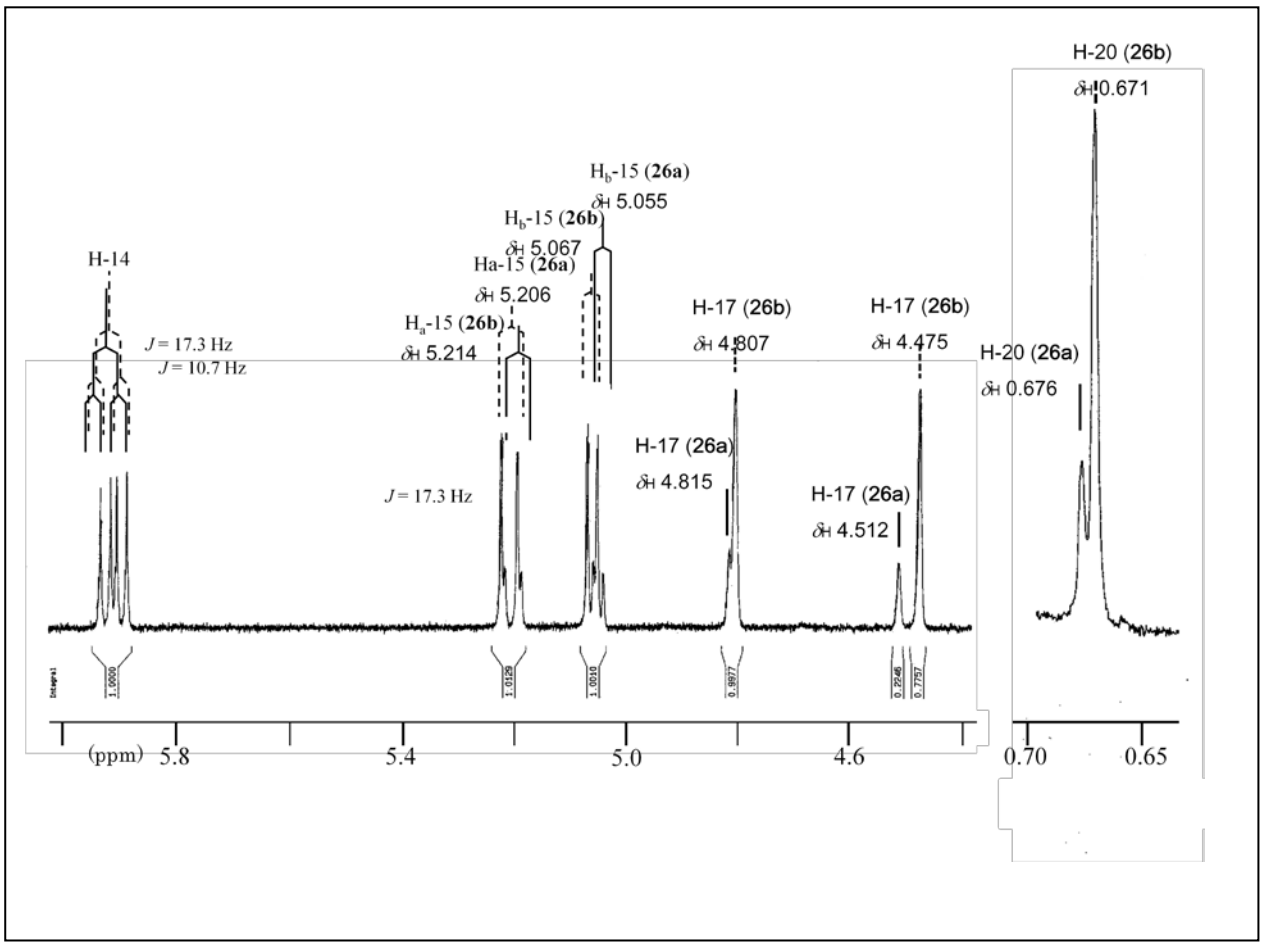


Table S1. Differences of proton chemical shifts between the isolated *ent*-manool and authentic *ent*-13-epi-manool measured in $CDCl_3$.

	δ_H (ppm) for the isolated product (a mixture of **26a**&**26b**)	δ_H (ppm) for authentic *ent*-13-epi-manool (13*R*)
Me-20	0.6765	0.6766
	0.6707	
H-17	4.512	4.512
	4.475	
H-17	4.815	4.813
	4.807	
Hb-15	5.055 (*J*=10.7 Hz)	5.050 (*J*=10.7 Hz)
	5.067(*J*=10.7 Hz)	
Ha-15	5.206 (*J*=17.3 Hz)	5.201 (*J*=17.1 Hz)
	5.214(*J*=17.3 Hz)	

As shown in this table, the proton chemical shifts (plain letters) of the minor peaks (ca. 1/5 peak intensities of the major product) were identical to those of authentic *ent*-13-*epi*-manool (13*R*), whose NMR data were kindly provided from Prof. Asakawa (Tokushima Bunri Univ.), thus major peaks (bold letters) indicate *ent*-manool (13*S*). No chemical shift difference of Me-16 was observed between **26a** and **26b** in the $CDCl_3$ solution.

Fig. S13. ^{1}H-NMR spectra of vitexifolin A in C_6D_6. (A), enzymatically synthesized vitexifolin A; (B), natural vitexifolin A.

A: ^{1}H NMR spectrum (400 MHz) of **28** (a mixture of **28a** and **28b**) in C_6D_6, which was obtained by incubating *syn*-CDP **9** with the Rv3378c enzyme. The ratio of **28a** to **28b** was 1:17.

B: ^{1}H NMR spectrum (400 MHz) of **28** isolated from *Vitex rotundifolia* in C_6D_6. (M. Ono, T. Yanaka, M. Yamamoto, Y. Ito and T. Nohara, *J. Nat. Prod.*, 2002, **65**, 537-541). The C(13)-stereochemistry of this natural product has not been reported, but determined to be 13*R* by us (see Fig. 5in the text). However a negligible amount of 13*S*-viterxifolin A was mixed as shown in the ^{1}H NMR spectrum (B).

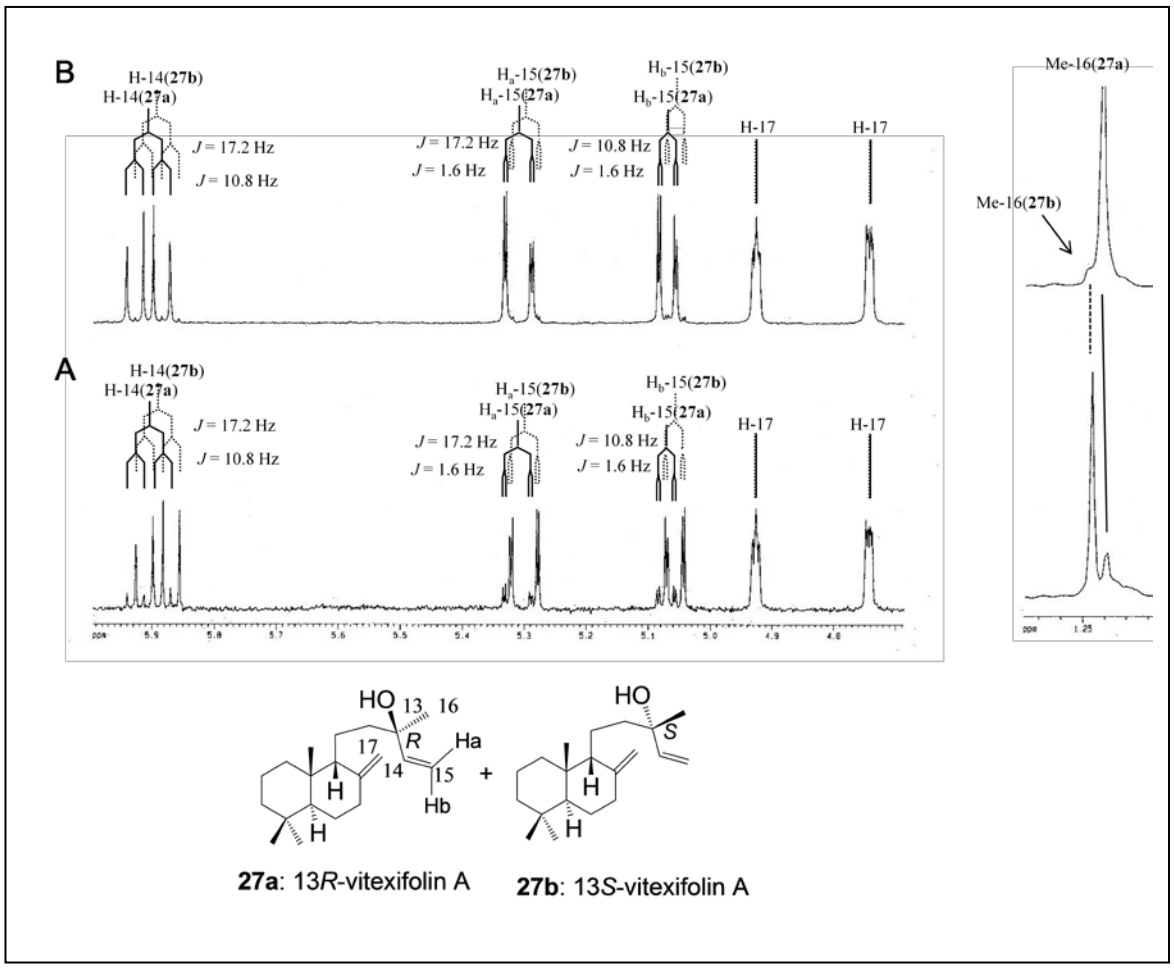


Fig. S14. Relationship between the logarithmic concentration of the tested compounds and the phagocytic activity of macrophage-like U937 cells.. R^2=0.96~0.98

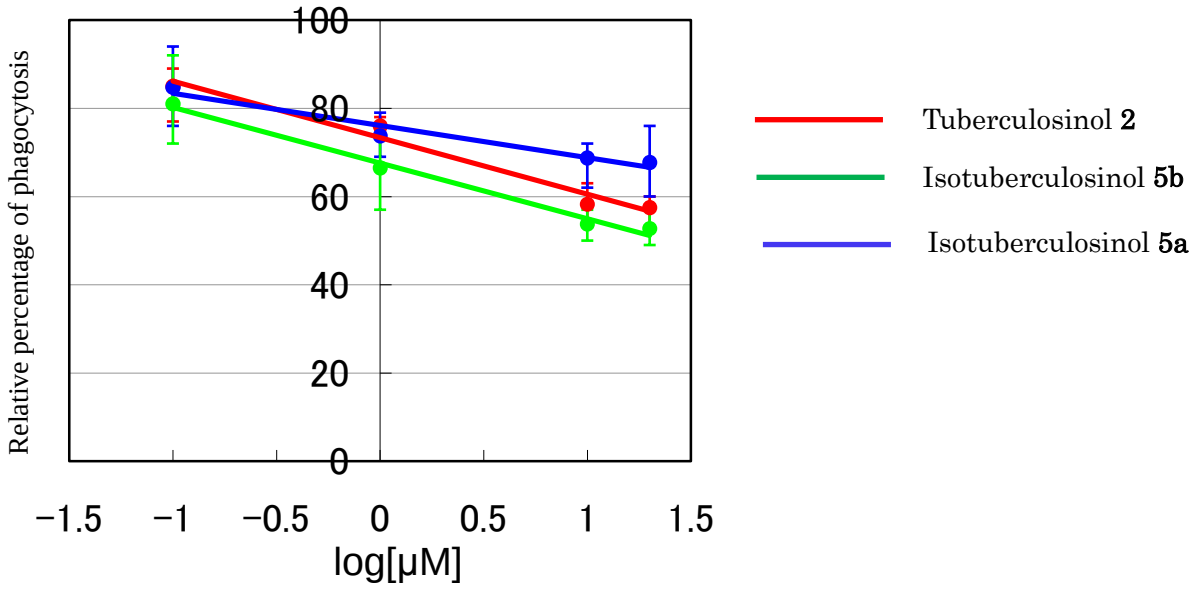


Fig. S15. Inhibition activities of **3**+**4a** and **3**+**4b** on the phagocytosis of FITC-OPZ by macrophage-like U937 cells. The cells were treated with 20 μM of **3**, **4a** and **4b,** 10 μM each of **3** and **4a** or **4b** (**3**+**4a**, **3**+**4b**) for 15 min and exposed to FITC-OPZ for 60 min at 37°C. The cells engulfing FITC-OPZ were counted by fluorescence microscopy. The data are normalized using the value of untreated cells (control) as 100% and expressed as mean ± SD (n=4). The synergistic inhibition was observed for **3**+**4b** with significantly lower percentage (32 ± 2.4%) compared to **3** (57 ± 3.2%), **4a** (68 ± 6.4%), **4b** (53 ± 3.3%) and **3**+**4a** (54 ± 2.1%) (P<0.01, Student's t-test).