Comparative Characterization of the Lactimidomycin and iso-Migrastatin Biosynthetic Machineries Revealing Unusual Features for Acyltransferase-less Type I Polyketide Synthases and Providing An Opportunity to Engineer New Analogues

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Supporting Information

Table S1. Bacterial strains and plasmids used in this study

Plasmids /Strains	Relevant characteristics	References
Plasmids		
pWHM79	pGEM-3zf-derived plasmid with the *ErmE** promoter	1
pHZ1358	*E. coli-Streptomyces* shuttle vector (a high-copy-number vector derived from the pIJ101 origin of replicon)	2
pIJ773	Plasmid carrying the 1398-bp Redirect cassette [*aac(3)IV* and *oriT* flanked by FRT sites] as an EcoRI/HindIII insert	3
pKC1139	*E. coli-Streptomyces* shuttle vector (a temperature sensitive vector derived from the $pSG5^{TS}$ origin of replicon)	4
pKC1218	*E. coli-Streptomyces* shuttle vector (a low-copy-number vector derived from the SCP2* origin of replicon)	4
pSET152	*E. coli-Streptomyces* shuttle vector that integrates into *att* site of *Streptomyces* chromosome	4
pBS3031	*E. coli-Streptomyces* shuttle vector (a high-copy-number vector derived from the pIJ101 origin of replicon)	5
pBS11016	pSET152-derived integrative vector with *neo* resistance gene replaced *aac(3)IV* gene	6
pBS15001	SuperCos1-derived cosmid carrying the upstream part of *ltm* cluster	This study
pBS15002	SuperCos1-derived cosmid carrying the central region of *ltm* cluster	This study
pBS15003	SuperCos1-derived cosmid carrying the downstream part of *ltm* cluster	This study
pBS15004	pBS15001-derived cosmid with the KS domain of PKS module-2 encoding region of *ltmE* replaced by the Redirect cassette	This study
pBS15005	pBS15003-derived cosmid with *ltmK* replaced by the Redirect cassette	This study
pBS15006	pWHM79-derived plasmid carrying the 2.5-kb SacI-MscI *ltmK* fragment	This study
pBS15007	pBS11016-derived plasmid with the 3.0-kb *ltmK* fragment from pBS15006 cloned into EcoRV site	This study
pBS15008	pBS15003-derived cosmid with *ltmH* replaced by the Redirect cassette	This study
pBS11006	SuperCos1-derived cosmid carrying the downstream part of *Streptomyces platensis* isomigrastatin gene cluster	7
pBS15009	pBS11016-derived plasmid carrying the *mgsIJK* fragment from pBS11006 cloned into EcoRV and XbaI sites	This study
pBS15010	pBS15009-derived plasmid carrying the *mgsK* fragment	This study
pBS15011	pBS15009-derived plasmid carrying the *mgsJK* fragment	This study
E. coli strains		
BW25113 /pIJ790	Host, expressing recombination promoting proteins from pIJ790, used for the Redirect PCR-targeting mutagenesis system	3
DH5α	General cloning host	8
ET12567 /pUZ8002	Methylation-deficient host, expressing transfer functions from pUZ8002, used for *E. coli-Streptomyces* intergeneric conjugation	9
S17-1	Methylation-proficient host used for *E. coli-Streptomyces* intergeneric conjugation	9
S. amphibiosporus strains		
ATCC 53964	Wild-type strain of *S. amphibiosporus*, LTM producing	10
SB15001	Δ*ltmE* mutant with the KS domain of PKS module-2 encoding region of *ltmE* replaced by the Redirect cassette (from pBS15004)	This study
SB15002	Δ*ltmK* mutant with *ltmK* replaced by the Redirect cassette (from pBS15005)	This study
SB15003	SB15002/pBS15007, restored production of LTM at level comparable to the wild-type *S. amphibiosporus*	This study
SB15004	Wild-type *S. amphibiosporus* with pBS15007, producing similar titers of LTM analogs comparable to wild-type strain	This study
SB15005	Δ*ltmH* mutant with *ltmH* replaced by the Redirect cassette (from pBS15008)	This study
SB15006	SB15002/pBS15010, producing two new compounds **16**	This study
SB15007	SB15002/pBS15011, producing one new compounds **16** and **17**	This study

Table S2. Oligonucleotide primers used in this study

Oligonucleotide	Sequence (DNA sequence identical to the Redirect cassette are underlined)	Function
For gene inactivation by λ-RED-mediated PCR-targeting mutagenesis		
ltmE-KS1-F1	5'-GCCGCCGACGGCGCGGCCGGCGGCCCGGTG GACGGGCAGATTCCGGGGATCGTCGACC-3'	Forward primer for *ltmE* redirect mutagenesis
ltmE-KS1-R1	5'-CGCCTCCGCGTCCGTGACCTCCGCCGCGTGT GTAGGCTGGAGCTGCTTC-3'	Reverse primer for *ltmE* redirect mutagenesis
ltmK-F1	5'-GTGACCACCCACCTCACCACCGACATCGACGA GATCGTCATTCCGGGGATCCGTCGACC-3'	Forward primer for *ltmK* redirect mutagenesis
ltmK-R1	5'-CGCCGGGGTGAGCCACATCGGGATGCGGGCG TAGCCGCGTGTAGGCTGGAGCTGCTTC-3'	Reverse primer for *ltmK* redirect mutagenesis
ltmH-F1	5'-ATGGAGGCGGTCGTCTTTCCCGGGCAGGGCG CGCAGCGATTCCGGGGATCCGTCGACC-3'	Forward primer for *ltmH* redirect mutagenesis
ltmH-R1	5'-GGTGCGGGCGGCCAGGGCGCGTTCCTCCGC GGGGGCGAATGTAGGCTGGAGCTGCTTC-3'	Reverse primer for *ltmH* re-direct mutagenesis
For sequencing or PCR amplification of probes for Southern analysis		
ltmE-KS1-F2	5'-GCTGTCCCCCTGTACGTCGTCGACCGG-3'	Forward primer for *ltmE*
ltmE-KS1-R2	5'-GCAGCCGCTCCTCGTCGTCGGCG-3'	Reverse primer for *ltmE*
ltmK-F2	5'-GCACCGGACCGGACCGTCCGG-3'	Forward primer for *ltmK*
ltmK-R2	5'-GTCCAAGTCGGAGACGGAGACG-3'	Reverse primer for *ltmK*
ltmH-F2	5'-TACGGACTCGACTCGATCTT-3'	Forward primer for *ltmH*
ltmH-R2	5'-GATGATGTCCACGTAGTAGGC-3'	Reverse primer for *ltmH*
Apra-F	5'-ATTCCGGGGATCCGTCGACC-3'	Forward primer for *aac(3)IV*
Apra-R	5'-TGTAGGCTGGAGCTGCTTC-3'	Reverse primer for *aac(3)IV*
For sequencing or PCR amplification for construction of pBS15009		
ErmE*-F1	5'-GTTAACGGTACCGCGAGTGTCCGTTCG-3'	Forward primer for *ErmE** at the upstream of *mgsI*
ErmE*-R1	5'-TCTAGATACGTAGCTGGATCCTACCA-3'	Reverse primer for *ErmE** at the upstream of *mgsI*
mgsI-F	5'-TACGTAGACATCCCACCGCAGTCG-3'	Forward primer for *mgsI*
mgsI-R	5'-TCTAGACTTAAGGCAGGGCGTGGTG-3'	Reverse primer for *mgsI*
ErmE*-F2	5'-CTTAAGGCGAGTGTCCGTTCG-3'	Forward primer for *ErmE** at the upstream of *mgsJ*
ErmE*-R2	5'-TGTACATACGTAGCTGGATCCTACCA-3'	Reverse primer for *ErmE** at the upstream of *mgsJ*
mgsJ-F	5'-TACGTAGGTTCACCAGACAG-3'	Forward primer for *mgsJ*
mgsJ-R	5'-TGTACATCTAGAGTTTAAACGGCGCCACCTCGTTCG-3'	Reverse primer for *mgsJ*
ErmE*-F3	5'-GTTTAAACGCGAGTGTCCGTTCG-3'	Forward primer for *ErmE** at the upstream of *mgsK*
ErmE*-R3	5'-TTAATTAATACGTAGCTGGATCCTACCA-3'	Reverse primer for *ErmE** at the upstream of *mgsK*
mgsK-F	5'-TACGTAGCACCCACCACCGATG-3'	Forward primer for *mgsK*
mgsK-R	5'-TTAATTAATCTAGATGTACAGTCATGACCGCCTG-3'	Reverse primer for *mgsK*

Table S3. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data for 8,9-dihydro-8*R*-hydroxy-LTM (**16**) and 8,9-dihydro-8*R*-methoxy-LTM (**17**) in $CDCl_3$[a]

position	8,9-dihydro-8*R*-hydroxy-LTM (**16**)		8,9-dihydro-8*R*-methoxy-LTM (**17**)	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	167.7 C		167.8 C	
2	125.0 CH	5.70 d (15.9)	124.8 CH	5.70 d (15.9)
3	150.3 CH	6.66 m	150.4 CH	6.66 m
4	30.6 CH_2	2.46 m*, 2.19 m	30.6 CH_2	2.44 m, 2.23 m
5	32.0 CH_2	2.56 m*, 1.98 m*	32.2 CH_2	2.58 m*, 2.03 m
6	125.8 CH	5.65 m	127.5 CH	5.55 m
7	138.4 CH	5.49 dd (15.6, 3.8)	134.5 CH	5.27 dd (15.8, 4.4)
8	68.9 CH	4.14 m*	78.4 CH	3.54 m
9	42.2 CH_2	2.00 m*, 1.48 m	40.7 CH_2	1.96 m, 1.48 m
10	33.7 CH	1.79 m*	33.7 CH	1.79 m*
11	82.0 CH	5.20 d (4.2)	82.1 CH	5.19 d (4.4)
12	134.1 C		134.1 C	
13	128.5 CH	5.36 d (9.4)	128.3 CH	5.36 br d (9.7)
14	46.6 CH	3.44 m	46.5 CH	3.43 m
15	212.1 C		212.2 C	
16	47.3 CH_2	2.58*	47.3 CH_2	2.57 d (5.7)
17	64.8 CH	4.12 m*	64.8 CH	4.11 m
18	40.7 CH_2	1.61 m, 1.35 m	40.7 CH_2	1.62 m, 1.34 m
19	27.1 CH	2.50 m*	27.1 CH	2.50 m
20	38.4 CH_2	2.78 m*, 2.35 m*	38.4 CH_2	2.79 m*, 2.34 m*
21	172.2 C		172.0 C	
22	17.4 CH_3	0.93 d (6.9)	17.3 CH_3	0.93 d (7.0)
23	15.0 CH_3	1.78 s	15.0 CH_3	1.77 d (1.3)
24	16.1 CH_3	1.19 d (6.8)	16.1 CH_3	1.19 d (6.8)
25	37.1 CH_2	2.78 m*, 2.35 m*	37.1 CH_2	2.79 m*, 2.34 m*
26	172.1 C		171.9 C	
NH		8.17 s		7.95 s
8-OCH_3			56.5 CH_3	3.29 s

[a]Assignments were based on COSY, HSQC and HMBC.
*Signals overlapped with others.

Figure S1. Migrastatin (**3**) and dorrigocins (**4**-**6**) are shunt metabolites of iso-migrastatin (**2**), and H_2O-mediated ring expansion and ring opening rearrangements of **2** to **3** and **4**-**6**, respectively.[6]

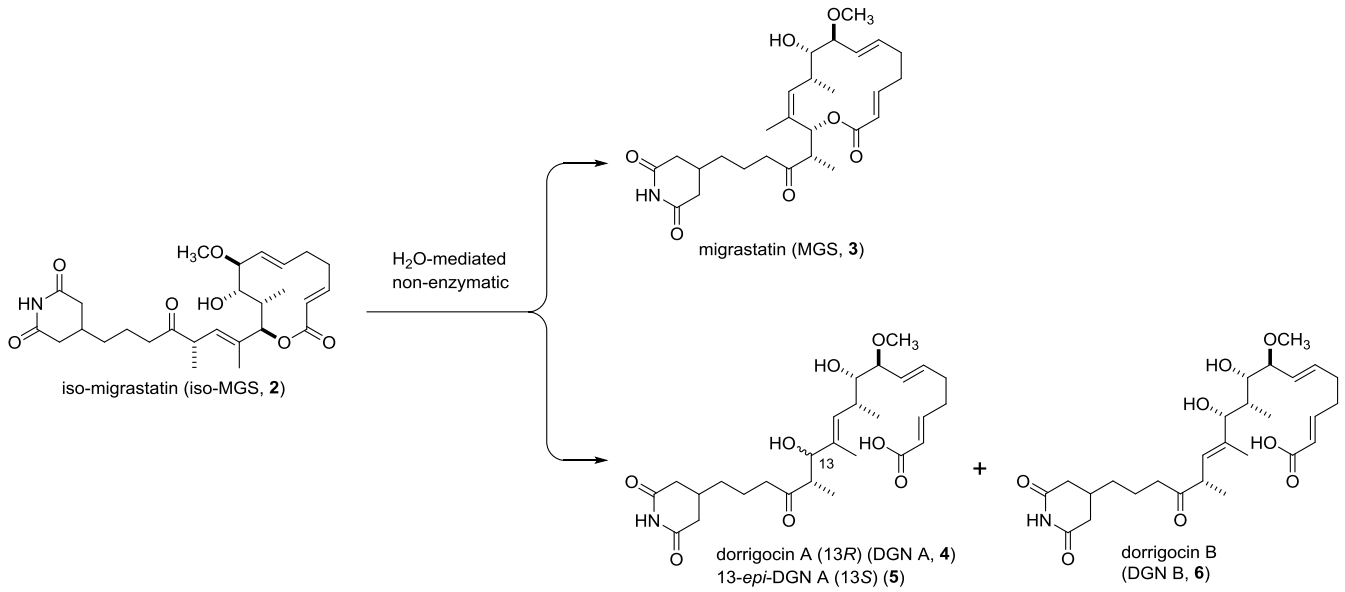
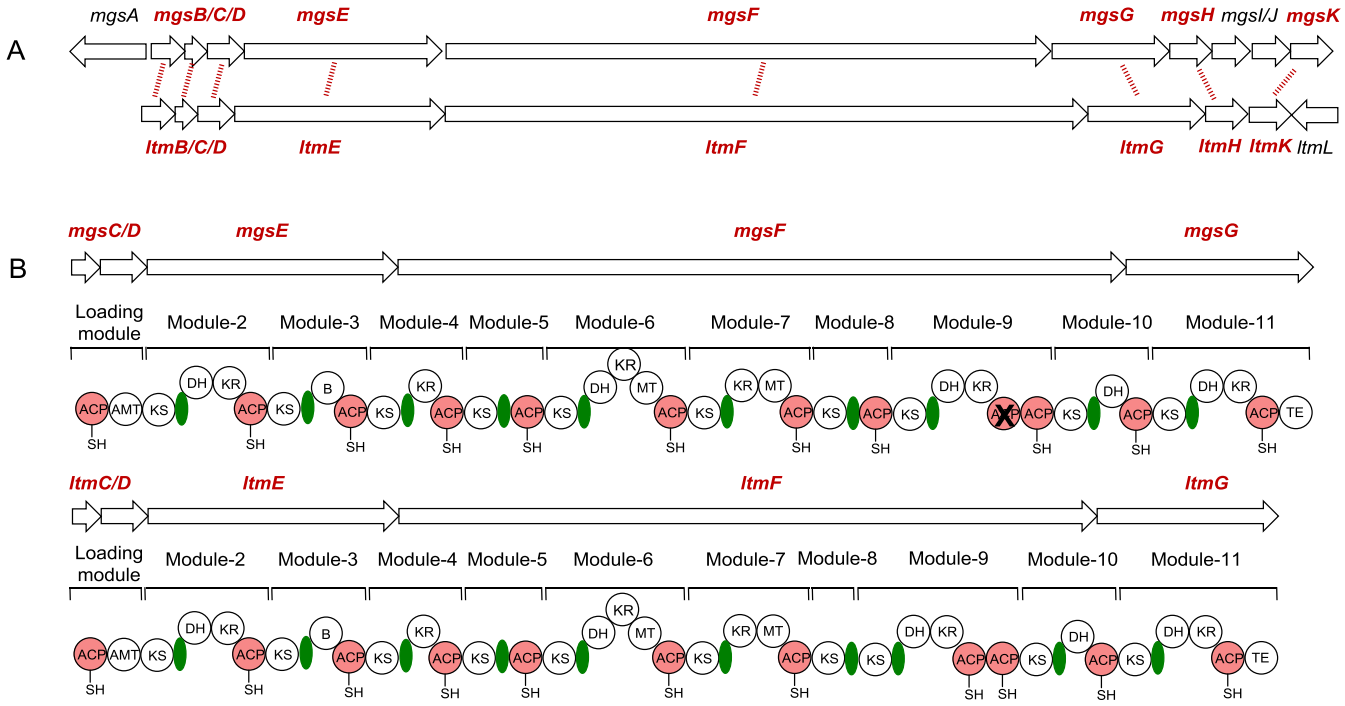
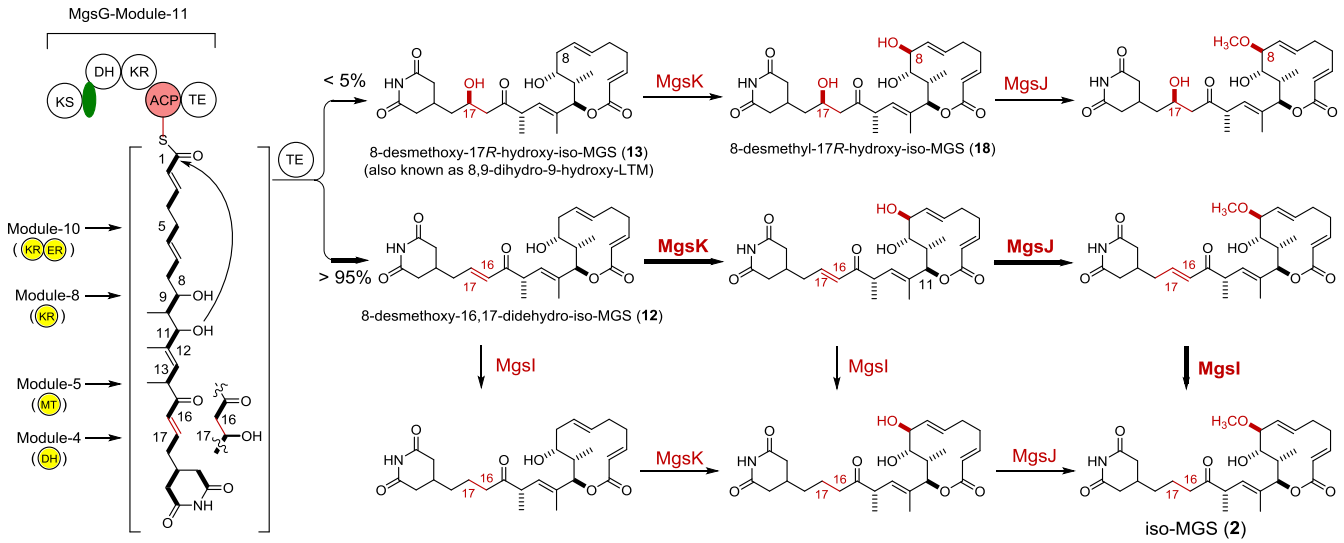


Figure S2. Comparison between LTM biosynthesis in *S. amphibiosporus* ATCC 53964 and iso-MGS biosynthesis in *S. platensis* NRRL 18993. (A) The *ltm* cluster consists of nine genes, while the *mgs* cluster consists of 11 genes. The two clusters show a highly conserved genetic organization. Homologs for eight of the genes are found in both clusters. The dashed lines depict homologues. (B) Doman and module organization of the LTM and iso-MGS type I AT-less PKSs. Except modules 8 and 9, the two PKSs have an identical domain and module organization and architecture. (C) The iso-MGS AT-less type I PKS biosynthesizes 8-desmethoxy-16,17-didehydro-iso-MGS (**12**) (>95%) and 8-desmethoxy-17*R*-hydroxy-iso-MGS (**13**) (<5%) as the nascent products and the three tailoring enzymes MgsIJK possess remarkable substrate promiscuity, accounting for the formation of all congeners of **2** known to *S. platensis NRRL* 18993 from **12** and **13**. (D) Sequence alignment of LtmK with selected bacterial cytochrome P450 enzymes. The conserved oxygen activation motif is shown in black box and the heme binding motif including the binding cysteine is shown in green box. The accession numbers are: LtmK (ACY01404), PldB (BAH02272), CYP107L3 (AAT45281), MgsK (ACY01396), and CYP199A4 (4EGP_A). Abbreviations are: ACP, acyl carrier protein; AMT, amidotransferase; B, branching; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; TE, thioesterase. Green ovals depict AT-docking domains. The yellow domains highlight the missing domains for the iso-MGS AT-less type I PKS to synthesize both 8-desmethoxy-16,17-didehydro-iso-MGS (**12**) and 8-desmethoxy-17*R*-hydroxy-iso-MGS (**13**) as nascent products and refer Figure 3B for comparison to the LTM AT-less type I PKS that biosynthesizes 8,9-dihydro-LTM (**14**) as the sole nascent product.



C


MgsG-Module-11
DH
KR
KS
ACP
TE
Module-10
(KR ER)
Module-8
(KR)
Module-5
(MT)
Module-4
(DH)
< 5%
> 95%
8-desmethoxy-17R-hydroxy-iso-MGS (13)
(also known as 8,9-dihydro-9-hydroxy-LTM)
8-desmethyl-17R-hydroxy-iso-MGS (18)
8-desmethoxy-16,17-didehydro-iso-MGS (12)
MgsK
MgsJ
MgsI
iso-MGS (2)

D

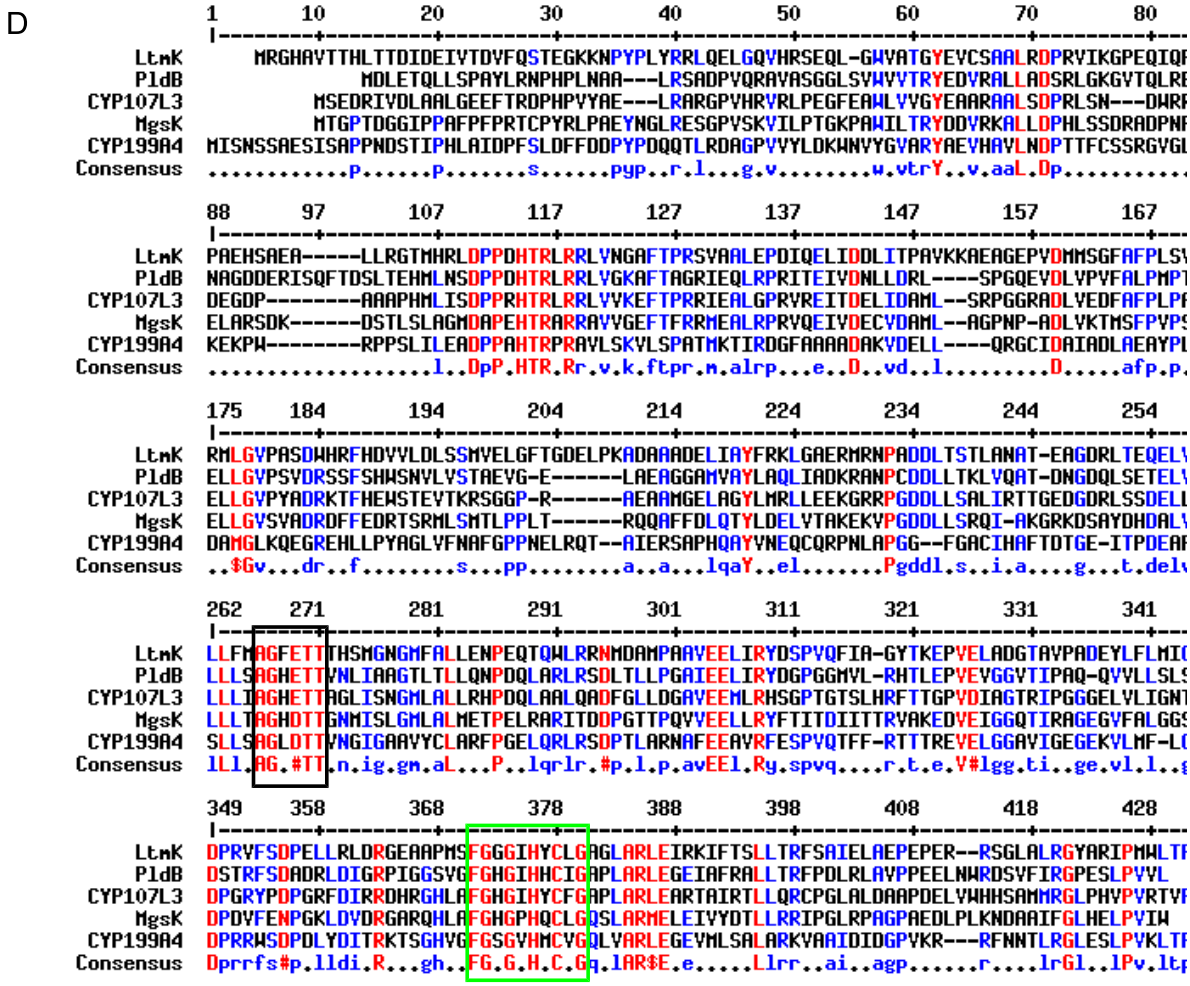
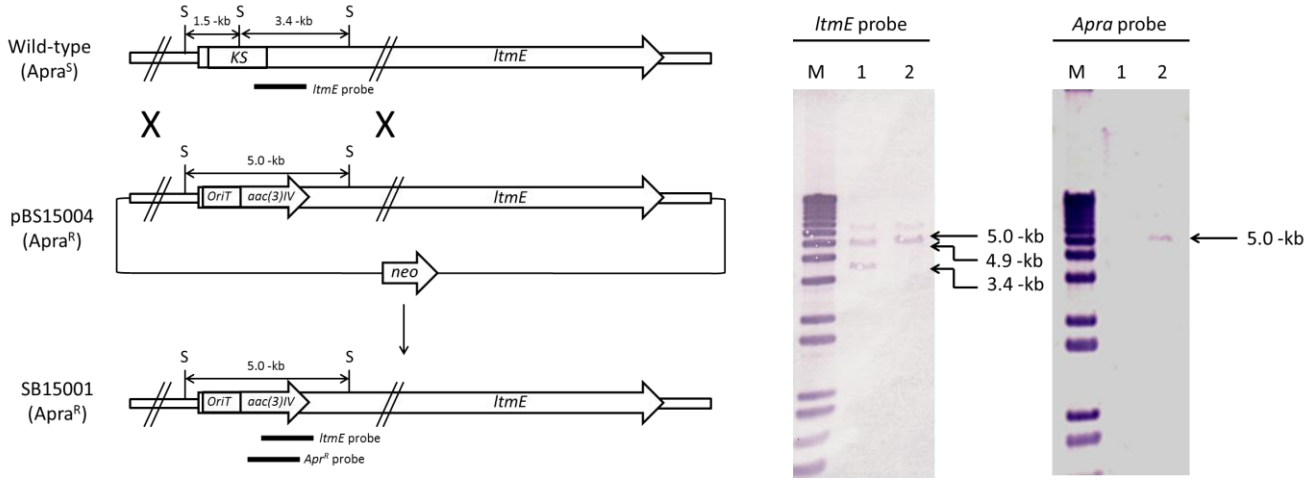
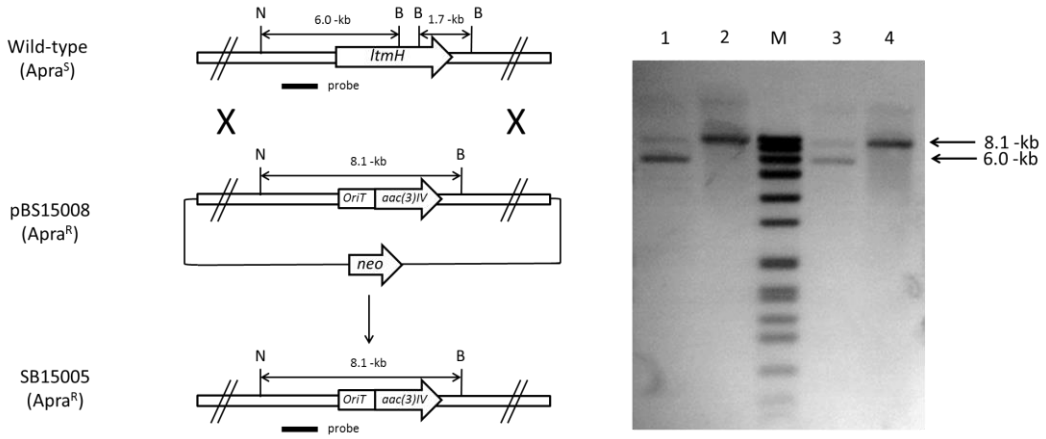

LtnK
PldB
CYP107L3
MgsK
CYP199A4
Consensus

Figure S3. Confirmation of the cloned gene cluster encoding LTM biosynthesis and functional assignment of the *ltm* cluster in *S. amphibiosporus* ATCC 53964 by gene inactivation with λ-RED-mediated PCR-targeting mutagenesis followed by HPLC analysis of metabolite profiles of the resultant mutant strains. (A) Construction of the Δ*ltmE* mutant strain SB15001. Restriction map showed the KS domain of PKS module-2 encoding region of *ltmE* gene was replaced by a RK2 origin of transfer (*oriT*) and apramycin resistance (*aac(3)IV*) gene cassette. The genomic DNAs isolated from resultant Δ*ltmE* mutant and wild-type strain were digested by SphI and hybridized with the two PCR amplified regions as probes (the *ltmE* probe hybridizes to partial KS domain encoding region and its downstream region and the *Apra* probe hybridizes to the *aac(3)IV* region) for Southern analysis. Southern analysis for the wild-type strain (lane 1) and Δ*ltmE* mutant (lane 2) using both probes showed correct fragment sizes. "S" represents SphI digestion site. (B) Construction of the Δ*ltmH* mutant strain SB15005. Restriction map showed the entire *ltmH* gene was replaced by a RK2 origin of transfer (*oriT*) and apramycin resistance (*aac(3)IV*) gene cassette. The genomic DNAs isolated from resultant Δ*ltmH* mutant and wild-type strain were digested by NdeI and BglII and hybridized with the PCR amplified upstream region of *ltmH* gene as a probe for Southern analysis. Southern analysis for the wild-type strain (lane 1 and 3) and Δ*ltmE* mutant (lane 2 and 4) showed correct fragment sizes. "N" represents NdeI digestion site and "B" represents BglII digestion site. (C) Construction of the Δ*ltmK* mutant strain SB15002. Restriction map showed the entire *ltmK* gene was replaced by a RK2 origin of transfer (*oriT*) and apramycin resistance (*aac(3)IV*) gene cassette. The genomic DNAs isolated from the resultant Δ*ltmK* mutant and the wild-type strain were digested by BglII and hybridized with the two PCR amplified regions as probes (*ltmK* probe hybridizes to partial *ltmK* gene and its downstream region, *Apra* probe only hybridizes to the *aac(3)IV* region) for Southern analysis. Southern analysis for wild-type strain (lane 1) and Δ*ltmK* mutant (lane 2) using both probes showed correct fragment sizes. "B" represents BglII digestion site.

A



B


Wild-type
(Apra^S)
N
6.0 -kb
B
B
1.7 -kb
B
ltmH
probe
X
X
pBS15008
(Apra^R)
N
8.1 -kb
B
OriT
aac(3)IV
neo
SB15005
(Apra^R)
N
8.1 -kb
B
OriT
aac(3)IV
probe
1
2
M
3
4
8.1 -kb
6.0 -kb

C

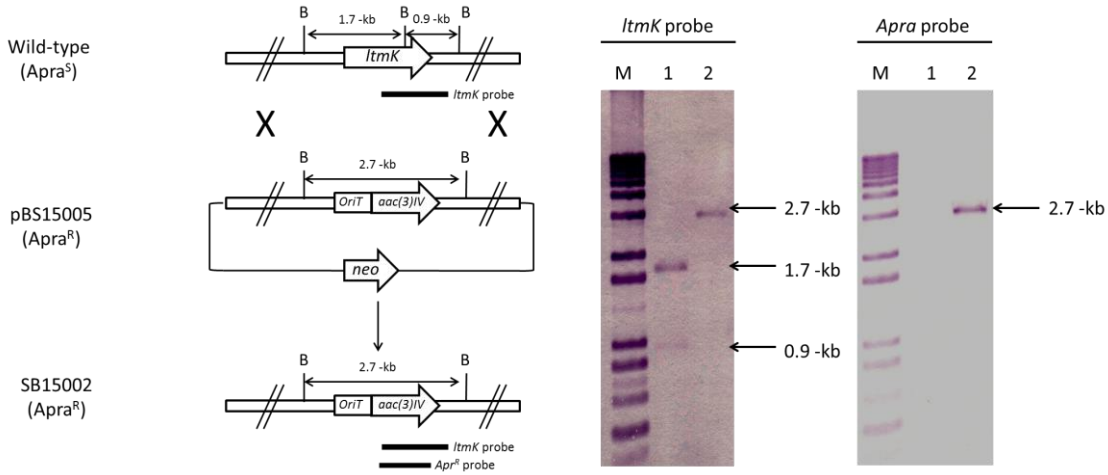

Wild-type
(Apra^S)
B
1.7 -kb
B
0.9 -kb
B
ltmK
ltmK probe
X
X
pBS15005
(Apra^R)
B
2.7 -kb
B
OriT
aac(3)IV
neo
SB15002
(Apra^R)
B
2.7 -kb
B
OriT
aac(3)IV
ltmK probe
Apr^R probe
ltmK probe
M
1
2
2.7 -kb
1.7 -kb
0.9 -kb
Apra probe
M
1
2
2.7 -kb

Figure S4. Construction of the recombinant strains SB15006 and SB15007 for production of the novel analogues **16** and **17**. The red lines show the ligation positions for constructing the expression vectors pBS15009 (*mgsIJK*), pBS15010 (*mgsK*), and pBS15011 (*mgsJK*). Introduction of pBS15010 and pBS15011 into the *ΔltmK* mutant strain of SB15002 yielded the recombinant strains SB15006 (*ΔltmK/mgsK*), producing **16**, and SB15007 (*ΔltmK/mgsJK*), producing **16** and **17**, respectively.

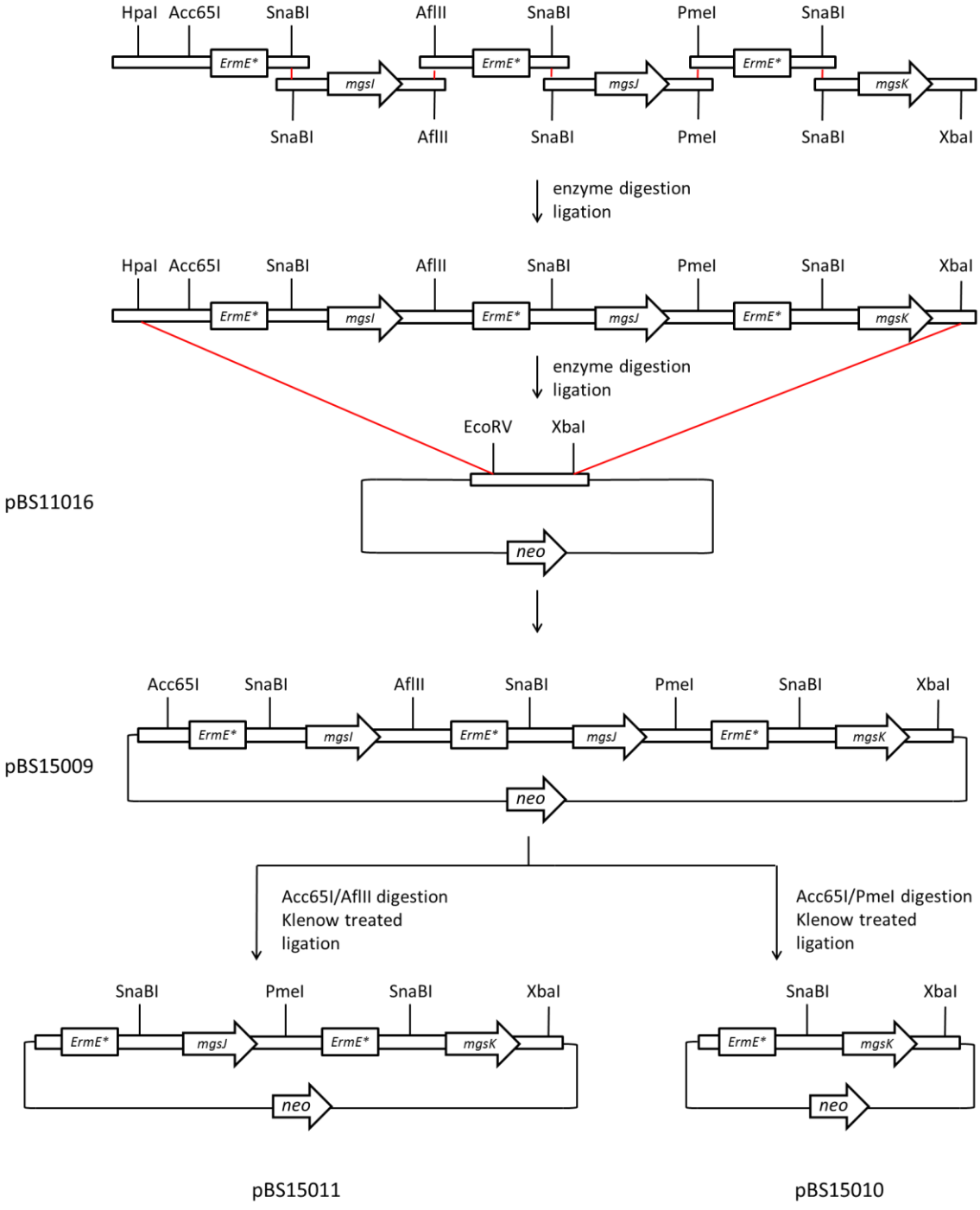
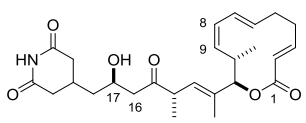
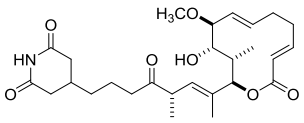


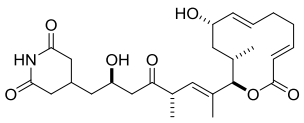
Figure S5. The cytotoxicity assay of **16** and **17** against MDA-MB-231, HeLa, and Jurkat cell lines in comparison with **1**, **2**, and selected congeners **13**, **15**, and **18**.



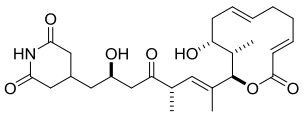
lactimidomycin (LTM, **1**)



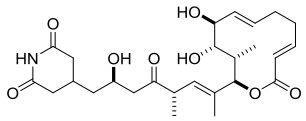
iso-migrastatin (iso-MGS, **2**)



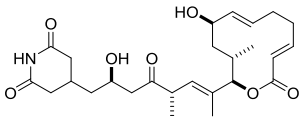
8,9-dihydro-8*S*-hydroxy-LTM (**15**)



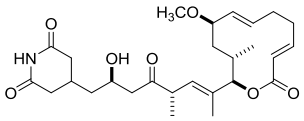
8,9-dihydro-9*R*-hydroxy-LTM (**13**)



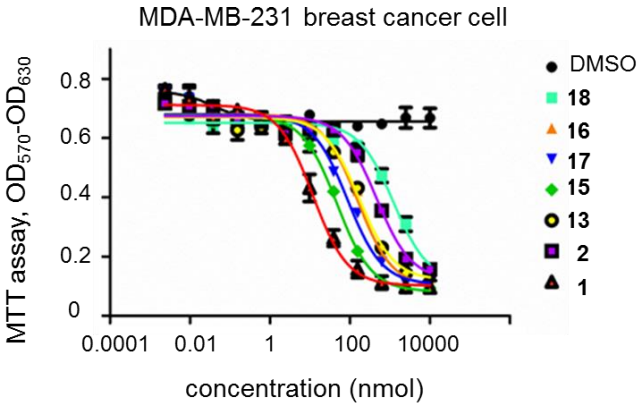
17*R*-hydroxy-8-desmethyl-iso-MGS(**18**)

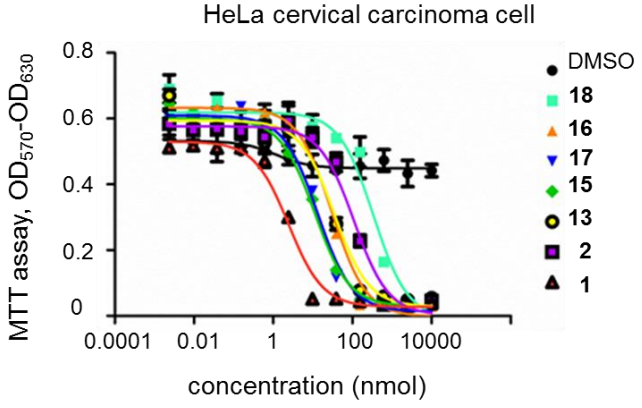


8,9-dihydro-8*R*-hydroxyl-LTM (**16**)



8,9-dihydro-8*R*-methoxyl-LTM (**17**)





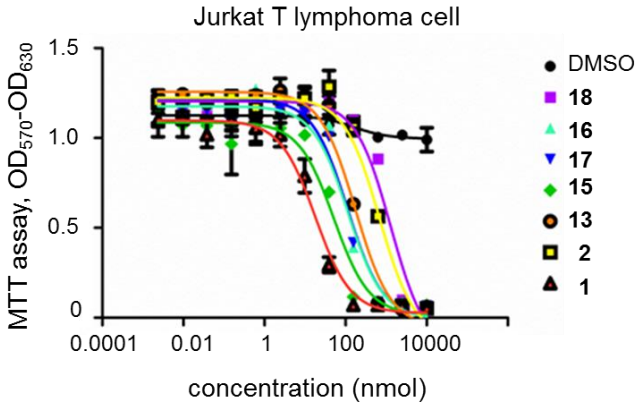
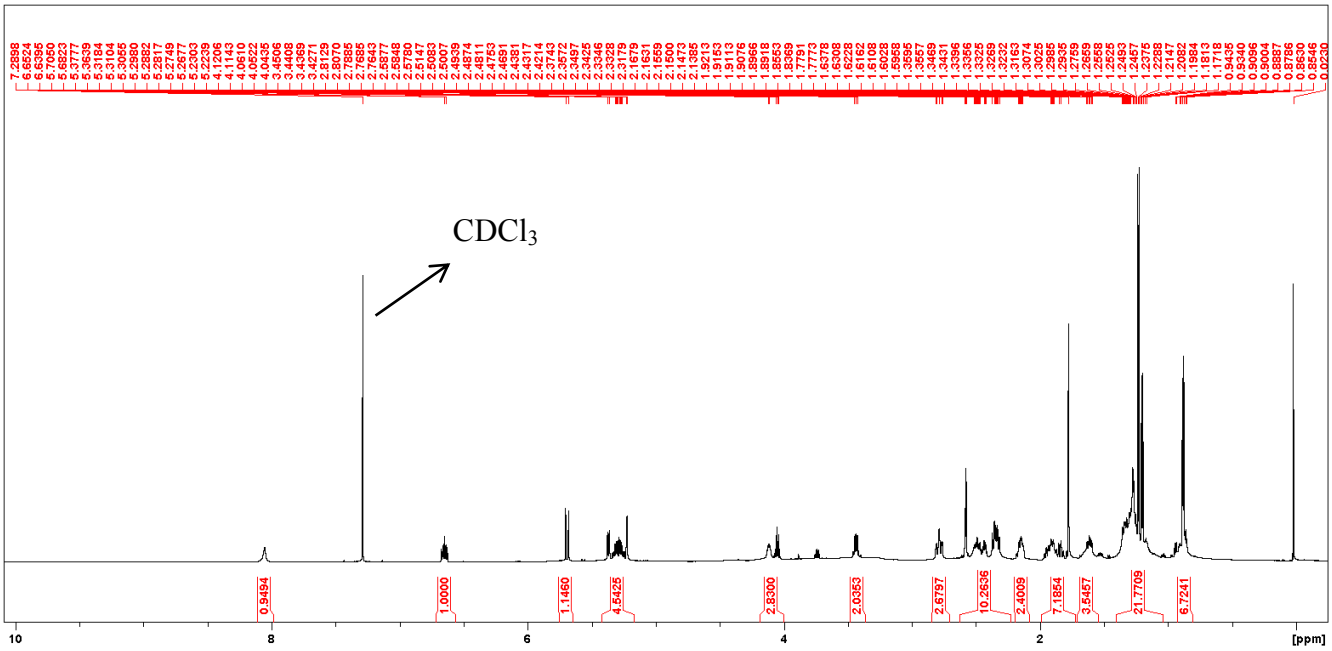
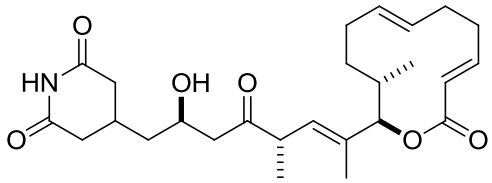


Figure S6. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR spectra of 8,9-dihydro-LTM (**14**) in $CDCl_3$





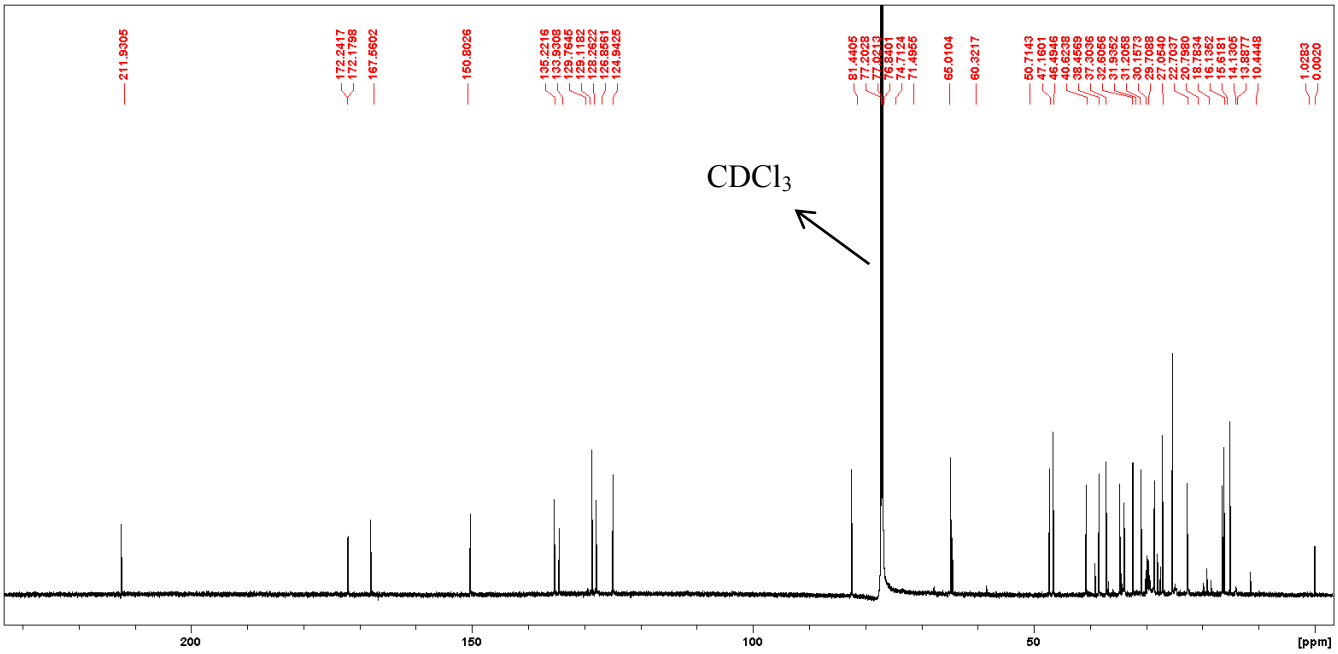
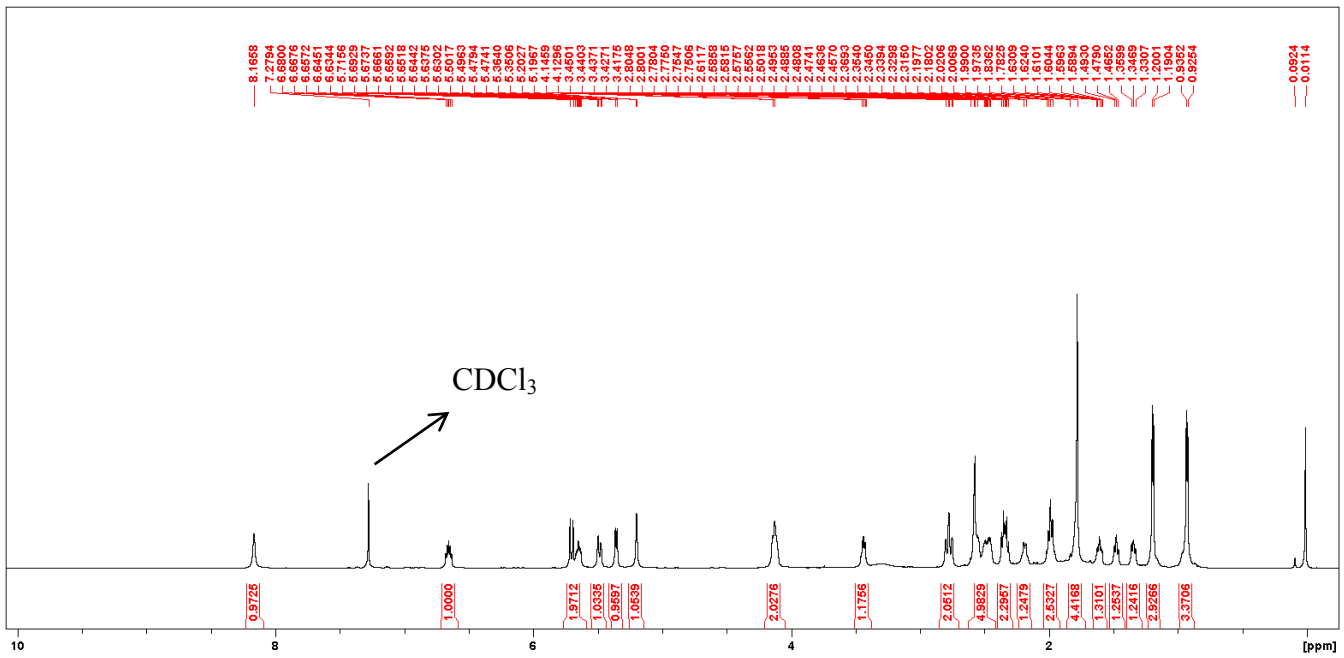
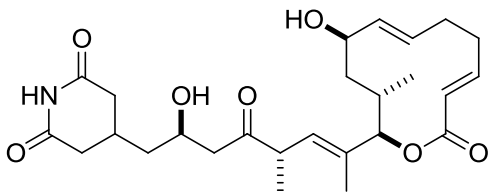


Figure S7. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR spectra of 8,9-dihydro-8*R*-hydroxyl-LTM (**16**) in $CDCl_3$





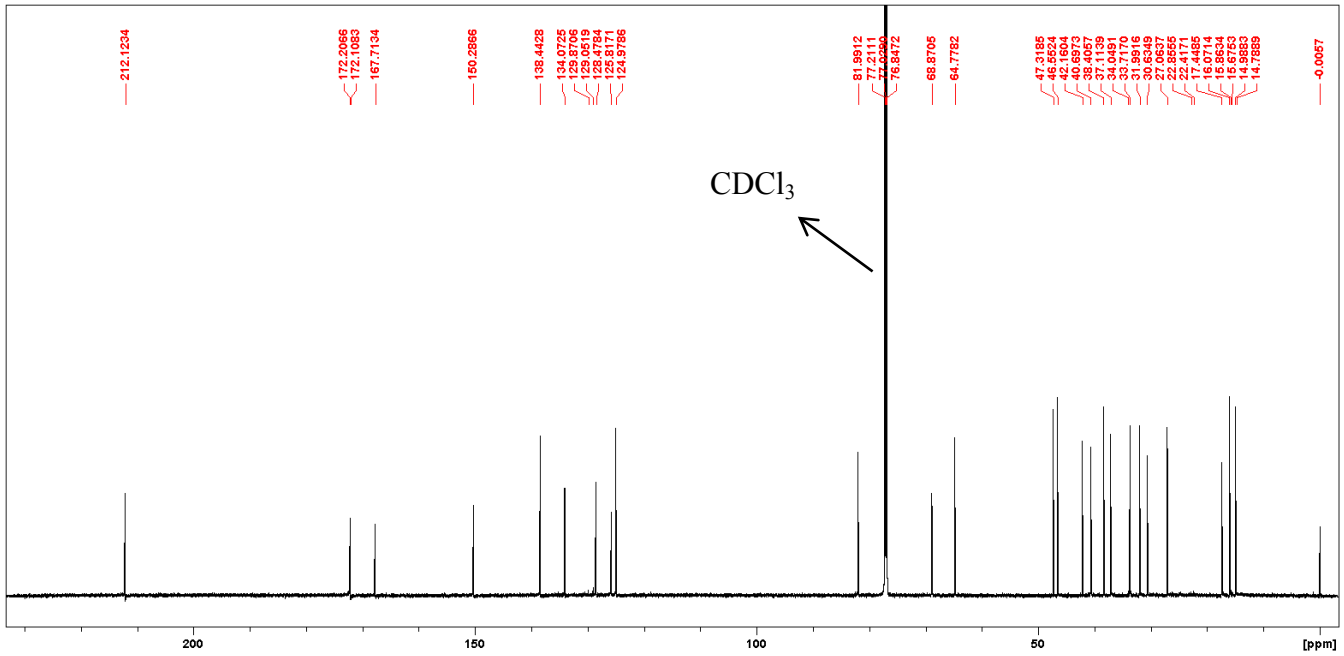
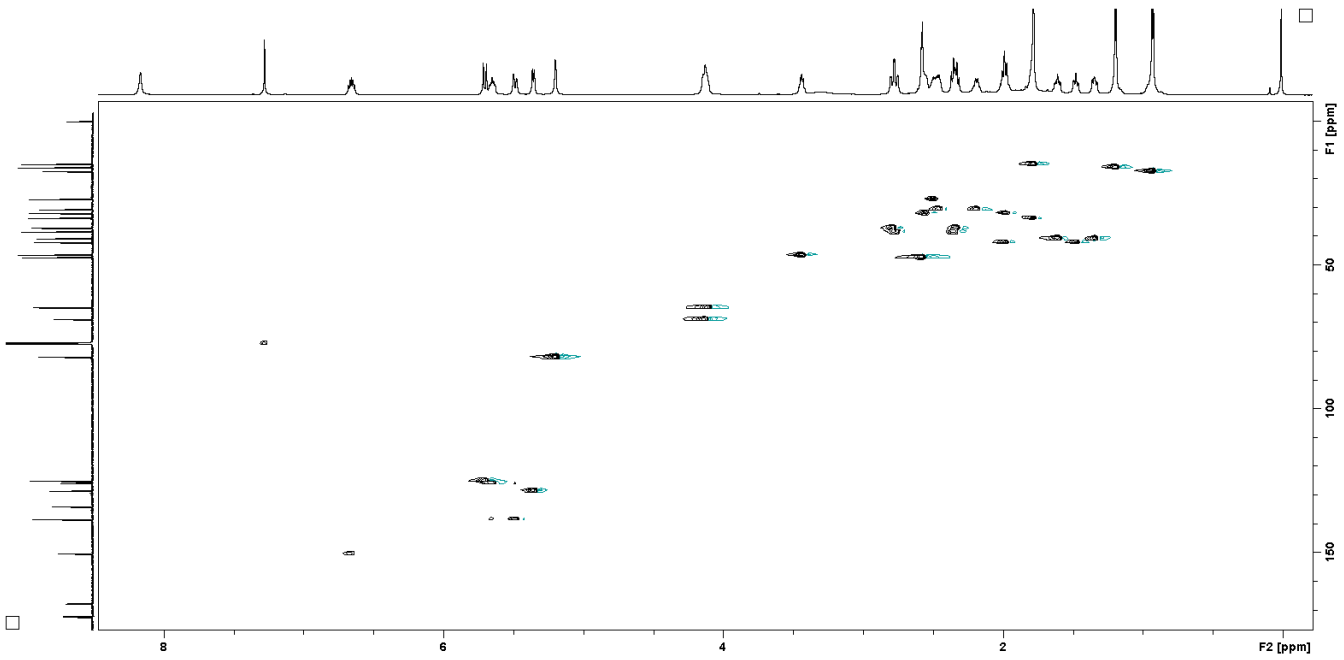
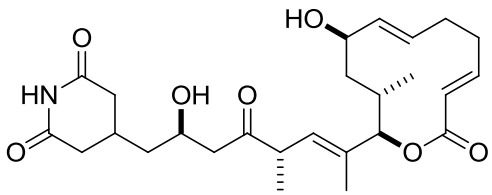


Figure S8. HSQC and HMBC spectra of 8,9-dihydro-8*R*-hydroxyl-LTM (**16**) in $CDCl_3$





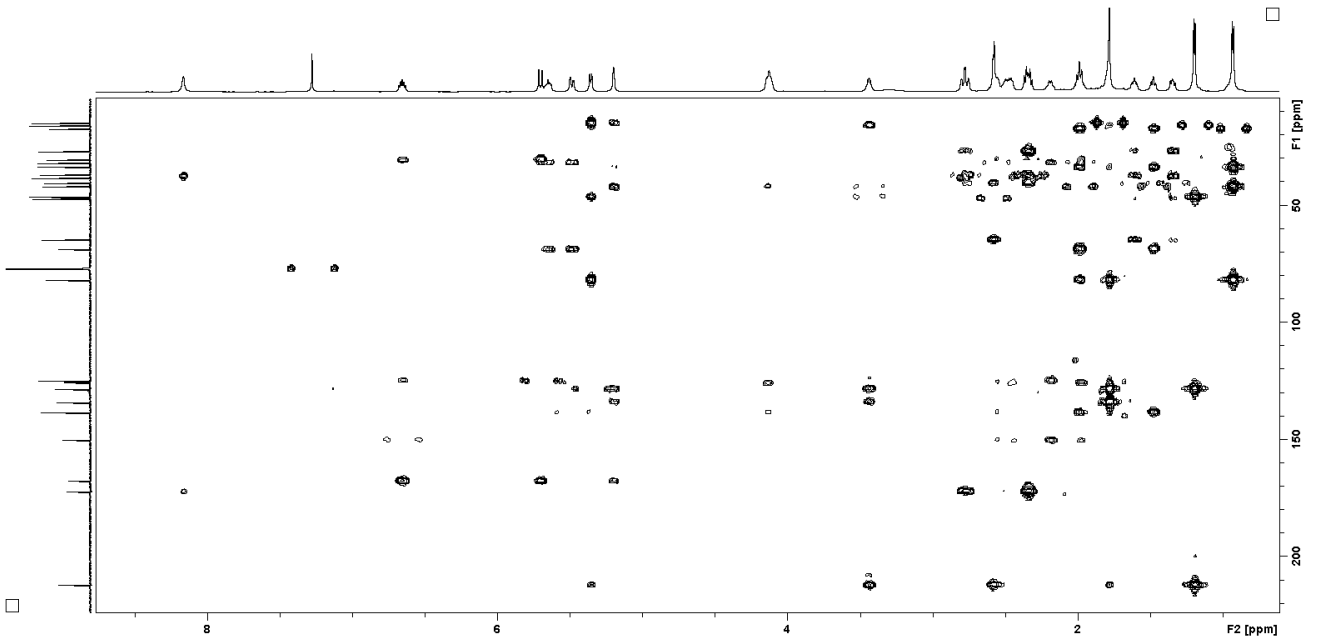
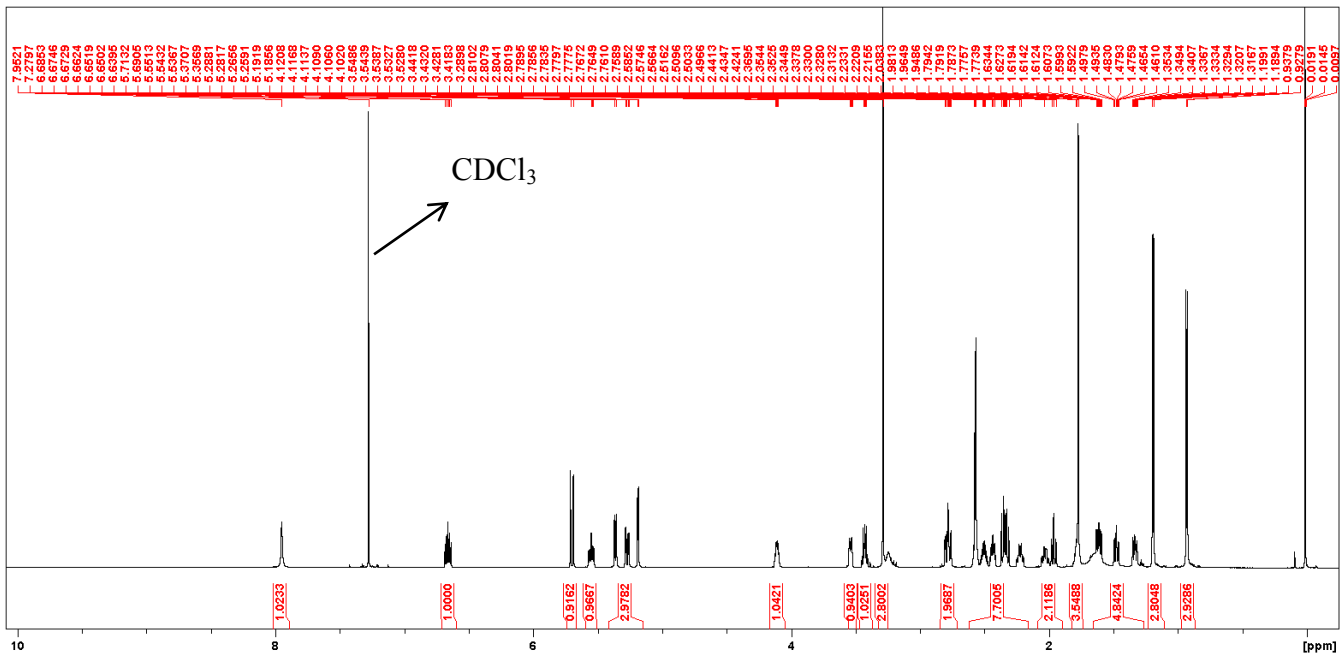
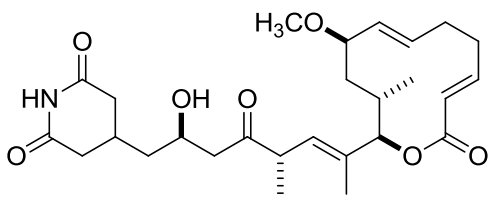


Figure S9. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR spectra of 8,9-dihydro-8*R*-methoxyl-LTM (**17**) in $CDCl_3$





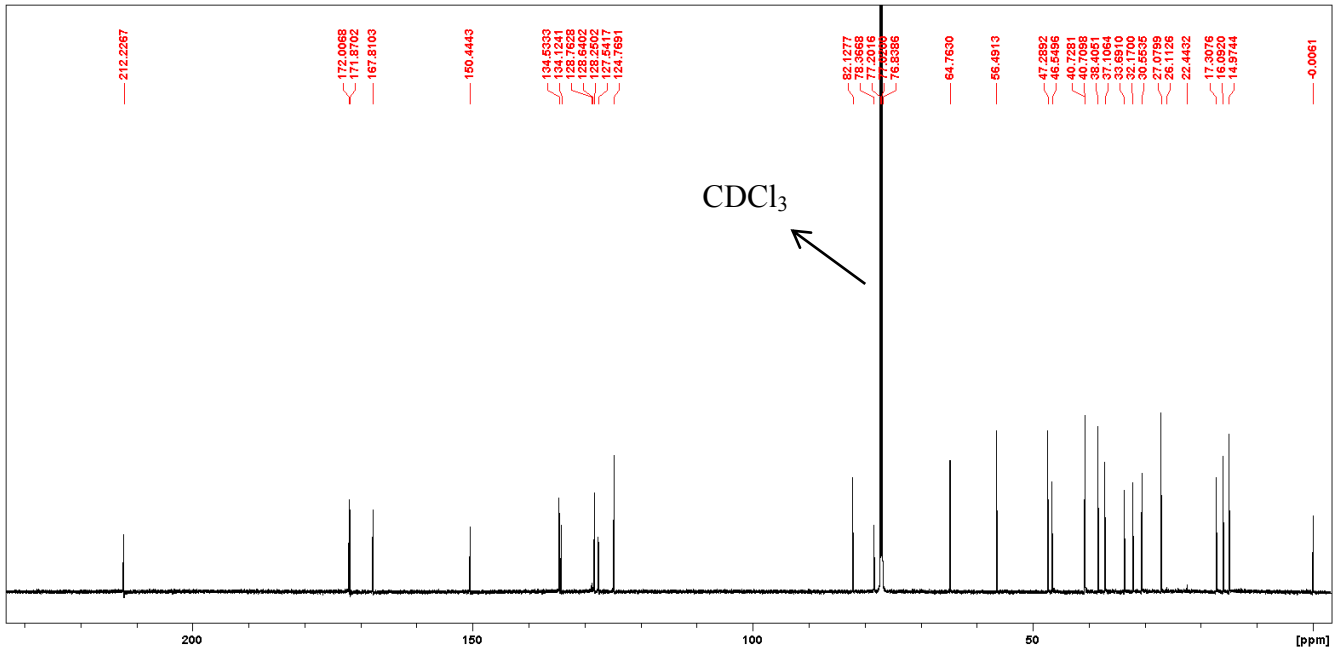
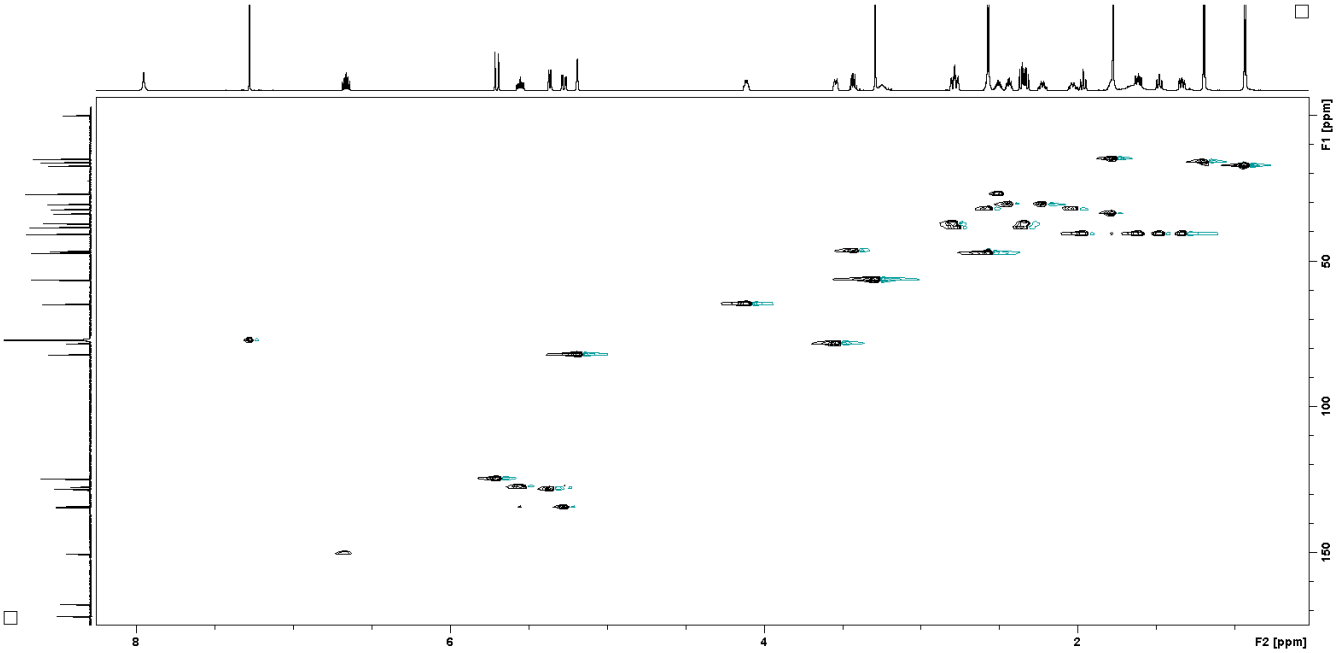
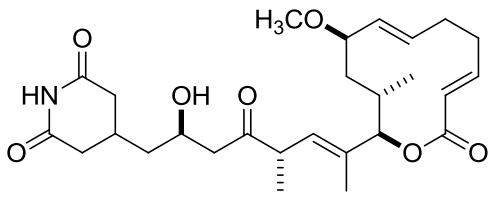
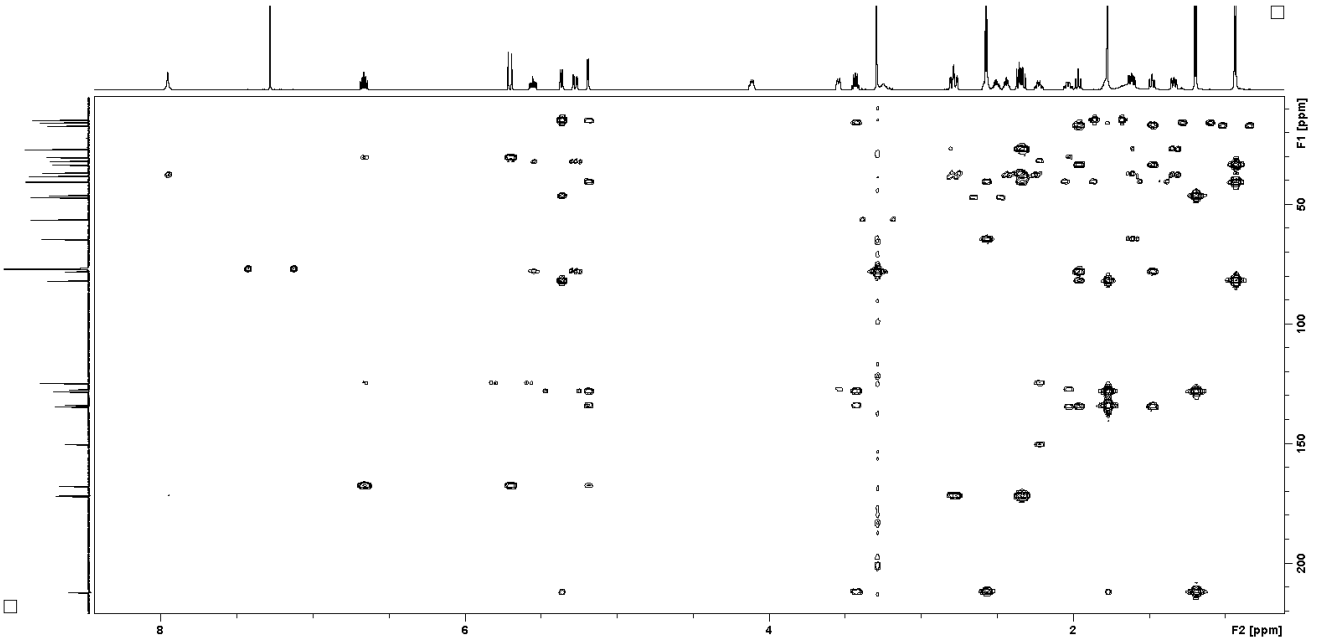


Figure S10. HSQC and HMBC spectra of 8,9-dihydro-8*R*-methoxyl-LTM (**17**) in $CDCl_3$







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