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for

A Polylinker Approach to Reductive Loop Swaps in Modular Polyketide Synthases

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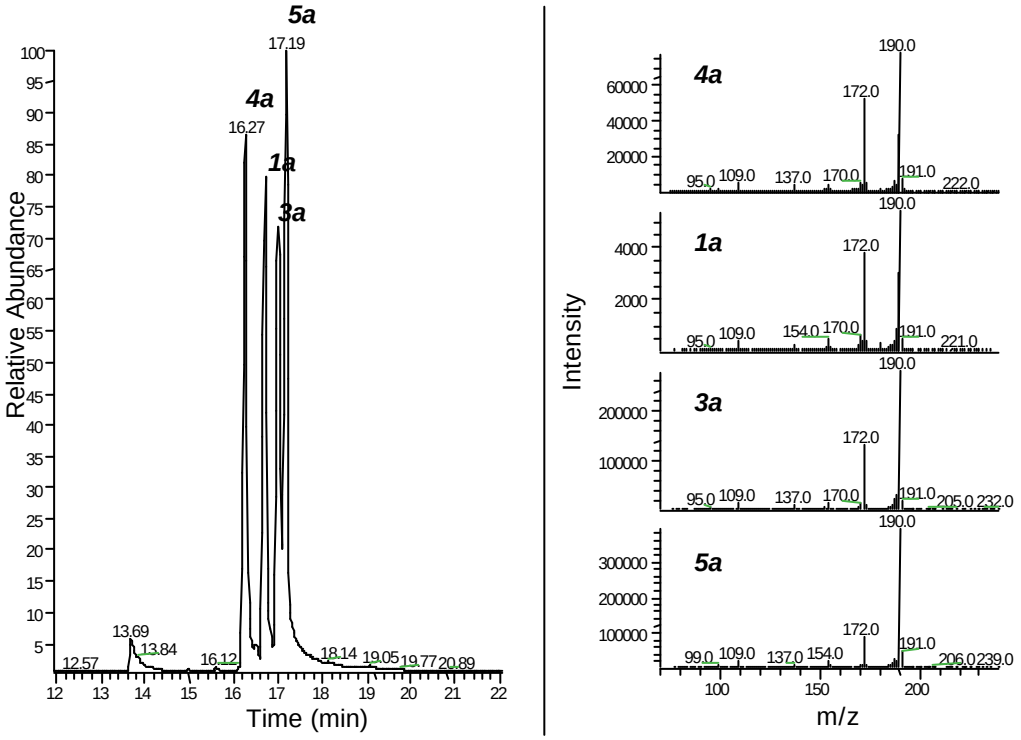

Figure S1. GC-MS of synthetic standard samples of each of the four diastereomeric 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactones **1a**, **3a**, **4a** and **5a** whose structures are shown in Fig. 3.

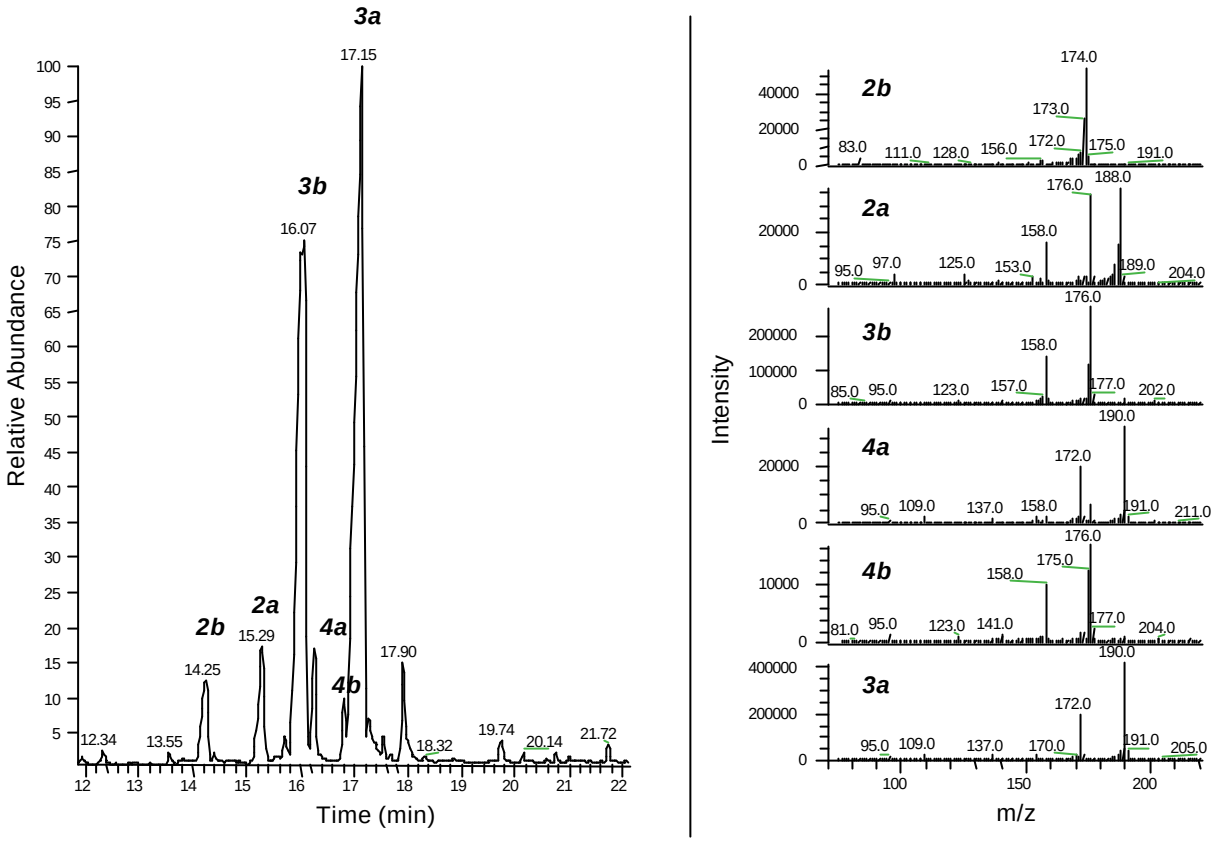

Figure S2. GC-MS separation and identification of triketide lactones from fermentation of *S. erythraea* JC2(pJLK27), containing a hybrid DEBS1-TE housing a reductive loop derived from the tylosin PKS (TYLS) module 1. The numbering of the compounds is exactly as in Figure 4, and their structures are given in Table 1 and Table 2. the **a** suffix refers to propionate starter unit-derived triketides and the **b** suffix refers to acetate unit-derived triketides.

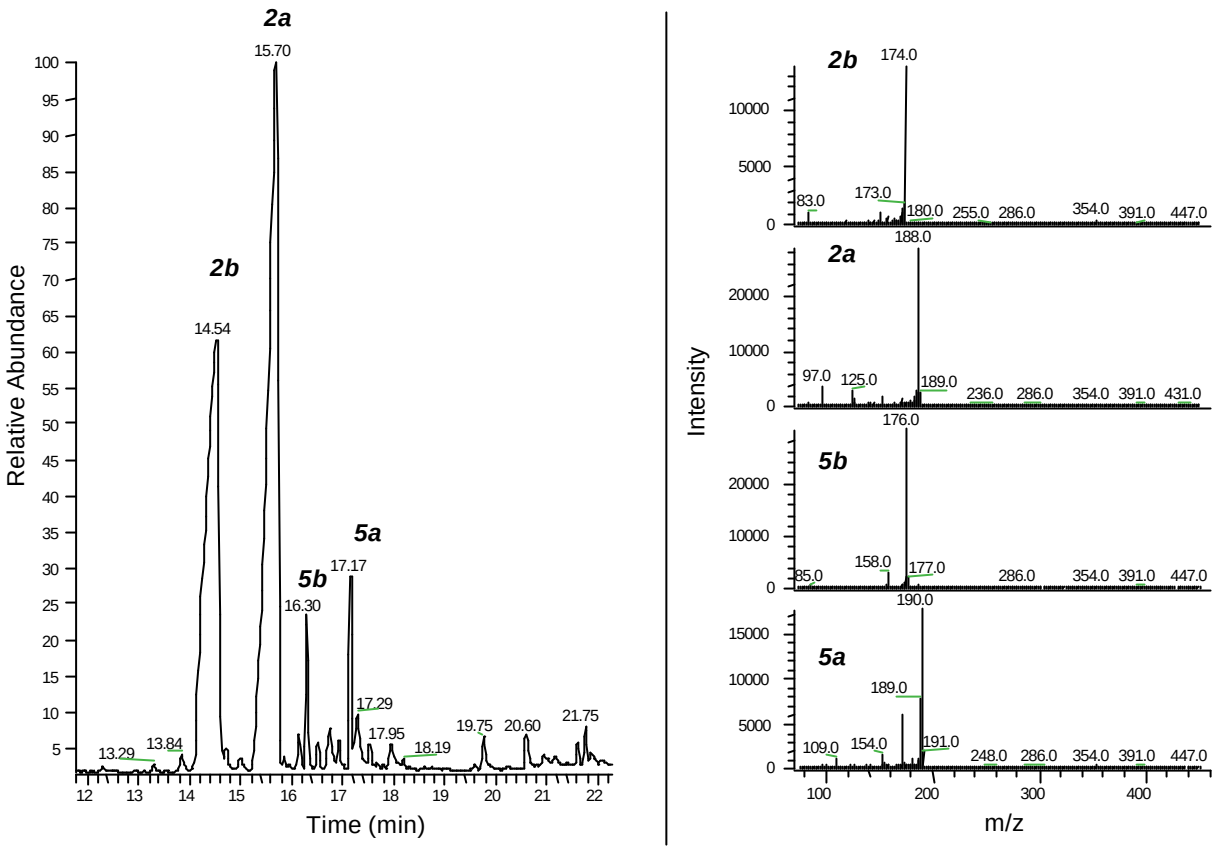

Figure S3. GC-MS separation and identification for triketide lactones from fermentation of *S. erythraea* JC2(pRIF7), containing a hybrid DEBS1-TE housing a reductive loop derived from rifamycin module 7. The numbering of the compounds is exactly as in Figure 5, and their structures are given in Tables 1 and 2. the **a** suffix refers to propionate starter unit-derived triketides and the **b** suffix refers to acetate unit-derived triketides.

Table S1. Synthetic oligonucleotides used in this study.

oLK1	5'-GGAGTACTGCGAGGGCGTGGGCAT-
oLK2	5'-GTGGATCCTGGCGAAGGGTCAGCTGG-
oLK3	5'-TACCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT-3'
oLK4	5'-ATGTTAACCGGTCGCGCAGGCTCTCCGTCT-3'
oLK5	5'-ATGTTAACGGGTCTGCCGCGTGCCGAGCGGAC-3'
oLK6	5'-CTTCTAGACTATGAATTCCCTCCGCCCAGC-3'
P1f	5'-CTAGGCCGGGCCGGACTGGTAGATCTGCCTACGTATCCTTTCCAG GGCAAGCGGTTCTGGCTGCAGCCGGACCGCACTAGTCCTCGTGAC GAGGGAGATGCATCGAGCCTGAGGGACCGGTT-3'
P1b	5'-AACCGGTCCCTCAGGCTCGATGCATCTCCCTCGTCACGAGGACTAGTGCGGTCCGGC TGCAGCCAGAACCGCTTGCCCTGGAAAGGATACGTAGGCAGATCTACCAGTCCGGCC CGG-3'
JLK25(ERY2)	
	5'-ATACTAGTTCCTCGTGACGAGCTCGACGG-3'
	5'-TAATGCATCCGGTTCTCCGGCCCGCTCGCT-3'
JCE1 (ERY1)	
	5'-CACCTAGGCACCGGAGCACGCCGGGTG-3'
	5'-ACGTTAACGCGCCCACCCGCGGTTCGG-3'
JCE5 (ERY5)	
	5'-GCCCTAGGCGACGCGCGGCCGGTCG-3'
	5'-CCGTTAACCGCTGGGCGAGCGCCGGG-3'
JLK27(RAP13)	
	5'-TACCTAGGCACCACCACAACCCGGGTA-3'
	5'-TACAATTGGCCCGCGAGTCCCCGACGCT-3'
JLK28 (RAP13)	
	5'-TAAGATCTTCCGACCTACGCCTTCCAAC-3'
	5'-TAATGCATCGACCTCGTTGCGTGCCGCGGT-3'
JLK41 (ERY4)	
	5'-ATAGATCTGCCTACGTACCCGTTCGAACACCAGCGCTTC-3'
	5'-ATCCTCAGGTTCGGCCCTGCCGCCTCGGCCTGCCCGGCGGCG CGCAGCTT-3'
JLK29 (RAP10)	
	5'-TAAGATCTTCCGACGTACGCGTTCCAGC-3'
	5'-ATGCTAGCCACTGCGCCGACGAATCACCGGTGG-3'
JLK35 (TYL1)	
	5'-TAAGATCTCCCTACGTACCCCTTCAACCAC-3'
	5'-GCTAGCCGCCGCGCCAGCTCGGGC-3'
JLK (RIF7)	
	5'-CCTACGTACGCCTTCGACCACCAGCACTT-3'
	5'-CGGCTAGCGGGCGTTCCAGGCCGCCGTCCT-3'
JLK (RIF8)	
	5'-CCTACGTACGCCTTCGACCACCAGCAC-3'
	5'-CGGCTAGCCGGCCCAGCAGCTGGTTG-3'
JLK30 (AVE1)	
	5'-CCTAGATCCGCCCACCTACCCCTTCCAACACCAG-3'
	5'-TGGGCTAGCGTTTTGTGCAACTCCGCCGGTGGAGTG-3'
GMS2 (AVE1)	
	5'-TGGCTGCAGAGCTCACAGCCGGGTGCCGGATCCGGTT-3'
	5'-TTTCCTCAGGTCCGCCGGTGGAGTGGGGCGCTGGAC-3'
JLK31 (AVE2)	
	5'-CCTAGATCTCCCCACCTACCCCTTCCAACACCACCACTACTG-3'
	5'-CCGGCTAGCCGGGCGTGCAGCTGGGCGCCGTTGTCCGCAC-3'
GMS4 (AVE2)	
	5'-CCCTACGTACCCCTTCCAACACCACTACTGGCTCGAAAG-3'
	5'-GGCCCTCAGGTGGGCGCCGTTGTCCGCACCACCGGTA-3'

Table S2. Strains and plasmids used in this study.

Plasmid	Description	Ref.[1]
CJR24	expression plasmid for DEBS1–TE	[1]
IG67		[2]
IG69		[2]
JLK01	PCR *Sca*I–*Avr*II of DEBS 1 (8206–8660) in pUC18	
JLK02	PCR *Avr*II–*Hpa*I of DEBS 1 (8655–10125) in pUC18	
JLK03	PCR *Hpa*I–*Xba*I of DEBS 1 (10119–11220) in pUC18	
JLK04	pJLK02 + pJLK03 (*Avr*II-*Xba*I fragment in pUC18)	
JLK05	pJLK01 + pJLK04 (*Sca*I-*Xba*I fragment in pUC18)	
JLK07	DEBS 1–TE with new *Avr*II (8663), *Hpa*I (10122) sites, based on pCJR24	
JLK08	PCR *Avr*II–*Bsu*36I of DEBS 1 (8655–10113) in pUC18	
JLK09	PCR *Bsu*36I/*Nhe*I–*Xba*I (10106–11220) in pUC18	
JLK10	pJLK08 + pJLK09 (inserts combined in pUC18)	
JLK13	DEBS 1–TE with new *Avr*II (8663), *Bsu*36I (10109) and *Nhe*I (10122) sites, based on pCJR24	
JLK114	pCJR24–based expression plasmid for DEBS 1–TE with polylinker (containing *Hpa*I) in place of KR_2 domain	
JLK115	as JLK114 but based on pUC18	
JLK116	as JLK115 but with *Nhe*I site instead of *Hpa*I site	
JLK117	pCJR24–based expression plasmid for DEBS 1–TE with polylinker (containing *Nhe*I) in place of KR_2 domain	
JLK118	PCR *Spe*I-*Nsi*I reductive loop of *ery* module 2 in pUC18	
JLK119.1	PCR *Avr*II-*Hpa*I reductive loop of *rap* module 13 in pUC18	
JLK120	PCR *Bgl*II-*Nsi*I reductive loop of *rap* module 13 in pUC18	
JLK121	PCR *Bgl*II *Nhe*I reductive loop of *rap* module 13 in pUC18	
JLK122	PCR *Avr*II-*Nhe*I reductive loop of *rap* module 13 in pUC18	
JLK23	PCR *Spe*I-*Nsi*I reductive loop of *ery* module 2 in pJLK115	
JLK24	PCR *Spe*I-*Nsi*I reductive loop of DEBS module 2 in pJLK116	
JLK25	as JLK23 but pCJR24-based	
JLK26	as JLK24 but pCJR24-based	
JLK27	JLK07 bearing *Avr*II-*Hpa*I reductive loop of *rap* module 13	
JLK28	JLK117 bearing *Bgl*II-*Nsi*I reductive loop of *rap* module 13	
JLK29	JLK117 bearing *Bgl*II-*Nhe*I reductive loop of *rap* module 13	
JLK30	JLK117 bearing *Bgl*II-*Nhe*I reductive loop of *ave* module 1, cloned from pIG67	
JLK31	JLK117 bearing BglII-*Nhe*I reductive loop of *ave* module 2, cloned from pIG69	
JLK32.3	PCR *Bgl*II-*Bsu*36I reductive loop *ery* module 4 cloned in pUC18	
JLK33.1	PCR *Bgl*II-*Nhe*I reductive loop of *tyl* module 1 cloned in pUC18	
JLK34	PCR *Pst*I-*Nsi*I reductive loop of *ery* module 4 in pUC18	
JLK35	JLK117 bearing *Bgl*II-*Nhe*I reductive loop of *tyl* module 1	
JLK38	JLK116 bearing *Bgl*II-*Bsu*36I insert from JLK32.3	
JLK41	pCJR24-based plasmid bearing *Bgl*II-Bsu36I insert from JLK32	
JLK42	pUC18-based plasmid bearing *Pst*I-*Nsi*I insert from JLK34	
JLK142	PCR *Bgl*II-*Nhe*I of *rap* module 1 in pUC18	
JCR1	JLK117 bearing *Bgl*II-*Nhe*I reductive loop of *rap* module 1	
JCR4	JLK114 bearing *Avr*II-*Hpa*I reductive loop of *rap* module 4	
JCE1	JLK114 bearing *Avr*II-*Hpa*I reductive loop of *ery* module 1	
JCRf7	JLK117 bearing *Sna*BI-NheI reductive loop of *rif* module 7	
JCRf8	JLK117 bearing *Sna*BI-NheI reductive loop of *rif* module 8	
GMS2	JLK114 bearing *Pst*I-*Bsu*36I reductive loop of *ave* module 1	
GMS4	JLK114 bearing *Pst*I-*Bsu*36I reductive loop of *ave* module 2	
S. erythraea JC2	deletion mutant of *S. erythraea* NRRL2338 lacking all *eryA* genes except for *eryAIII* TE [5]	

[1]All plasmids and strains were generated in this study unless otherwise referenced.

Construction of plasmid pJLK114

First, a 1.47 kbp DNA fragment (encoding the KR2 domain) was amplified by PCR from the *eryAI* gene encoding DEBS 1, using plasmid pNTEP2[3] as template, and the mutagenic oligonucleotides oLK3 and oLK4 (Table S1) which introduced *Avr*II and *Hpa*I restriction sites respectively, treated with T4 polynucleotide kinase and cloned into *Sma*I-linearised pUC18 to give pJLK02. A 1.12 kbp 3' flanking region of the *eryAI* gene was similarly amplified by PCR using the mutagenic oligonucleotides oLK5 and oLK6 (Table S1), introducing *Hpa*I and *Xba*I sites, and inserted into *Sma*I-cut pUC18 to give pJLK03. The insert from pJLK02 was excised with *Pst*I and *Hpa*I and cloned into similarly cut pJLK03 to give pJLK04. A 460 bp 5' flanking region of the *eryAI* gene was amplified, flanked by a natural *Sca*I restriction site and an engineered *Avr*II site, using mutagenic oligonucleotides oLK1 and oLK2 and likewise cloned into *Sma*I-cut pUC18 to give pJLK01. The insert from this was then excised as a *Pst*I-*Avr*II fragment and ligated into similarly-cut pJLK04 to give pJLK05. The *Sca*I-*Xba*I insert from pJLK05, encoding the C-terminal portion of DEBS 1–TE, was then used in a three-way ligation with the *Nde*I-*Sca*I fragment of pNTEP2, encoding the N–terminal portion of DEBS 1–TE, and the plasmid backbone excised from pCJR24 using *Nde*I–*Xba*I, to obtain plasmid pJLK07. This plasmid was digested with *Avr*II and *Hpa*I in order to replace the *Avr*II–*Hpa*I fragment with a polylinker created by annealing together synthetic oligonucleotides P1f and P1b (Table S1), to create plasmid pJLK114.

Construction of plasmid pJLK117

To do this, the *Avr*II–*Hpa*I insert of pJLK02 was amplified by PCR to give an *Avr*II–*Bsu*36I fragment, which was cloned into pUC18 to give pJLK08; while the *Hpa*I–*Xba*I fragment of pJLK03 was amplified by PCR using oligonucleotides to give a *Bsu*36I–*Xba*I fragment, introducing an *Nhe*I site next to the 5' *Bsu*36I site, and cloned into pUC18 to give pJLK09. The insert from pJLK08 was then excised as a *Pst*I–*Bsu*36I fragment and ligated into similarly cut pJLK09 to give pJLK10, which thus contains the desired modified *Avr*II-*Xba*I fragment encoding the C-terminal portion of DEBS 1–TE. This was used to replace the corresponding DNA fragment of pJLK07 to give pJLK13. The 9.9 kbp insert from pJLK114 was excised with *Nde*I and *Xba*I and cloned into pUC18 previously cut with the same enzymes, to give pJLK115. This plasmid was digested with *Bsu*36I and *Xba*I and the corresponding Bsu36I–*Xba*I fragment from pJLK13 was ligated in its place to yield pJLK116. Finally, the full-

length DEBS 1–TE gene was excised from pJLK116 as an *Nde*I–*Xba*I fragment and cloned into similarly–cut pCJR24 to obtain expression plasmid pJLK117, which contains a second version of the polynucleotide linker in place of the KR_2 domain.

Use of plasmids pJLK114 and pJLK117 for construction of *S. erythraea* JC2/ pJLK114 and JC2/pJLK117 and the production of triketide lactone compounds

Approximately 5 μg plasmid pJLK114 was used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton-resistant colonies were isolated. From several colonies, total DNA was isolated and analysed by Southern blot hybridisation to confirm that the plasmid had integrated into the chromosomal *ery* TE gene. JC2/pJLK114 was plated onto SM3 agar (5 g glucose,50 g MD30E maltodextrin, 25 g Arkasoy soy flour, 3 g molasses (beet), 0.25 g K_2HPO_4, 2.5 g $CaCO_3$, 22 g agar and distilled water to 1 L (pH 7.0) containing 50 μg/L thiostrepton and allowed to grow for 12 days at 30 °C, before extraction of triketides for GC-MS analysis..

Construction of plasmid pJLK25

Plasmid pJLK25 is a pJLK114–based plasmid in which the DNA fragment encoding the reductive loop of the second module of the erythromycin PKS (DEBS) is inserted into the multiple cloning site. It was constructed via several intermediate plasmids as follows:

The ~1.4 kbp DNA fragment of the *eryAI* gene of *S. erythraea* encoding the reductive loop of module 2 was amplified by PCR using the mutagenic primers given in Table S1. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and individual colonies were screened for their plasmid content. The desired plasmid pJLK118 was identified by its restriction pattern and by DNA sequencing.

Plasmid pJLK118 was digested with *Nde*I and *Xba*I and the ~11.2 kbp fragment was ligated with plasmid pCJR24 which had been digested with *Nde*I and *Xba*I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and the desired plasmid pJLK25 was identified by its restriction pattern.

Approximately 5 μg of plasmid pJLK25 was used to transform protoplasts of *S. erythraea* JC2 and stable thiostrepton-resistant colonies were isolated and checked for

correct integration of the DEBS 1-TE gene. The growth and conditions of analysis of the recombinant *S. erythraea* were as for JC2/JLK114 (nomenclature). The major products were identified by GC-MS analysis and comparison with authentic material as (2*R*,3*S*,4*S*,5*R*)- 3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactone and (2*R*,3*S*,4*S*,5*R*)- 3,5-dihydroxy-2,4-dimethyl-*n*-hexanoic acid δ-lactone.

Construction of plasmid pJLK28, of strain JC2/pJLK28 (nomenclature) and fermentation to produce triketides

Plasmid pJLK28 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 13 of the rapamycin PKS has been inserted in the multiple cloning site. It was constructed as follows:

The ~3.2 bp DNA fragment of the *rapC* gene[4] of *Streptomyces hygroscopicus* encoding the reductive loop of the rapamycin PKS module 13 was amplified by PCR using the appropriate mutagenic primers as shown in Table S1. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK120 was identified by its restriction pattern and by DNA sequencing.

Plasmid pJLK120 was digested with *Bgl*II and *Nsi*I and the ~3.2 kbp fragment was ligated with plasmid pCJR24 which had been digested with *Bgl*II and *Nsi*I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B cells and the desired plasmid pJLK28 was identified by its restriction pattern. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pJLK28 on SM3 agar for 12 days, extraction of the triketide products and GCMS analysis showed that the major products were (2*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-heptanoic acid *d*-lactone and (2*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid *d*-lactone.

Construction of plasmid pJLK41, of strain JC2/pJLK41 and fermentation to produce triketides

Plasmid pJLK41 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 4 of the erythromycin PKS has been inserted into the multiple cloning site. It was constructed as follows:

The ~3.2 kbp DNA segment of the *eryAII* gene of *S. erythraea* encoding the reductive loop of module 4 was amplified by PCR using the mutagenic primers as listed in Table S1 and cosmid 4B (cosmid containing the erythromycin PKS) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK32.3 was identified by its restriction pattern and by DNA sequencing.

Plasmid pJLK32.3 was digested with *Bgl*II and *Bsu*36I and the 3.2 kbp fragment was ligated with plasmid pJLK116 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK38 was identified by its restriction pattern and by DNA sequencing. Plasmid pJLK38 was digested with *Nde*I and *Xba*I and the ~13 kbp fragment was ligated with plasmid pCJR24 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK41 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pJLK41 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed that the major products were (2*S*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactone and (2*S*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid δ-lactone.

Construction of plasmid pJLK29, of strain JC2/pJLK29 and fermentation to produce triketides

Plasmid pJLK29 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 10 of the rapamycin PKS has been inserted into the multiple cloning site. It was constructed as follows:

The ~2.2 kbp DNA segment of the *rapB* gene of *S. hygroscopicus* encoding the reductive loop of module 4 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1 and using as a template a ~7 kbp restriction fragment obtained by cutting cosmid cos 26[4] with *Sca*I and *Sph*I. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the de-

sired plasmid pJLK121.1 was identified by its restriction pattern and by DNA sequencing.

Plasmid pJLK121.1 was digested with *Bgl*II and *Nhe*I and the 2.2 kbp fragment was ligated with plasmid pJLK117 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK29 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pJLK29 in 30 mL of SM3 medium containing 5 μg/ml thiostrepton in a 250 mL flask with a single spring to reduce clumping, shaken at 250 rpm and at 30 °C for 8 days, the broth was centrifuged, the supernatant adjusted to pH 3 and extracted three times with an equal volume of ethyl acetate. The solvent was removed by evaporation and the residue dissolved in methanol for GC-MS analysis after conversion to the methyl ester with either diazomethane or trimethylsilyl diazomethane. GC-MS analysis showed that the major products were (4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hept-2-enoic acid and (2*S*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hex-2-enoic acid.

Construction of plasmid pRIF7, of strain JC2/pRIF7 and fermentation to produce triketides

Plasmid pJLK35 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 7 of the rifamycin PKS has been inserted into the multiple cloning site. It was constructed as follows:

The ~2.1 kbp DNA segment of the rifamycin PKS genes of *Amycolatopsis mediterranei* encoding the reductive loop of module 7 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1 and cosmid 6 (a cosmid starting at 35727 and going beyond 76199, numbering according to accession number AF-040570) as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pUCRIF7 was identified by its restriction pattern and by DNA sequencing.

Plasmid pUCRIF7 was digested with *Sna*BI and *Nhe*I and the 2.1 kbp fragment was ligated with plasmid pJLK117 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the de-

sired plasmid pRIF7 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pRIF7 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed that the major products were (2*S*,3*S*,4*S*,5*R*)-3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid *d*-lactone and (2*S*,3*S*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid *d*-lactone.

Construction of plasmid pJLK30, of strain JC2/pJLK30 and fermentation to produce triketides

Plasmid pJLK30 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 1 of the avermectin PKS has been inserted into the multiple cloning site using *Bgl*II and *Nhe*I sites. It was constructed as follows:

The ~1.7 kbp DNA segment of the gene of the avermectin PKS of *Streptomyces avermitilis* encoding the reductive loop of module 1 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1, and as template plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pIG67 was identified by its restriction pattern and by DNA sequencing.

Plasmid pIG67 was digested with *Bgl*II and *Nhe*I and the 1.7 kbp fragment was ligated with plasmid pJLK117 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK30 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pJLK30 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed that the major products were (2*R*,3*R*,4*S*,5*R*)-3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactone and (2*R*,3*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid δ-lactone.

Construction of plasmid pGMS2, of strain JC2/pGMS2 and fermentation to produce triketides

Plasmid pGMS2 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 1 of the avermectin PKS has been inserted into the multiple cloning site using *Pst*I and *Bsu*36I sites. It was constructed as follows:

The ~1.7 kbp DNA segment of the gene of the avermectin PKS of *Streptomyces avermitilis* encoding the reductive loop of module 1 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1 and as template plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pIG68 was identified by its restriction pattern and by DNA sequencing.

Plasmid pIG68 was digested with *Pst*I and *Bsu*36I and the 1.7 kbp fragment was ligated with plasmid pJLK117 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pGMS1 was identified by its restriction pattern and by DNA sequencing. Plasmid pGMS1 was digested with *Nde*I and *Xba*I and the ~11.5 kbp fragment was ligated with plasmid pCJR24 digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pGMS2 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pGMS2 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed that the major products were (2*R*,3*R*,4*S*,5*R*)-3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lac-tone and (2*R*,3*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid δ-lactone.

Construction of plasmid pJLK31, of strain JC2/pJLK31 and fermentation to produce triketides

Plasmid pJLK31 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 2 of the avermectin PKS has been inserted into the multiple cloning site using *Bgl*II and *Nhe*I sites. It was constructed as follows:

The ~2.4 kbp DNA segment of the gene of the avermectin PKS of *Streptomyces avermitilis* encoding the reductive loop of module 1 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1, and as template plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pIG69 was identified by its restriction pattern and by DNA sequencing.

Plasmid pIG69 was digested with *Sna*BI, *Bsu*36I and *Dra*I and the 2.4 kbp fragment was ligated with plasmid pJLK117 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid was identified by its restriction pattern and by DNA sequencing. This plasmid was digested with *Nde*I and *Xba*I and the ~12.4 kbp fragment was ligated with pCJR24 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK31 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pJLK31 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed that the major products were (2*R*,3*R*,4*S*,5*R*)-3,5-dihydroxy-2,4-dimethyl-*n*-heptanoic acid δ-lactone and (2*R*,3*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid δ-lactone.

Construction of plasmid pGMS4, of strain JC2/pGMS4 and fermentation to produce triketides

Plasmid pGMS4 is a pJLK117-based plasmid except that the DNA fragment encoding the reductive loop of module 2 of the avermectin PKS has been inserted into the multiple cloning site using *Sna*BI and *Bsu*36I sites. It was constructed as follows:

The ~2.4 kbp DNA segment of the gene of the avermectin PKS of *Streptomyces avermitilis* encoding the reductive loop of module 2 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1 and as template plasmid pIG155, which contains the first two modules of the avermectin PKS cloned into plasmid pT7-7. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated

with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pIG70 was identified by its restriction pattern and by DNA sequencing.

Plasmid pIG70 was digested with *Sna*BI, *Bsu*36I and *Dra*I and the 2.4 kbp fragment was ligated with plasmid pJLK116 which had been digested with *Sna*BI and *Bsu*36I. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pGMS3 was identified by its restriction pattern and by DNA sequencing. Plasmid pGMS3 was digested with *Nde*I and *Xba*I and the ~12.4 kbp fragment was ligated with pCJR24 digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pGMS4 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done as for JC2/pJLK114. After growth of the recombinant strain JC2/pGMS4 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed only faint traces of triketide lactones.

Construction of plasmid pJLK27, of strain JC2/pJLK41 and fermentation to produce triketides

Plasmid pJLK27 is a pJLK114-based plasmid except that the DNA fragment encoding the reductive loop of module 13 of the rapamycin PKS has been inserted into the multiple cloning site. It was constructed as follows:

The ~3.2 kbp DNA segment of the *rapC* gene of *S. hygroscopicus* encoding the reductive loop of module 13 was amplified by PCR using the appropriate mutagenic primers as listed in Table S1 and cosmid 31[4] as template. The PCR product was treated with T4 polynucleotide kinase and then ligated with plasmid pUC18 which had been linearised by digestion with *Sma*I and then treated with alkaline phosphatase. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK120a was identified by its restriction pattern and by DNA sequencing.

Plasmid pJLK120a was digested with *Avr*II and *Hpa*I and the 3.2 kbp fragment was ligated with plasmid pJLK114 which had been digested with the same enzymes. The ligation mixture was used to transform electrocompetent *E. coli* DH10B and the desired plasmid pJLK27 was identified by its restriction pattern and by DNA sequencing. Transformation of *S. erythraea* JC2 and selection for the desired integrants was done

as for JC2/pJLK114. After growth of the recombinant strain JC2/pJLK41 on SM3 agar for 12 days, extraction of the triketide products and GC-MS analysis showed that the major products were (2*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-heptanoic acid *d*-lactone and (2*R*,4*S*,5*R*)-5-hydroxy-2,4-dimethyl-*n*-hexanoic acid *d*-lactone.

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