



Tandem assembly of the epothilone biosynthetic gene cluster by *in vitro* site-specific recombination

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We describe a site-specific recombination-based tandem assembly (SSRTA) method for reconstruction of biological parts in synthetic biology. The system was catalyzed by *Streptomyces* phage ϕ BT1 integrase, which belongs to the large serine recombinase subfamily. This one-step approach was efficient and accurate, and able to join multiple DNA molecules *in vitro* in a defined order. Thus, it could have applications in constructing metabolic pathways and genetic networks.

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Synthetic biology is more ambitious than conventional genetic engineering, and aims to design and reconstruct biological systems or even entire bacterial genomes. An essential technique in synthetic biology is the physical assemble of multiple small DNA fragments into large constructs with a defined order and orientation^{1,2}. Traditional methods rely on restriction enzyme digestion followed by ligation, and while this approach works well for the insertion of a single DNA sequence into a vector, as well as being time-consuming, it is often hard to find enough distinct restriction sites for the cloning of multiple DNA fragments¹. Two techniques, BioBrickTM developed at MIT³ and BglBrick developed at UC Berkeley⁴, standardized the process of DNA assembly by using standard restriction sites. Biological parts were flanked by four restriction sites (two on each side) and the assembly products of the first round can subsequently be used in the next round, thus permitting the assembly of multiple parts.

The BioBrick² method is limited by a number of ‘forbidden sites’. This has resulted in the development of a series of ligation-independent cloning (LIC) strategies that offered sequence-independent assembly. These include SLIC (sequence and ligase independent cloning)⁵, In-FusionTM (Clontech) technologies⁶, the ‘Gibson’ isothermal assembly method⁷ and CPEC (circular polymerase extension cloning)^{8,9}. These methods are dependent on overlapping homologous sequences at the ends of the DNA fragments^{1,2}. SLIC, In-FusionTM and the Gibson isothermal assembly method use a mechanism called ‘chew back and anneal’^{2,7,10,11}, while CPEC relies on annealing of complimentary DNA regions and a single high-fidelity PCR cycle⁹. Using the isothermal assembly method, Gibson and his colleagues successfully assembled three 5-kb DNA molecules using fragments of only 40 bp overlaps. This method was also used to assemble a 900-kb molecule *in vitro*⁷. The Gibson method was also effective in gene synthesis, and an entire 16.3-kilobase mouse mitochondrial genome was synthesized through three rounds of assembly from 600 overlapping 60-mers¹².

Several competing approaches using type II restriction enzymes have also been developed, such as Golden Gate cloning^{13,14} and Pairwise Selection Assembly (PSA)¹⁵. Since type II restriction enzymes cut outside of their recognition sequence, through proper design of the cleavage sites this approach is scarless¹⁴. Using Golden Gate cloning, Weber *et al*¹³ assembled a 33-kb DNA molecule from 44 individual parts in three steps. Blake *et al*¹⁵ used PSA to reconstruct a completely synthetic 91-kb molecule in six rounds of assembly. In general, assembly of DNA fragments occurs on three scales – ‘parts to genes’, ‘genes to pathway’ and ‘pathways to genomes’² – and each assembly strategy described above has its own advantages and disadvantages at different scales^{1,2}.

We have developed an alternative strategy for the assembly of multiple DNA segments in one reaction based on ϕ BT1 integrase-mediated recombination *in vitro*, termed site-specific recombination-based tandem assembly (SSRTA). We previously established a site-specific recombination system based on *Streptomyces* phage ϕ BT1 integrase-mediated integration *in vitro*¹⁶, and identified 16 pairs of non-compatible *attB* and *attP* recombination sites¹⁷. The ϕ BT1 integration system is highly efficient and accurate because no recombination can occur between

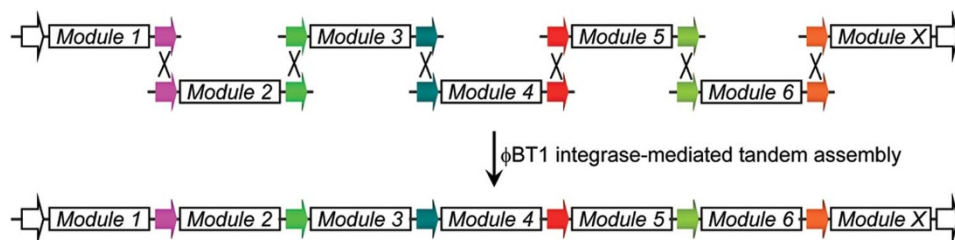


Figure 1 | Site-specific recombination-based tandem assembly *in vitro*. Multiple DNA modules were flanked by pairs of non-compatible recombination sites of the ϕ BT1 integration system. A series of mutated *attB* sites were placed upstream of each module, and mutated *attP* sites were located downstream. After incubation of all the DNA modules with ϕ BT1 integrase, tandemly assembled products are produced in a one-step reaction. Differently coloured arrows represented different pairs of recombination sites.

att sites with different central di-nucleotides¹⁷. The SSRTA method is sequence-independent, and excellent for the combinatorial assembly of multiple biological parts in a defined order, even with those with high GC contents (70%). The utility of SSRTA was demonstrated in the assembly of an entire epothilone biosynthetic gene cluster (62.4 kb) from ten individual parts.

Results

The recombination efficiencies of ϕ BT1 integrase-catalyzed reactions between *attB* and *attP* sites *in vitro* were over 90% in our previous studies^{16,17} and could therefore be used for establishing a robust DNA assembly approach. This system contains 16 pairs of mutated recombination sites, the purified ϕ BT1 integrase and a simple buffer (see Materials and Methods). Once the target DNA modules have been flanked by a pair of non-compatible *att* sites, multiple fragments can be assembled in one reaction simultaneously at 30°C *in vitro* (Fig. 1). Because site-specific recombination occurs by precise breakage-joining events and do not involve any DNA synthesis or loss, this method is highly accurate, and no errors are introduced after assembly.

We selected the epothilone biosynthetic gene cluster to test this SSRTA method. Epothilone polyketides are promising anti-cancer drugs with remarkable microtubule-stabilizing activity. The total length of the gene cluster is nearly 56 kb, and consists of six open reading frames (*epoA* to *epoF*) and ten modules, including one loading module (LM), one non-ribosomal peptide synthetase (NRPS) module and eight polyketide synthase (PKS) modules. Modules 3 to 6 form a whole ORF defined as *epoD*, while modules 7 and 8 form *epoE*¹⁸ (see Supplemental Fig. S1). *EpoK*, a cytochrome P450 involved in epoxidation of epothilones C and D to A and B¹⁹, was not included in this work. The purified genomic DNA of the epothilone-producing myxobacterial strain *Sorangium cellulosum* So0157-2 was used as the template for PCR amplification of the modules²⁰. Due to the high GC content (69.5%) of the epothilone biosynthetic gene cluster, we adopted an ‘entry clone’ strategy to place individual modules into a series of vectors containing the appropriate pairs of recombination sites beforehand, instead of engineering the recombination sites into the 5′-terminus of PCR primers.

We constructed a series of ‘entry vectors’ with the apramycin resistance gene (*aac(3)IV*) flanked by a pair of recombination sites. We also put two *XcmI* recognition sites close to the recombination sites. After the vectors were digested with *XcmI*, the target DNA modules were inserted into the ‘entry vectors’ through TA cloning (Fig. 2a). The whole epothilone biosynthetic gene cluster was divided into small parts to facilitate amplification by high-fidelity PCR (PrimerSTARTM HS DNA polymerase, Takara) (Supplemental Fig. S1). Through PCR amplification and sub-cloning, we constructed nine independent ‘entry clones’ which contained all the essential genes for epothilones production. The ribosomal binding site sequences were fused in the 5′-terminus of each open reading frame (ORF) by PCR amplification (Supplemental Fig. S1). After

linearization by restriction enzymes, these ‘entry clones’ were ready for tandem assembly *in vitro*. It should be noted that the genes in each entry clone can be replaced by any other desired module. The details of the construction process are described in Materials and Methods, Supplemental Fig. S1 and Tables S1 and S2. Plasmid pZLE10 was designed to propagate the circular assembly products in *Escherichia coli*, and then to integrate into the *Streptomyces* genome for epothilone production (Fig. 2b).

The construction process was divided into two steps: first we combined *epoD* DNA sequences from five individual clones and removed the scar sequences inside the ORF. Then we assembled all the epothilone biosynthetic genes from seven individual clones in one reaction. First we tested the ability to combine *epoD* from four ‘entry clones’ (Fig. 2c). The plasmids pTA0006-M3-*aphII*, pTA0613-M4, pTA1303-M5, pTA0315-M6 and pZLE10 were linearized (Supplemental Fig. S2) and incubated with ϕ BT1 integrase overnight (or over 8 hrs) at 30°C. As shown in Fig. 3a, multiple assembly products were obtained (the correct, complete assembly product was a 29.8-kb plasmid). The *in vitro* reaction products were transformed into *E. coli* strain DH10B by electroporation. After selection with apramycin and neomycin, resistant clones were isolated and the plasmid DNA was extracted. PCR analysis of the linker regions between modules and restriction enzyme digestion indicated that the five segments had assembled correctly (Fig. 3b, c, Supplemental Fig. S3 and S4). We next explored the possibility of combining seven segments in a one-step reaction to generate pZLE10-*epo* (Fig. 2d, Supplemental Fig. S2 and S4). As shown in Fig. 3d-f, we obtained the assembled plasmid (62.4 kb) successfully. Thus, this tandem assembly system can be used to assemble at least seven DNA segments into a circular molecule *in vitro*.

Discussion

DNA assembly provides a means for extensively changing the genetics of entire biological pathways and cells. This may be useful for creating organisms capable of more than overproduce individual proteins – a common goal of cut-and-paste cloning techniques. Several successful approaches have been described that achieve this aim, such as BioBrick standard assembly³, the ‘Gibson’ isothermal assembly method^{7,12} and the Golden Gate assembly method¹⁴. In this manuscript we have demonstrated the utility of the SSRTA method by reconstructing the epothilone biosynthetic gene cluster. Seven independent DNA fragments were assembled into a circular plasmid (62.4 kb) through a one-step *in vitro* incubation. An ideal assembly method should have no forbidden sites, would be suitable for combinatorial assembly of different parts from standard modules, and importantly, would allow assembly of the parts in a defined order². The SSRTA method described here is based on site-specific recombination between a series of non-compatible *attB* and *attP* sites and so concerns about forbidden sites are not relevant. It is notable that the epothilone biosynthetic gene cluster contains ten direct repeat sequences larger than 100 bp, including a 554-bp direct repeat. Many

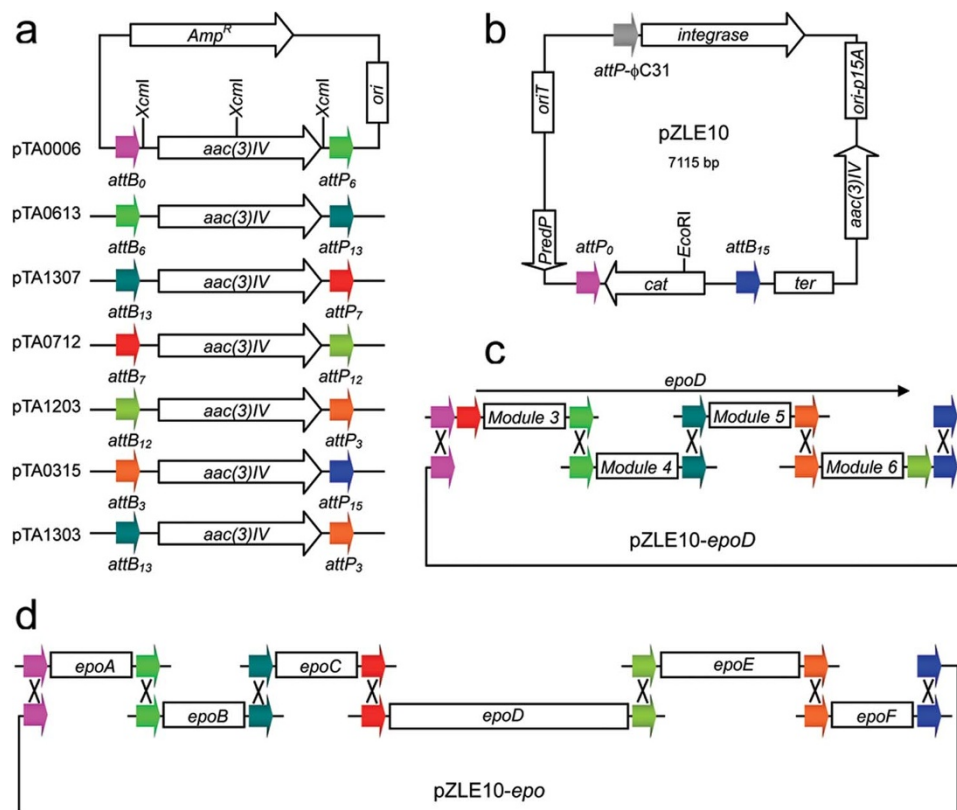


Figure 2 | Strategy for assembling the epothilone biosynthetic gene cluster by SSRTA. (a) Map of seven ‘entry vectors’ used in this study. Arrows in pink, green, dark green, red, lime, orange and blue represent pairs of recombination sites numbered 0, 6, 13, 7, 12, 3, and 15, respectively. (b) Map of plasmid pZLE10. Details are available in the Online Methods and Supplementary Table 2. (c) Schematic of the tandem assembly of pZLE10-*epoD*. Five groups of recombination reactions occur simultaneously, between *attB*₀/*attP*₀, *attB*₆/*attP*₆, *attB*₁₃/*attP*₁₃, *attB*₇/*attP*₇ and *attB*₁₅/*attP*₁₅. (d) Schematic of pZLE10-*epo* assembly. Seven groups of recombination reactions take place, between *attB*₀/*attP*₀, *attB*₆/*attP*₆, *attB*₁₃/*attP*₁₃, *attB*₇/*attP*₇, *attB*₁₂/*attP*₁₂, *attB*₃/*attP*₃ and *attB*₁₅/*attP*₁₅.

antibiotic biosynthetic gene clusters contains such direct repeats^{21,22}. Homology-based assembly methods may not be appropriate in these cases, but SSRTA has proved successful. Furthermore, each ‘entry vector’ could be used to build libraries of biological parts. These parts could be reconstructed in a defined order through combinatorial assembly (Supplemental Fig. S5). PCR products could also be directly used in this SSRTA method. Details of the pairs of non-compatible recombination sites and the sequences of PCR primers are described in Supplemental Fig. S6. The shortcoming of the SSRTA method is that it introduces scar sequences (*attR*, 42 bp) between modules after assembly, although scar sequences between genes may not be problematic².

Synthetic biology holds promise in the development of cheaper drugs and ‘green’ biofuels, efficient environmental remediation and targeted therapies for diseases^{23,24}. This SSRTA method provides an alternative way to rebuild genetic pathways and networks based on a phage-encoded large serine recombinase. Since nearly ten of these recombinases have been biochemically characterized^{25,26}, this method could be widely adopted in other systems. This would also allow applications that combine multiple SSRTAs.

Methods

System design and construction of the ‘entry vectors’. A major feature of this SSRTA system is the use pairs of non-compatible recombination sites. Non-compatible sites cannot recombine with each other, and the stringent control that this offers is the main strength of the system (Fig. 1). To assemble the full-length epothilone biosynthetic gene cluster (see supplemental Fig. S1) from six independent clones, seven pairs of *attB*/*attP* sites are needed. We chose pairs 0, 3, 6, 7, 12, 13 and 15, the sequences of which are given in our previous report¹⁷. These recombination sites were used to construct seven ‘entry vectors’, named pTA0006, pTA0613, pTA1307, pTA0712, pTA1203, pTA0315 and pTA1303 (Fig. 2a). pTA0006 was

generated using primers PT1 (containing *attB*₀) and PTF (containing *attP*₆) to amplify the apramycin-resistance gene (*aac*(3)*IV*), and the PCR product was inserted into pMD19-T by TA cloning. Similarly, primers PTB6 (containing *attB*₆) and PTP13 (containing *attP*₁₃) were used to construct pTA0613; primers PTB13 (containing *attB*₁₃) and PTP7 (containing *attP*₇) were used to construct pTA1307; primers PTB7 (containing *attB*₇) and PTP12 (containing *attP*₁₂) were used to construct pTA0712; primers PTB12 (containing *attB*₁₂) and PTP3 (containing *attP*₃) were used to construct pTA1203; primers PTB3 (containing *attB*₃) and PTP15 (containing *attP*₁₅) were used to construct pTA0315; and primers PTB13 (containing *attB*₁₃) and PTP3 (containing *attP*₃) were used to construct pTA1303 (Supplemental Table S1 and S2). The apramycin-resistance gene was cloned into the ‘entry vectors’ in the same orientation in each case, as shown in Fig. 2a.

Construction of the ‘entry clones’ and destination vector. The complete epothilone biosynthetic gene cluster was divided into 17 parts for PCR amplification (Supplemental Fig. S1). To clone *epoA*, a 4289-bp DNA fragment was amplified using primers 00a and 00b, and then inserted into pTA0006 by TA cloning to generate pTA0006-M0; for *epoB*, a 4256-bp DNA fragment was amplified using primers 01a and 01b, and then inserted into pTA0613 to generate pTA0613-M1; for *epoC*, a 5525-bp DNA fragment was amplified using primers 02a and 02b, and then inserted into pTA1307 to generate pTA1307-M2; for module 3, a 4766-bp DNA fragment was amplified using primers 03a and 03b, and then inserted into pTA0006 to generate pTA0006-M3. Module 4 was divided into two parts, M4a (primers 04a/ZE05, 3252 bp) and M4b (primers ZE06/04b, 3220 bp); these were amplified and cloned into pTA0613 separately, and then combined into one plasmid to generate pTA0613-M4 by enzyme digestion and ligation. Modules 5, 6, 7 and 8 were each divided into two parts. M5a (primers 05a/ZE07, 3607 bp) and M5b (primers ZE08/05b, 2767 bp) were PCR amplified and cloned into pTA1303 separately, and then combined into one plasmid to generate pTA1303-M5. M6a (primers 06a/ZE09, 2490 bp) and M6b (primers ZE10/06b, 2611 bp) were PCR amplified and cloned into pTA0315 separately, and then combined into one plasmid to generate pTA0315-M6. M7a (primers 07a/ZE11, 3506 bp) and M7b (primers ZE12/07b, 2683 bp) were PCR amplified and cloned into pTA1203 separately. M8a (primers 08a/ZE13, 2649 bp) and M8b (primers ZE14/08b, 3188 bp) were PCR amplified and cloned into pTA1203 separately. Plasmids containing genes from modules 7 and 8 were combined together by ligation after enzyme digestion to generate pTA1203-*epoE*. Module 9 was divided

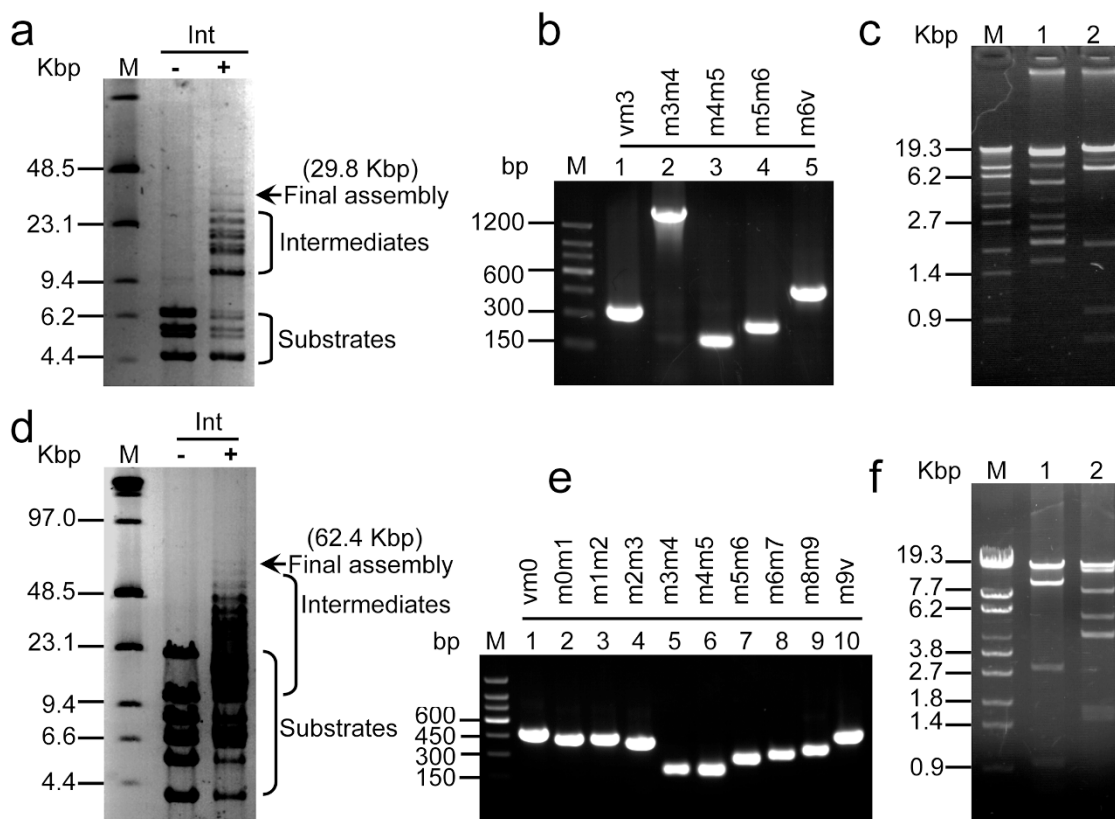


Figure 3 | Identification of the assembly products. (a) Analysis of assembled pZLE10-*epoD* by pulsed-field gel electrophoresis. The sizes of the substrates were 6316 bp, 6610 bp, 7417 bp, 5178 bp and 7115 bp. The final assembly product was a 29-kb plasmid. (b) Identification of pZLE10-*epoD* by PCR of the linker regions. The predicted sizes were 322 bp (vm3), 1615 bp (m3m4), 177 bp (m4m5), 249 bp (m5m6) and 468 bp (m6v). (c) *Apa*I (lane 1), *Hind*III & *Nhe*I (lane 2) digests of pZLE10-*epoD*. (d) Analysis of assembled pZLE10-*epo* by pulsed-field gel electrophoresis. The sizes of substrates were 4447 bp, 5749 bp, 7019 bp, 24857 bp, 11568 bp, 8840 bp and 7115 bp and the final assembly product was a 62.4-kb plasmid. (e) Identification of pZLE10-*epo* by PCR of the linker regions. The predicted sizes were 467 bp (vm0), 420 bp (m0m1), 420 bp (m1m2), 386 bp (m2m3), 177 bp (m4m5), 249 bp (m5m6), 277 bp (m6m7), 306 bp (m8m9) and 411 bp (m9v). (f) *Hind*III (lane 1) and *Nde*I (lane 2) digests of pZLE10-*epo*.

into three parts, M9a (primers 09a/ZE15, 3088 bp), M9b (primers ZE16/ZE19, 2229 bp) and M9c (primers ZE20/09b, 2443 bp); they were PCR amplified and cloned into pTA0315 separately, and then combined into one plasmid to generate pTA0315-M9. The correct orientation of all inserts in the 'entry clones' was confirmed. To facilitate selection in the tandem assembly process, a PCR fragment (primers Oxl28R/Oxl29, 1338 bp) containing the *aph*II gene (conferring resistance to kanamycin and neomycin) was inserted into pTA0006-M3 through *Spe*I digestion and ligation (see Supplementary Table S1 and S2).

The destination vector pZLE10 used for integration and expression of the assembled gene cluster in *Streptomyces* was constructed as follows. A PCR fragment (primers ZE03/ZE04, 1956 bp) containing the *redP* promoter, the terminator from pIJ6021²⁷, the chloramphenicol resistance gene, and the *attP*₀ and *attB*₁₅ sites were inserted into the pMD-19T vector (Takara) to generate an intermediate plasmid. This was digested with *Xba*I and *Bgl*II to release a 1962-bp fragment. Plasmid T-Bxbatt1-Bxbatt2 (B. Zhang, L. Zhang, R. Dai, M. Yu, G. Zhao, and X. Ding, manuscript in preparation) was digested with *Nhe*I and *Bgl*II to release a 4441-bp fragment containing apramycin and ampicillin resistance genes and the replication origin of pUC19 and p15A. These two fragments were ligated to generate pZLE12. pZLE12 was then digested with *Xba*I to release a 3713-bp fragment and plasmid pSET152 was digested with *Nhe*I and *Xba*I to release a 3402-bp fragment; these two fragments were ligated to generate pZLE10 (see Supplemental Table S2).

Tandem assembly of *epoD* and the complete epothilone biosynthetic gene cluster.

The enzymes used to linearize the 'entry clones' for *in vitro* assembly procedures are described in Supplemental Fig. S2. DNA segments under 10 kb were isolated by 0.8% agarose gel electrophoresis in 1×TAE buffer, and purified using an agarose gel DNA extraction kit (Genlayer); segments larger than 10 kb were purified by phenol-chloroform extraction and ethanol precipitation. For pZLE10-*epoD* assembly, the five DNA segments containing modules 3, 4, 5 and 6 and pZLE10 were incubated with Φ BT1 integrase overnight (or over 8 hrs) at 30°C. The buffer contained 10 mM Tris-HCl, 100 mM KCl, 50 mM NaCl, 2 mM EDTA and 1 mM DTT. Heterologous expression, purification and preservation of Φ BT1 integrase were described in our previous work¹⁶. The tandemly assembled products were purified by phenol-chloroform extraction and ethanol precipitation, and analysis by pulsed-field gel electrophoresis (PFGE) performed on 1% agarose gels (BioLabs) in 0.5×TBE buffer,

with the following parameters: switch time, 1–6 seconds; run time, 11 hours; angle, 120°; and voltage gradient, 6 V/cm. Recombination events were detected by PCR analysis. The assembled products were transformed into *E. coli* strain DH10B by electroporation (25 μ F capacitance, 1.8 kV/mm, and 200 ohm resistance).

Transformants were selected with apramycin and neomycin. The plasmid from surviving clones were identified by PCR using primers ES53/54, then isolated and purified. The plasmid was identified by further PCR reactions of the linker regions between modules and by restriction digestion (Fig. 3b, c, and Supplemental Fig. S3). Correct clones were propagated for the next round of assembly. For assembly of the total epothilone biosynthetic gene cluster, the seven DNA segments containing *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, *epoF* and pZLE10 were incubated with Φ BT1 integrase, and the subsequent steps were as described above.

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Author contributions

L.Z., G.-P.Z. and X.-M.D. designed research; L.Z. performed research; L.Z. and X.-M.D. analyzed data; and L.Z., G.-P. Z. and X.-M.D. wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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