

# Cloning and Elucidation of the FR901464 Gene Cluster Revealing a Complex Acyltransferase-less Polyketide Synthase Using Glycerate as Starter Units

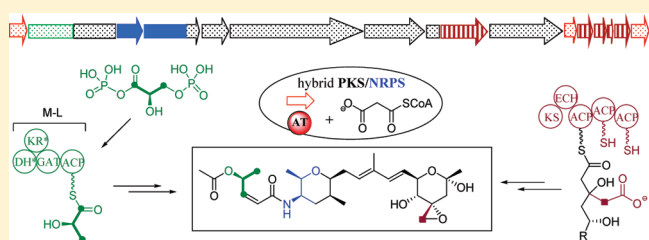
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**S** Supporting Information

**ABSTRACT:** FR901464, an antitumor natural product, represents a new class of potent anticancer small molecules targeting spliceosome and inhibiting both splicing and nuclear retention of pre-mRNA. Herein we describe the biosynthetic gene cluster of FR901464, identified by degenerate primer PCR amplification of a gene encoding the 3-hydroxy-3-methylglutaryl-CoA synthase (HCS) postulated to be involved in the biosynthesis of a  $\beta$ -branched polyketide from *Pseudomonas* sp. No. 2663.

This cluster consists of twenty open reading frames (ORFs) and was localized to 93-kb DNA segment, and its involvement in FR901464 biosynthesis was confirmed by gene inactivation and complementation. FR901464 is biosynthesized by a hybrid polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS), HCS, and acyltransferases (AT)-less system. The PKS/NRPS modules feature unusual domain organization including multiple domain redundancy, inactivation, and tandem. Biochemical characterization of a glyceryl transferase and an acyl carrier protein (ACP) in the start module revealed that it incorporates *D*-1,3-bisphosphoglycerate, which is dephosphorylated and transferred to ACP as the starter unit. Furthermore, an oxidative Baeyer–Villiger reaction followed by chain release was postulated to form a pyran moiety. On the basis of in silico analysis and genetic and biochemical evidences, a biosynthetic pathway for FR901464 was proposed, which sets the stage to further investigate the complex PKS biochemically and engineer the biosynthetic machinery for the production of novel analogues.



## INTRODUCTION

FR901464 is one of the most potent members of antitumor natural products that lower the mRNA levels of oncogenes and tumor suppressor genes with new modes of action.<sup>1–3</sup> This natural product, and its analogs FR901463 and FR901465 (Figure 1), were isolated from the fermentation broth of the bacterium *Pseudomonas* sp. No. 2663 as novel transcriptional activators that cause cell cycle arrest at the G1 and G2/M phases.<sup>1</sup> FR901464 exhibits potent antitumor activity against a number of human cancer cell lines with  $IC_{50}$  values in the range of 0.6–3.4 nM in vitro, and prolongs the life of human solid tumor bearing mice in vivo at 0.056–1 mg/kg dosage. Despite its high potency in inducing SV40 promoter, this compound paradoxically lowers the mRNA levels of *p53*, *p21*, *c-myc*, and *E2F-1* in human breast adenocarcinoma MCF-7 cells at 20 nM and induces apparent apoptosis in MCF-7 cells with the impressive  $LC_{50}$  of 0.5 nM.<sup>2</sup> Recently, FR901464 and its methylated derivative spliceostatin A (Figure 1) were identified as general pre-mRNA inhibitors, which target SF3b subcomplex in the U2 small nuclear ribonucleoprotein particle of spliceosome, block splicing and prompt nuclear export of intron-bearing precursors.<sup>4,5</sup> Pre-mRNA splicing is a critical step in the expression of nearly all eukaryotic genes in which intron sequences are

removed and exons are joined together to generate a mature translatable mRNA. This process is mediated by the spliceosome, a RNA-based machinery containing five small nuclear RNAs and numerous associated proteins.<sup>6</sup> Thus, understanding the spliceosome assembly, composition, and the regulation is essential for deciphering post-transcriptional control of the genome. The discovery of spliceosome targeting natural products, such as FR901464, provides not only valuable molecular tools to explore the biological questions, but also potentially new avenue for the development of novel anticancer drugs.<sup>4,7–9</sup>

Despite its linear hybrid peptide/polyketide framework, FR901464 is characterized with three discrete chiral fragments joined by conjugated, unsaturated linkages. The unique structure, unprecedented pharmacological profile and novel mode of action have drawn considerable interest to further development of FR901464 as a potential anticancer drug.<sup>10,11</sup> Total synthesis of FR901464 and its analogues have been extensively investigated.<sup>12–16</sup> Meayamycin (Figure 1), a more stable analogue of FR901464, showed remarkable antitumor activity against MCF-7 cells with a  $GI_{50}$  of 10 pM, making it one of the most potent anticancer agents known to date.<sup>16,17</sup>

**Received:** June 28, 2010

**Published:** February 3, 2011

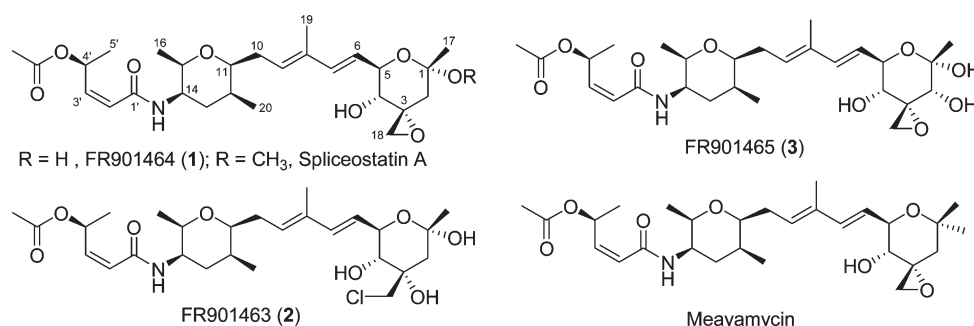


Figure 1. Structures of FR901464 and derivatives.

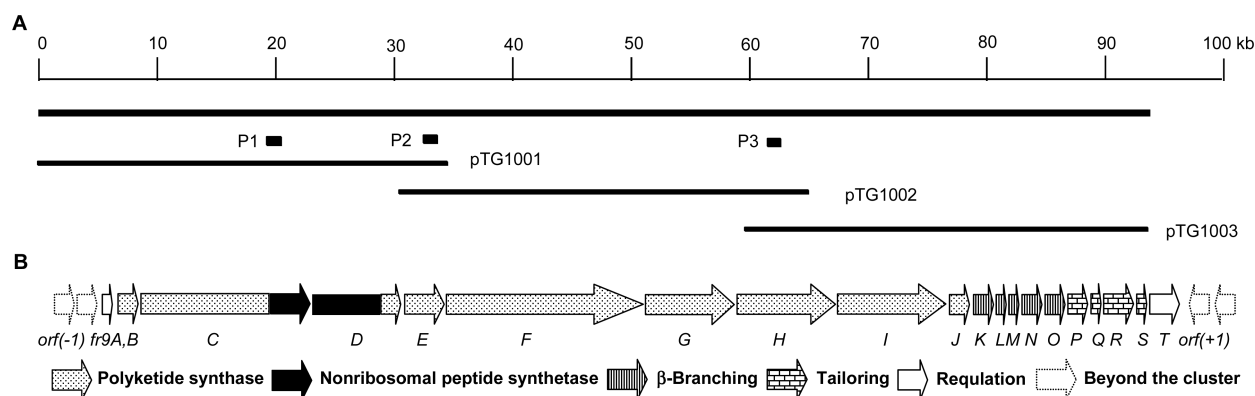


Figure 2. Localization and organization of the FR901464 gene cluster. (A) Localization and restriction map of the cloned 93.03 kb DNA region from *Pseudomonas* sp. No. 2663 as represented by three overlapping fosmid clones; (B) Organization of the gene cluster.

Complementary to achieving structural diversity of natural products chemically, combinatorial biosynthesis of microbial secondary metabolites offers a promising alternative to prepare complex “un-natural” natural products biosynthetically.<sup>18–20</sup> The success of this strategy depends on the cloning and characterization of the biosynthetic pathway of microbial natural product genetically and biochemically. Though the biosynthetic origin of FR901464 has not been established, its linear hybrid peptide/polyketide backbone supports a hybrid nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) assembly on the basis of the emerging paradigm for hybrid NRPS/PKS system.<sup>21,22</sup> Typically, modular PKSs contain a  $\beta$ -ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain and catalyze one cycle of chain elongation that extended the growing polyketide chain by a C<sub>2</sub> unit using Claisen condensation of malonate units.<sup>22,23</sup> Recently,  $\beta$ -branching in polyketides was characterized in several natural products<sup>24–27</sup> and revealed a conserved enzyme cassette including 3-hydroxy-3-methylglutaryl-CoA synthase (HCS) responsible for  $\beta$ -branch incorporation.<sup>28</sup> FR901464 contains a  $\beta$ -branched structure at C-18 position (Figure 1), which is likely derived by a similar process. We hypothesized that the gene cluster responsible for the biosynthesis of FR901464 could be identified by degenerate primer-based PCR of highly conserved HCS and KS from genomic DNA of *Pseudomonas* sp. No. 2663. Herein, we used this strategy to clone the FR901464 gene cluster, which reveals an unprecedented molecular architecture of hybrid PKS/NRPS/PKS assembly line. Despite of isoprenoid-like alkylation by HCS and an “AT-less” PKS with trans-acting ATs, the most striking features of this megasynthetase system involve: (i) incorporation of a glycolytic intermediate as the PKS start unit;

(ii) an oxidative Baeyer–Villiger (B–V) domain in PKS module likely catalyzing the polyketide chain release; (iii) employment of different strategies to form two pyran moieties in one molecule; and (iv) three ATs with high homology involved in the biosynthesis of FR901464.

## MATERIALS AND METHODS

**General.** A FR901464 producer, *Pseudomonas* sp. No. 2663 (FERM BP-3421), was purchased from the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, and was cultured as described.<sup>1</sup> *Escherichia coli* DH5 $\alpha$  competent cells were used for routine subcloning and plasmid preparations. pCC1FOS Vector (Epicenter) was used for fosmid library preparations. *E. coli* cells were grown in LB medium with appropriate antibiotics when necessary. All other common biochemicals and chemicals were from standard commercial sources. PCR amplification was carried out using either *Taq* DNA polymerase or PfuUltra DNA polymerase with genomic DNA or fosmid as a template and degenerate or specific primers listed in Table S1. Primers were ordered from Invitrogen Shanghai Center. DNA sequencing was performed at the Shanghai GeneCore Biotechnology Inc. The general genetic manipulations of *Pseudomonas* were preformed following described methods.<sup>29,30</sup>

**Genomic Library Construction and Screening.** A genomic library of *Pseudomonas* sp. 2663 was constructed in fosmid vector pCC1FOS-1 (Epicenter) according to the manufacturer's protocol. The HCS gene probe (P1) for library screening was obtained by PCR amplification with primers HCS-For and HCS-Rev-1 or HCS-Rev-2 (Table S1) confirmed by sequencing. The genomic library ( $6.0 \times 10^3$  clones) was screened by colony hybridization and the resultant positive clones were further confirmed by PCR amplification with primers PKS-For and PKS-Rev and Southern blotting. To extend the cloned DNA

region on the chromosome, the library was further screened by colony hybridization with probe P2 (a 1.55-kb PCR fragment generated with F-walk-For1 and F-walk-Rev1 as primers) from pTG1001 and probe P3 (a 1.56-kb PCR fragment produced with F-walk-For2 and F-walk-Rev2 as primers) from pTG1002 (Figure 2A, all the PCR primers are summarized in Table S1).

**Sequence Analysis.** The open reading frames (ORFs) were deduced from the sequence with FramePlot 4.0beta program (<http://nocardia.nih.gov/jp/fp4>) and BLAST methods. Amino acid sequence alignments were performed by CLUSTALW method in the BIOLOGYWORKBENCH 3.2 package (<http://workbench.sdsc.edu>). Identification of functional domains of PKSs was carried out with a program available at <http://www.nii.res.in/searchall.html>. Prediction of amino acid specificity of individual NRPS domain was performed with a program available at <http://bix.umbi.umd.edu/Projects/nrps/>. The nucleotide sequence reported in this paper has been deposited into GenBank under accession no. HM047288.

**Construction of Gene Replacement and Complementation Mutants.** To inactivate the HCS gene *fr9k* of *Pseudomonas* sp. 2663, a 1.4-kb *XbaI/KpnI* fragment generated with FR9K-L-For and FR9K-L-Rev as primers (Table S1) and a 1.5-kb *KpnI/HindIII* fragment produced with FR9K-R-For and FR9K-R-Rev as PCR primers (Table S1) were successively ligated and cloned into *HindIII/XbaI* sites of pTL2028<sup>29</sup> to yield pTG1004 in which *fr9k* was inactivated by in-frame deletion. The resultant plasmid pTG1004 was introduced into *Pseudomonas* sp. 2663 by electroporation and kanamycin-resistant colonies were identified as single crossover mutants. Further incubation and screening for sucrose-resistant and kanamycin-sensitive colonies were identified as double crossover mutant strain TG1001.

To express *fr9k* in mutant *Pseudomonas* TG1001, a 1.4-kb *XbaI-HindIII* fragment containing the intact *fr9K* were cloned into pVLT33 vector<sup>30</sup> to yield pTG1005, in which the expression of FR9K is under the control of *Ptac* promoter. The expression construct was introduced into *Pseudomonas* sp. TG1001 by electroporation, and the resultant *Pseudomonas* sp. TG1002 strain that harbors pTG1005, along with the wild-type strain as a control, were cultured and analyzed by high performance liquid chromatography (HPLC) for FR901464 production as described below.

Further in vivo generation of *fr9B*, *fr9J*, *fr9O*, and *fr9Q* gene replacement mutants in *Pseudomonas* sp. 2663 was achieved by a new lambda Red-based technique using one-step PCR product flanked by short homology regions.<sup>31</sup> The knockout cassettes were constructed using PCR with respective PCR primer pairs listed in Table S1, and the kanamycin resistance gene was amplified using pET28a as template. The PCR respective products and plasmid pRKaraRed<sup>31</sup> were introduced into *Pseudomonas* sp. 2663 by electroporation and kanamycin-resistant colonies were identified as double crossover mutant strains TG1003 ( $\Delta fr9B$ ), TG1004 ( $\Delta fr9J$ ), TG1005 ( $\Delta fr9O$ ), and TG1006 ( $\Delta fr9Q$ ), respectively.

**Production and Analysis of FR901464.** *Pseudomonas* sp. 2663 wild-type and recombinant strains were cultured in LB (supplemented with appropriate antibiotic for recombinant strains) at 30 °C for 24 h. For fermentation, 2.5 mL of seeding culture of each strain was transferred into a 250 mL flask containing 50 mL of MPM-2 medium (yeast extract 0.23%, glucose 0.26%, Na<sub>2</sub>HPO<sub>4</sub> 0.24%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.05%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.006%) and incubated at 25 °C for 72 h. For FR901464 isolation, each 50 mL of the culture broth was filtered and extracted twice with 50 mL of ethyl acetate, and the combined extract was concentrated to 100  $\mu$ L in a vacuum.

HPLC analysis was carried out on a Microsorb-MV C18 column (5  $\mu$ , 4.6 mm  $\times$  250 mm, ser. no. 0170300853, GraceSmart RP18). The column was equilibrated with 50% solvent A (H<sub>2</sub>O, 0.1% TFA) and B (CH<sub>3</sub>CN, 0.1% TFA) and eluted with the following program: 0–35 min, a linear gradient from 85% A/15% B to 45% A/55% B; 35–55 min,

a linear gradient from 45% A/55% B to 5% A/95% B; 55–58 min, a linear gradient from 5% A/95% B to 85% A/15% B, 58–60 min, constant 85% A/15% B. This was carried out at a flow rate of 1 mL/min and UV detection at 235 nm using an Agilent 1100 HPLC system. The identity of target compound was confirmed by liquid chromatography–mass spectrometry (LC-MS) analysis performed on a LCMS-2010 A (SHIMADZU, JP). FR901464 showed (M + H)<sup>+</sup> ion at *m/z* of 508.62, consistent with the molecular formula C<sub>27</sub>H<sub>41</sub>NO<sub>8</sub>.

**PKS Domain Cloning, Expression, and Purification.** The genes encoding GAT and ACP<sub>L</sub> domains of Fr9C were amplified by PCR from fosmid pTG1001 with primer set FR9C-G-For and FR9C-G-Rev, and FR9C-LT-For and FR9C-LT-Rev, respectively (Table S1). PCR products were purified, cloned and confirmed by sequencing, then a *NdeI/HindIII* fragment for GAT and a *NdeI/XhoI* fragment for ACP were cloned into the same sites of pET37b to make the expression plasmid pTG1006 (for *fr9C-GAT*) and pTG1007 (for *fr9C-ACP<sub>L</sub>*). The resulting plasmids were transformed separately into *E. coli* BL21 (DE3) for protein expression. For overproduction of proteins, cells harboring the desired plasmid were grown in LB medium supplemented with 50  $\mu$ g/mL of kanamycin. Cultures (1 L) were grown to an OD<sub>600</sub> of 0.4–0.6 at 37 °C and then cooled to 16 °C for 30 min, and protein expression was induced by the addition of 50  $\mu$ M IPTG (final concentration). Cultures were grown for an additional 24 h. Purification of the His-tagged fusion protein with Ni-NTA affinity resin was performed according to manufacturer's manual (Qiagen, Valencia, CA), and the resultant proteins were dialyzed against 50 mM MOPS (pH 7.5), 50 mM NaCl, and 10% glycerol, and stored at –80 °C.

**Biochemical Assays of Glyceryl Transfer.** The generation of holo-ACP from apo-ACP and production of glyceryl-S-ACP were performed according to literature procedures.<sup>32,33</sup> To prepare holo-ACP from apo-ACP, each reaction (50  $\mu$ L) containing 50 mM MOPS (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM TCEP, 5  $\mu$ M Sfp, 0.25 mM CoA, and 80  $\mu$ M apo-ACP were incubated for 2 h at 30 °C. The reaction was quenched by adding 20  $\mu$ L of 10% formic acid and then was analyzed by HPLC. Control reactions were performed in the absence of Sfp or CoA. To prepare D-1,3-bisphosphoglycerate (1,3-BPG) in situ, a reaction (50  $\mu$ L) containing 50 mM MOPS (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM TCEP, 5 mM ATP, 0.25 mM D-3-phosphoglycerate (PG), 50 U PG kinase, and 10  $\mu$ M GAT was incubated for 30 min at 30 °C, a portion of the reaction (20  $\mu$ L) mixture was then added to a reaction (30  $\mu$ L) containing holo-ACP as described above and incubated at 30 °C for 10 min. The reaction was quenched by adding 20  $\mu$ L of 10% formic acid and analyzed by HPLC. Control reactions were performed in the absence of ATP, GAT or PGK. HPLC analysis was carried out on a GraceVydac protein and peptide C18 column. The column was equilibrated with 80% solvent A (H<sub>2</sub>O, 0.1% formic acid) and 20% solvent B (CH<sub>3</sub>CN, 0.1% formic acid) and developed with the following program: 0 to 3 min, constant 80% A/20% B; 3 to 60 min, a linear gradient to 32% A/68% B; 60 to 63 min, a linear gradient to 5% A/95% B; 63 to 65 min, a linear gradient to 5% A/95% B; 65 to 68 min, a linear gradient to 80% A/20% B, and 68 to 70 min, constant 80% A/20% B. It was carried out at a flow rate of 1 mL/min with UV detection at 220 nm, using an Agilent 1200 HPLC system. MS analysis was carried out with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS.

## RESULTS

**Cloning and Sequencing of the FR901464 Biosynthetic Gene Cluster.** Studies of isoprenoid-like alkylations in polyketide biosynthesis revealed a conserved enzyme cassette including HCS responsible for  $\beta$ -branch incorporation.<sup>28</sup> To specifically probe genes encoding this type HCS, we adopted a PCR approach using degenerate primers (Table S1) to access the gene fragment which was assumed for incorporating a  $\beta$ -branched



**Table 1. Deduced Functions of ORFs in FR901464 Biosynthetic Gene Cluster**

| gene                       | amino acid | protein homologue  | similarity/identity (%) | proposed function  |
|----------------------------|------------|--|-------------------------|--|
| <i>orf(-3)<sup>a</sup></i> | 119        | (EDS88398) ; <i>Burkholderia pseudomallei</i>                          | 90/81                   | hypothetical protein                                     |
| <i>orf(-2)<sup>a</sup></i> | 95         | BTH_II1883 (ABC35934); <i>Burkholderia thailandensis</i>               | 75/45                   | hypothetical protein                                     |
| <i>orf(-1)<sup>a</sup></i> | 128        | (ZP_02465790); <i>Burkholderia thailandensis</i>                       | 71/48                   | hypothetical protein                                     |
| <i>fr9A</i>                | 294        | DNA binding protein (AAV52815);<br><i>Burkholderia glumae</i>          | 67/52                   | transcriptional regulator                                |
| <i>fr9B</i>                | 360        | MmpIII (AAM12912); <i>Pseudomonas fluorescens</i>                      | 53/35                   | acyltransferase  |
| <i>fr9C</i>                | 5061       | PKS (CAN93347) ; <i>Sorangium cellulosum</i> 'So ce 56'                | 50/36                   | PKS/NRPS(DH-KR-GAT-ACP-KS-KR-ACP-KS-ACP-ACP-TE-KS-ACP-C) |
| <i>fr9D</i>                | 1770       | OnnI (AAV97877); <i>Theonella swinhoei</i>                             | 54/38                   | NRPS/PKS(C-A-PCP-KS)                                     |
| <i>fr9E</i>                | 1188       | RhiD (CAL69891); <i>Burkholderia rhizoxina</i>                         | 51/36                   | PKS(DH-KR-MT)  |
| <i>fr9F</i>                | 4904       | BaeM (ABS74063) ; <i>Bacillus amyloliquefaciens</i> FZB42              | 54/38                   | PKS(ACP-ER-KS-DH-KR -ACP-KS-DH-KR-MT-ACP-KS)             |
| <i>fr9G</i>                | 2218       | BryB (ABKS1300); <i>Candidatus Endobugula sertula</i>                  | 48/32                   | PKS(DH-KR-ACP-KS-KR-ACP)                                 |
| <i>fr9H</i>                | 2662       | OnnB (AAV97870); <i>Theonella swinhoei</i>                             | 52/37                   | PKS(KS-ECH-ACP-ACP-ACP-KS-OX)                            |
| <i>fr9I</i>                | 3052       | RhiB (CAL69889); <i>Burkholderia rhizoxina</i>                         | 31/47                   | PKS(KR-ACP-KS-DH-ACP-KS-TE)                              |
| <i>fr9J</i>                | 296        | BryP (ABKS1299); <i>Candidatus Endobugula sertula</i>                  | 62/44                   | acyltransferase  |
| <i>fr9K</i>                | 429        | TaC (CAB46502); <i>Myxococcus xanthus</i>                              | 77/64                   | HMG-CoA synthase   |
| <i>fr9L</i>                | 261        | BaeH (ABS74059); <i>Bacillus amyloliquefaciens</i>                     | 68/52                   | enoyl-CoA hydratase                                      |
| <i>fr9M</i>                | 79         | CurB (AAT70097); <i>Lyngbya majuscula</i>                              | 71/52                   | ACP  |
| <i>fr9N</i>                | 421        | MupG (AAM12921); <i>Pseudomonas fluorescens</i>                        | 67/53                   | KS <sup>S</sup>  |
| <i>fr9O</i>                | 377        | MmpIII (AAM12912); <i>Pseudomonas fluorescens</i>                      | 63/48                   | acyltransferase  |
| <i>fr9P</i>                | 341        | KtzO (ABV56595); <i>Kutzneria</i> sp. 744                              | 46/32                   | dioxygenase  |
| <i>fr9Q</i>                | 145        | StiJ (CAD19093); <i>Stigmatella aurantiaca</i>                         | 48/26                   | cyclase  |
| <i>fr9R</i>                | 482        | AufA (CAO98845); <i>Stigmatella aurantiaca</i> DW4/3-1                 | 57/37                   | cytochrome P450  |
| <i>fr9S</i>                | 152        | GCNS-N-acyltransferase (EED71453);<br><i>Variovorax paradoxus</i> S110 | 75/63                   | acyltransferase  |
| <i>fr9T</i>                | 467        | Diguanylate cyclase (ABB05776); <i>Burkholderia</i> sp. 383            | 90/84                   | diguanylate cyclase                                      |
| <i>orf(+1)<sup>a</sup></i> | 248        | Rmet_6270 (ABF13129); <i>Ralstonia metallidurans</i>                   | 54/45                   | hypothetical protein                                     |
| <i>orf(+2)<sup>a</sup></i> | 342        | Integrase (ACA89979); <i>Burkholderia cenocepacia</i>                  | 96/94                   | integrase  |

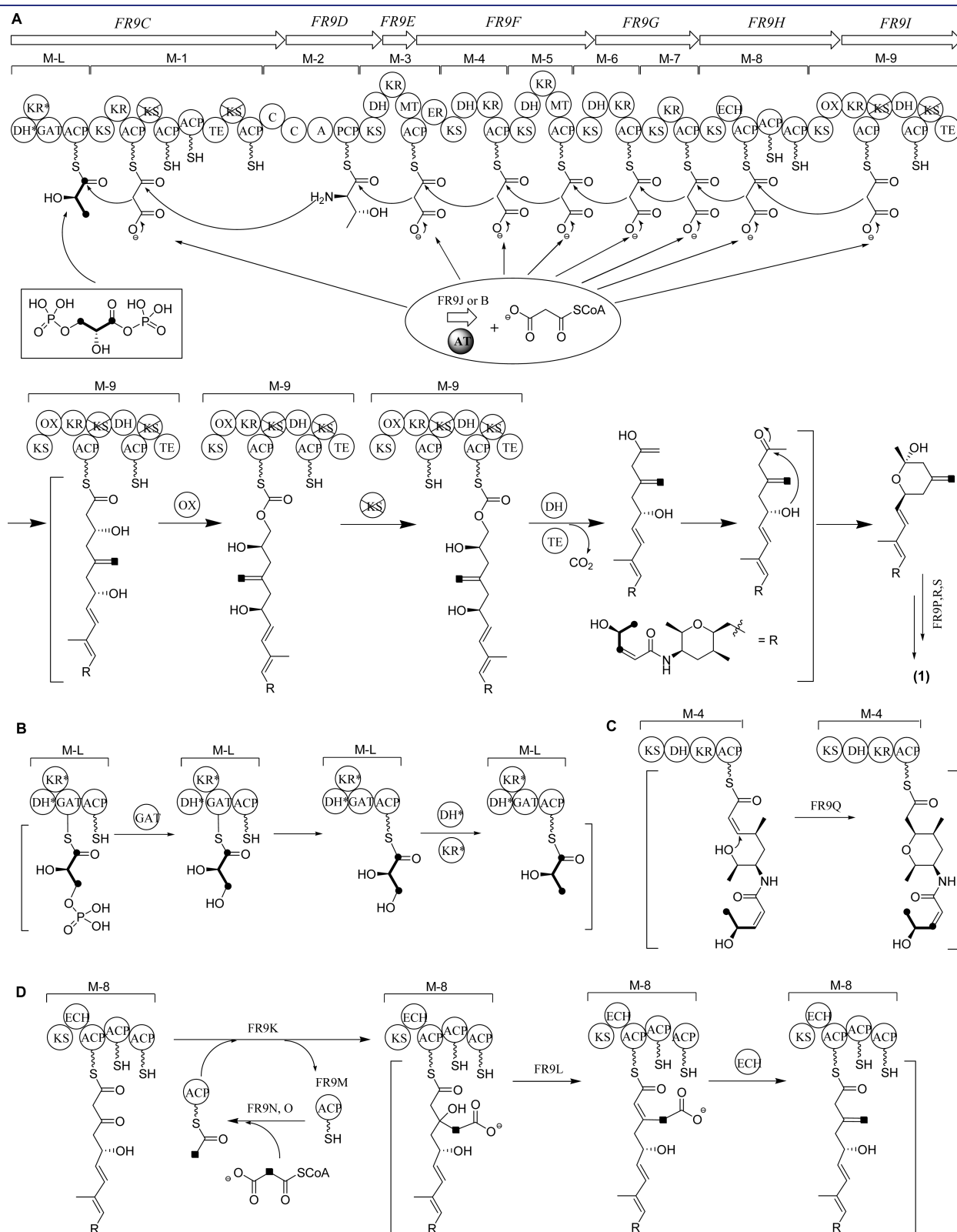
<sup>a</sup> *orfs* beyond FR901464 gene cluster.

structure of C18 in FR901464. With the genomic DNA of *Pseudomonas* sp. 2663 as template, distinct products with the expected size were both amplified with two pairs of primers. After cloning and sequencing, analysis of randomly selected clones confirmed that most of them contained an identical DNA fragment P1, the deduced amino acid sequence of which is highly homologous to known HCSs. Using the P1 product as a probe, approximately 6,000 clones of the *Pseudomonas* sp. 2663 genomic library were screened, resulting in 10 overlapping fosmids that span a 50-kb DNA region. To further confirm this DNA region containing PKS genes, we utilized these fosmids as template and degenerate primer set PKS-For and PKS-Rev (Table S1) based on conserved regions of KS domain to amplify successfully the distinct products with expected size. Sequence analysis of randomly selected clones confirmed that the deduced amino acid sequence is highly homologous to known KSs. To ensure full coverage of the entire FR901464 gene cluster, chromosome walking from the right end of fosmid pTG1001 was carried out by using a PCR product P2 as a probe. Further chromosome walking using a similar strategy led to a 90-kb contiguous DNA region on the chromosome, which was mapped to three overlapping fosmids, pTG1001, pTG1002, and pTG1003 (Figure 2A).

DNA sequencing of the three fosmids yielded a 93,029-bp contiguous DNA sequence with 65.4% of overall GC content, characteristic of *Pseudomonas* DNA.<sup>34</sup> Bioinformatic analysis of

the region revealed 29 ORFs (Figure 2B and Table 1), 20 of which, from *fr9A* to *fr9T*, were proposed to constitute the FR901464 gene cluster according to functional assignment of their deduced products. Genes beyond this region encode hypothetical proteins and lack significant similarity to those involved in secondary metabolite biosynthesis.

**Genes Encoding Hybrid PKS/NRPS/PKS.** The *fr9C* gene encodes a hybrid PKS/NRPS with unusual architecture and PKS loading module. This gene product contains fourteen identifiable domains organized into a loading module, an extension module of PKS, and a typical NRPS condensation (C) domain (Figure 3A). Both modules are quite unusual. The first domain in loading module is most similar to a dehydratase (DH), and the second is similar to a ketoreductase (KR); however, neither is an exact match throughout the sequence. The third domain is highly homologous to FkbH, a bifunctional glyceryl transferase/phosphatase (GAT) belonging to the haloacid dehalogenase superfamily, which was recently characterized to divert D-1,3-bisphosphoglycerate (1,3-BPG) into PKS biosynthesis.<sup>32</sup> This four-domain (DH\*-KR\*-GAT-ACP) loading module is most similar to the loading module of BryA (32% identity and 52% similarity), a PKS in bryostatin biosynthesis from the uncultivated bacterial symbiont.<sup>35</sup> The first extension module of FR9C is predicted to have a total of nine domains with the KS-KR-ACP-KS-ACP-ACP-TE-KS-ACP characteristic organization.



**Figure 3.** Proposed biosynthetic pathway of FR901464. Proposed model for AT-less PKS/NRPS and chain termination by B–V oxidation process (A), loading mechanism (B), formation of the first tetrahydropyran (C), and  $\beta$ -methylation of C-18 (D). A, adenylation; ACP, acyl carrier protein; AT, acyltransferase; C, condensation; DH, dehydratase; ECH, enoyl-CoA hydratase; ER, enoyl reductase; GAT, glyceryl transferase/phosphatase; KR, ketoreductase; KS, ketosynthetase; MT, methyltransferase; OX, FAD-dependent monooxygenase; PCP, peptidyl carrier protein; SAM, S-adenosyl-methionine; TE, thioesterase.

However, the second and the third KS domain are likely inactive because the conserved motif C–H–H was mutated into C–A–H or C–N–H (Figure S1). Residing at the C terminus of FR9C is a NRPS C domain with an intact highly conserved signature motif HHxxxDA/G which is commonly present in C domains in the databases.

The *fr9D* gene encodes a hybrid NRPS/PKS with C-adenylation (A)-peptidyl carrier protein (PCP)-KS domain organization. This C domain is also believed to be functional because of the present of conserved motif HHxxxDA/G (Figure S1). The specificity-conferring codes<sup>36,37</sup> for the A domain were identified as D-235, F-236, W-239, N-278, V-299, G-301, M-322, V-330, H-331, and K-517, which shown 100% amino acid specificity toward Thr. It suggests that Thr is activated by this A domain and subsequently loaded to the PCP.

Five PKS genes, *fr9E*, *fr9F*, *fr9G*, *fr9H*, and *fr9I*, encode seven PKS modules containing a total of eight KSs, six KR, five DHs, two methyltransferases (MTs), one enoyl reductase (ER) and five ACPs (Figure 3A).

All ACPs contain a signature motif around the invariable 4'-phosphopantetheine attachment site Ser residue (Figure S1) and DHs contain a conserved active site of H-D "catalytic dyad".<sup>38</sup> The KR contains a signature NADPH binding site, active site of S–Y and LDD motif of B-type KR to produce a hydroxyl group of "R" stereochemistry (Figure S1).<sup>39</sup> Both FR9H and FR9I possess some unusual domain organizations for PKS. An enoyl-CoA hydratase (ECH) domain and tandem ACP domains were identified in module 8 of FR9H, which are typical features of  $\beta$ -branch incorporation of polyketide. Additionally, an FAD-dependent monooxygenase (OX) domain is located at the C terminus of FR9H. Finally, FR9I contains a total of seven domains KR-ACP-KS-DH-ACP-KS-TE, but both KS domains were assigned inactive for decarboxylation-chain elongation due to a conserved catalytic triad of C–H–H replaced by C–Q–H or C–H–A (Figure S1).

All eight PKS modules lack the cognate AT domains suggesting that those PKSs belong to "AT-less" PKSs, also known as *trans*-AT PKSs family.<sup>40–43</sup> Three genes, *fr9B*, *fr9J*, and *fr9O*, are found in FR901464 gene cluster that all encode proteins with high sequence homology to AT domains and contain a highly conserved active site of GHSxG and substrate binding motif of AFHS that is specific for malonyl-CoA (Figure S1).<sup>44</sup> The FR9B is a protein of 360 amino acids, FR9O of 377 amino acids, both of them are most similar to MmpIII (35–48% identity and 52–63% similarity), a *trans*-AT in mupirocin biosynthetic pathway.<sup>45</sup> Meanwhile, FR9J contains 296 amino acids, and shows more sequence homology to BryP (44% identity and 62% similarity), a *trans*-AT in the bryostatin biosynthetic pathway. Interestingly, recent *in vivo* studies showed that BryP and MmpIII can functionally complement.<sup>46</sup>

**Genes Encoding Proteins for  $\beta$ -Branch Incorporation.** The *fr9KLMNO* genes constitute an operon required for the isoprenoid-like alkylation in FR901464 biosynthesis. FR9K is a HCS homologue showing high identity (60–70%) to PksG,<sup>24</sup> TaC,<sup>25</sup> CurD and JamH,<sup>27</sup> which were characterized as the key enzymes catalyzing the transfer of  $-\text{CH}_2\text{COO}^-$  from acyl-S-ACP to a  $\beta$ -keto thioester polyketide intermediate. The following genes, *fr9L*, *fr9M*, *fr9N*, and *fr9O*, encode for a freestanding ECH, a freestanding ACP, a freestanding KS and a freestanding AT, respectively. A conserved Cys residue of KS (FR9N) required for C–C bond formation was mutated into Ser (Figure S1),

requiring for only decarboxylation to generate acetyl-S-ACP. In addition to the ECH-ACP-ACP-ACP domain located on FR9H, all these components are predicted essential for  $\beta$ -branch incorporation.

**Genes Encoding Tailoring Enzymes and Regulatory Proteins.** FR9P contains a conserved HxD/E---H iron(II)-binding motif and shows high sequence homology (32% identity and 46% similarity) to KtzO, a nonheme iron hydroxylase from the Kutzneride biosynthetic pathway.<sup>47</sup> This enzyme is postulated to catalyze the stereospecific hydroxylation at the relatively unreactive position, possibly the hydroxylation at C-4 of FR901464. FR9Q is similar to a number of putative proteins, further sequence analysis did reveal similarity to the cyclase domain of StjJ<sup>48</sup> and Cyc11 of IdmP,<sup>49</sup> which were assigned to form the tetrahydropyran ring. FR9R, containing a typical heme binding motif FGHGAHxCLG and homologous to known cytochrome P450 oxidase, is a candidate enzyme responsible for oxidation of C3–C18 double bond to form epoxide. FR9S, highly homologous to a family of GCN5-related N-acetyltransferase,<sup>50</sup> may be responsible for acetylation of the hydroxyl group at C-4' position.

The *fr9A* and *fr9T* genes encode putative regulatory proteins. FR9A resembles the LysR family of regulators. FR9T shows high homology (80–90% identity) to diguanylate cyclase, which catalyzes the dimerization of two molecules of GTP to form bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), the second messenger for signal transduction and regulation in bacteria.<sup>51</sup>

**Validation of the FR901464 Gene Cluster.** To probe the role of FR9K in FR901464 production, a gene replacement plasmid pTG1004 was constructed, in which *fr9K* was inactivated by in-frame deletion. Plasmid pTG1004 was introduced into *Pseudomonas* sp. No. 2663 by electroporation to select for the resulting double crossover mutants *Pseudomonas* sp. TG1001, which were sucrose-resistant and kanamycin-sensitive. The genotype of this mutant strain was confirmed by PCR analysis (Figure S2). The *fr9K* gene deleted mutant strain, TG1001, completely lost its ability to produce FR901464, FR901463, and FR901465 (Figure 4 II), which was confirmed by HPLC and LC-MS analysis. To genetically complement the mutant strain, a construct pTG1005 expressing a functional copy of *fr9K* in *trans* was made and introduced into TG1001 to afford strain TG1002. The analysis of the metabolite accumulated by this mutant was performed by HPLC (Figure 4 III) and LC-MS, which revealed that the production of FR901464 was restored. The fraction of FR901464 was collected and further subjected to analysis by high-resolution MS yielded  $(\text{M} + \text{H})^+$  and  $(\text{M} + \text{Na})^+$  ions at  $m/z = 508.2928$  and  $530.2731$ , consistent with the molecular formula  $\text{C}_{27}\text{H}_{41}\text{NO}_8$  of FR901464 [calculated 508.2911 for  $(\text{M} + \text{H})^+$  and 530.2730 for  $(\text{M} + \text{Na})^+$ ]. These results confirmed that the *fr9K* gene is necessary for the biosynthesis of FR901464.

Further gene replacement experiments were carried out to test the necessity of several other genes in the biosynthesis of FR901464. The knockout targets include three freestanding ATs, FR9B, FR9J, and FR9O and the putative cyclase, FR9Q. All of these genes were inactivated through double crossover using a new developed method<sup>31</sup> based on the Red recombination system, and the resulting mutants TG1003, TG1004, TG1005, and TG1006 were confirmed by Southern analysis (Figure S3). The production of FR901464 in these mutants was all completely abolished by HPLC (Figure 4 IV–VII) and LC-MS analysis, demonstrating that these four genes are necessary for the biosynthesis of FR901464.

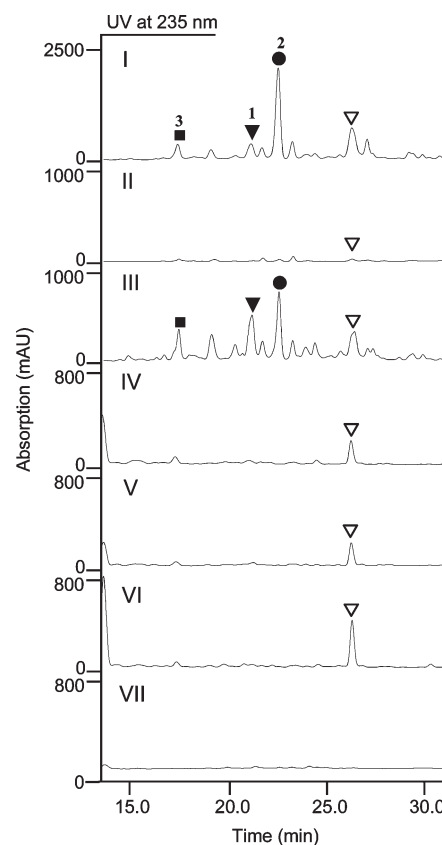


**Characterization of the Unusual Starter Unit of PKS.** To characterize the genes/proteins associated with chain initiation of PKS, the genes encoding GAT and ACP domain of loading module were cloned and heterologously overexpressed in *E. coli* BL21 (DE3) and respective proteins were purified to homogeneity (Figure S4). HPLC and electrospray ionization (ESI)-MS analysis confirmed that ACP protein is apo-form. When it was incubated with CoA and the Sfp PPTase, apo-ACP was completely converted into the functional holo-form (Figure S5). To verify glyceryl transfer reactions catalyzed by GAT, the purified protein was incubated with ATP,  $Mg^{2+}$ , 3-PG, and 3-PG kinase for generation of 1,3-BPG in situ, then the holo-ACP was added to directly test glyceryl loading. Reaction mixtures were subjected to HPLC analysis (Figure 5B), the reaction products were also subjected to Q-TOF-MS analysis. As expected, the addition of 88.0 Da to holo-ACP is exactly in agreement with the mass increase for glycerate (Figure 5C and Figure S5). All those in vitro results suggested that 1,3-BPG could be effectively activated by GAT, and subsequently dephosphorylated, and transferred onto holo-ACP to form glyceryl-S-ACP intermediate to start polyketide chain biosynthesis (Figure 5A).

## DISCUSSION

**Cloning the Biosynthetic Gene Cluster of FR901464.** Methods of PCR cloning PKS genes based on conservation of the KS domain in PKSs have been widely used to clone the biosynthetic gene cluster of polyketides. However, the amplified KS fragments often represent a pool of heterologous products from different polyketide biosynthesis gene clusters because of multiple polyketide pathways commonly existing in the genome of a given microorganism. We recognized that structurally, FR901464 is a hybrid polyketide/peptide framework containing a  $\beta$ -branched structure. Taking advantage of the emerging paradigm for HCS-catalyzed isoprenoid-like alkylation for  $\beta$ -branch incorporation,<sup>28</sup> we reasoned that the first choice of amplifying an HCS domain rather than the KS domain of a PKS module as a probe would greatly increase our chance to isolate the target gene cluster. The PCR products appear to be very specific, which led us to successfully identify FR901464 gene cluster combined with PCR amplification of KS fragments. In fact, there was one previous example of PCR amplification of HCS fragment to distinguish jamaicamides cluster from four groups of PKS-containing gene clusters.<sup>52</sup> This method should now be taken into consideration in formulating strategy to clone biosynthesis gene clusters of other  $\beta$ -branched polyketide natural products.

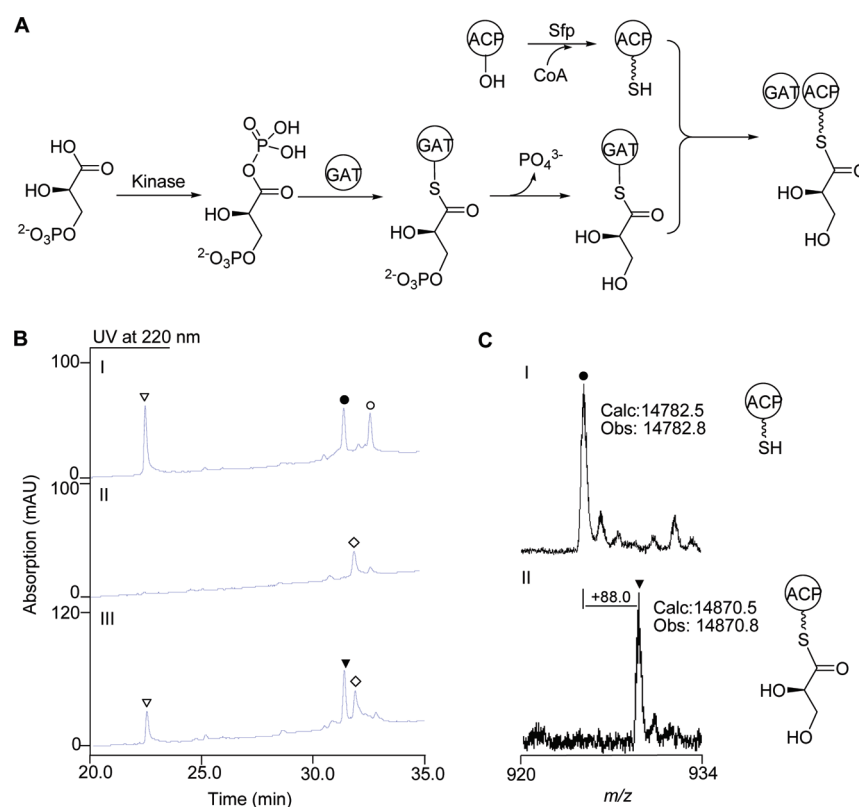
**Hybrid Biosynthetic System of AT-less PKS, NRPS, and HCS.** FR901464 biosynthesis joins the growing family of AT-less PKSs featuring with *trans*-acting ATs, hybrid PKS/NRPS, and isoprenoid-like alkylation by HCS. In contrast to classical type I PKSs, AT-less PKSs lack the cognate AT domains in PKS modules but require the standing-alone AT(s) to act iteratively *in trans* to load the extender unit to each of the PKS modules for polyketide biosynthesis.<sup>40–43</sup> Several complex natural products, exemplified by leinamycin,<sup>53</sup> myxovirescin A,<sup>54</sup> kalimantacin,<sup>55</sup> and coralopyronin A,<sup>56</sup> whose biosynthetic pathway are also directed by hybrid PKS/NRPS, HCS, and AT-less PKS (Figure S6 and Table S2). Additionally, classical ER domains, which are used by type I PKSs to generate completely reduced intermediates, are largely absent in AT-less PKSs and replaced by an external oxidoreductase that is often fused with an AT.<sup>57,58</sup> However, an ER domain of FR9F shows significant homology to known ER domains with the signature NADPH binding site



**Figure 4.** HPLC analysis of FR901464 production by *Pseudomonas* wide-type and recombinant strains. (I) wide-type *Pseudomonas* sp. No. 2663; (II) mutant *Pseudomonas* sp. TG1001 ( $\Delta$ fr9K); (III) mutant *Pseudomonas* sp. TG1002 (TG1001 harboring the fr9K expression plasmid pTG1005); (IV) mutant *Pseudomonas* sp. TG1003 ( $\Delta$ fr9B); (V) mutant *Pseudomonas* sp. TG1004 ( $\Delta$ fr9J); (VI) mutant *Pseudomonas* sp. TG1005 ( $\Delta$ fr9O); and (VII) mutant *Pseudomonas* sp. TG1006 ( $\Delta$ fr9Q). (▼), FR901464 (1); (●), FR901463 (2); (■), FR901465 (3); (▽), an unknown metabolite whose production is independent of FR901464 biosynthesis.

and a unique Tyr residue (LxAxGxY)<sup>59</sup> in the active site to produce the S-methyl branch of C-12 in the AT-less PKSs of FR901464. In most cases of AT-less PKS gene cluster, there is one gene encoding AT, occasionally with one gene encoding two tandem AT domains exemplified by bryostatin<sup>46</sup> and mupirocin,<sup>45</sup> or two discrete genes encoding two ATs exemplified by pederin.<sup>60</sup> Until recently, two ATs (three AT domains) encoded by two genes have been speculated to incorporate both malonyl-CoA and ethylmalonyl-CoA for kirromycin,<sup>61</sup> and both malonyl-CoA and methoxymalonyl-CoA for oxazolomycin<sup>62</sup> biosynthesis. However, three genes located in FR901464 gene cluster all encode ATs with high homology and all contain the conserved active site of GHSxG and substrate binding motif of AFHS that is specific for malonyl-CoA, which is very similar to bacillaene gene cluster but without *trans*-ER domain.<sup>63</sup> The *in vivo* inactivation of each one of the ATs abolished the production of FR901464, which suggested the loading function of each AT could not be replaced each other. Further determination the specific function and the specific ACP partners of each AT is on the way, it will be reported in due course.

**Unusual Domain Organization of PKS and NRPS.** The hybrid PKS/NRPS, HCS, and AT-less PKS system in FR901464



**Figure 5.** In vitro loading and transfer of glycerate to ACP in loading module. (A) Chemical mechanism of reaction; (B) HPLC analysis of purified apo-ACP and holo-ACP modified by Sfp (I), the generation of glyceryl-S-GAT (II), and holo-ACP incubated with glyceryl-S-GAT (III); (C) Q2-TOF-MS analysis of holo-ACP (I) and glyceryl-S-ACP (II). (o), apo-ACP; (●), holo-ACP; (◇), D-3-phosphoglycerate kinase; (▽), Sfp; (▼), glyceryl-S-ACP.

biosynthetic pathway also characterizes with an unprecedented molecular architecture and unusual domain organization. First, a surprising finding was the NRPS module consisting of tandem C domains, both of which seem to be functional because of the presence of conserved motif HHxxxDA/G. This is rare in NRPS. For the thiazole-forming NRPS module, tandem cyclization (Cy) domains have been identified in VibF<sup>64</sup> and LnmI,<sup>53</sup> in which the second Cy domain is responsible for condensation step and the first one for cyclization to afford the thiazolanyl-S-PCP intermediate. Second, there are totally four unprecedented KS domains characterized by the mutated catalytic triad of C–H–H. Since the H–H residues are necessary for malonyl-S-ACP decarboxylation to generate the corresponding carbon anion, and the Cys residue catalyzes condensation between the resultant carbon anion and the acyl-S-KS to form a C–C bond, those mutated KSs all may have lost the function of decarboxylation and C–C bond formation. However, they may all catalyze the transfer of the growing chain intermediate of acyl-S-ACP from the upstream module. The similar hypothesis of KS mediated A(P)CP-to-ACP transfer has been proposed in the biosynthetic pathway of leinamycin<sup>53</sup> and nigericin.<sup>65</sup> Third, the PKS contains nine modules but fifteen ACP domains including two groups of ACP-ACP and ACP-ACP-ACP consecutive ACPs, all containing the signature motif around the invariable 4'-phosphapantetheine attachment site Ser residue (Figure S1). Commonly, tandem ACP domains are often located in modules on which  $\beta$ -branch incorporation is predicted to occur, suggesting that this type of domain organization may have some role in recruiting some *trans*-acting biosynthetic machinery

to the appropriate site on the assembly line.<sup>28</sup> Recently, tandem ACP domains of mupirocin<sup>66</sup> and polyunsaturated fatty acid<sup>67</sup> pathway were proved to increase final production and apparently function either in parallel or in series. Additionally, a polyketide chain “skipping” mechanism was also proposed based on in vivo and in vitro data in leinamycin pathway.<sup>68</sup> Several ACP domains in FR901464 pathway could be functionally classified into these two types, but the exact roles of those “excrecent” ACP domains need to be further investigated.

**Biosynthesis of FR901464 Using Glycerate as Starter Units for PKS.** The complex hybrid PKS megasynthetase in FR901464 pathway contains an unusual PKS initiation module that incorporates glycolytic intermediate to start FR901464 biosynthesis. For a typical PKS initiation module, an AT domain loads acyl-CoA and transfers to an ACP domain to form thioester.<sup>69</sup> Alternatively, a modified KS called KS<sup>Q</sup> in which the active site Cys mutated into Glu (Q) residue was found in PKS loading module. This type of loading modules will load decarboxylated starter units such as malonyl-CoA and catalyzes their decarboxylation directed on the PKS. Additionally, starter units produced as free carboxylic acids seem to be activated and loaded by an NRPS like A-A(P)CP didomain at the N-terminus of the PKS.<sup>69</sup> However, the loading module of FR901464 PKS is an unusual DH\*-KR\*-GAT-ACP domain architecture and similar to the loading module of PKS in bryostatin biosynthesis, which is proposed to divert 1,3-BPG into PKS biosynthesis based on sequence analysis.<sup>35</sup> The FkbH homologue GAT domain, a bifunctional glyceryl transferase/phosphatase, contains the conserved nucleophilic Asp (D) critical for dephosphorylation and



active site Cys (Figure S1). The biochemical evidence has proven that 1,3-BPG was loaded, dephosphorated, and transferred to ACP domain to start polyketide biosynthesis. Similar mechanisms have only been biochemically characterized including polyketide chain elongation steps involved in the biosynthesis of oxazolomycin<sup>32</sup> and zwittermixin A,<sup>70</sup> and polyketide chain release process involved in the biosynthesis of tetronate.<sup>33,71,72</sup>

**An Oxidative Baeyer–Villiger Domain in PKS for Polyketide Chain Release.** The PKS in FR901464 pathway also revealed an unprecedented chain termination module. Usually, the TE-mediated product release mechanisms are the best-understood systems and are also regarded as the canonical mechanisms for most polyketide biosynthesis.<sup>73</sup> Indeed, there is a C-terminal TE domain in PKS, while an OX domain is predicted to release polyketide chain via a Baeyer–Villiger (B–V) oxidative mechanism like the process in aurafuron<sup>74</sup> and pederin<sup>60</sup> biosynthetic pathways. This OX domain shows a significant sequence similarity to PedG, AufJ, and MtmOIV, the B–V monooxygenase (BVMO) in the biosynthesis of pederin, aurafuron, and mithramycin. MtmOIV is a 56 kDa homodimeric FAD- and NADPH-dependent monooxygenase, which catalyzes the key frame-modifying step of the mithramycin biosynthetic pathway and currently is the only BVMO proven to react with its natural substrate via a B–V reaction.<sup>75</sup> However, in the FR901464 biosynthetic pathway, the FR9H-OX domain not only contains conserved cofactor binding motif GxGxxG at the N-terminus, but also possess a motif with the amino sequence FxGxxxHxxx(Y/F) (Figure S1). This motif might be implicated in conformational changes of the enzyme during the catalytic cycle.<sup>76,77</sup>

**Proposed Biosynthetic Pathway of FR901464.** Cloning and characterization of the gene cluster of FR901464 allowed us to propose a model for FR901464 biosynthesis (Figure 3). First, GAT domain of the loading module, a bifunctional glyceryl transferase/phosphatase, specifically sequesters 1, 3-BPG, covalently tethers 3-PG to the conserved Cys as a thioester, and subsequently dephosphorylates, transfers the resultant glyceryl moiety to the ACP, yielding glyceryl-S-ACP. A dehydration reaction catalyzed by DH\* and a reduction by KR\* then occur, which is ready for the first condensation cycle (Figure 3B). A discrete AT, encoded by *fr9B* or *fr9J* or *fr9O*, provides AT activity *in trans* to FR9C and loads malonyl CoA extender unit to the first ACP domain of module-1. The active KS of FR9C will then catalyze decarboxylation and condensation reactions, followed by reduction of the  $\alpha$ -keto group by KR domain. Next, the mutated KS-ACP-ACP-TE-KS may mediate the polyketide intermediate transfer from the first ACP to the last ACP in module-1. A dehydration reaction may occur during this transfer process to generate the C2'=C3' double bond of FR901464. The second NRPS module selects, activates, and loads a Thr to its cognate PCP and catalyzes the condensation between the aligned acyl-S-ACP and Thr-S-PCP. At this point, the growing acyl-peptidyl-S-PCP intermediate is switched from the NRPS to the PKS assembly line. The discrete AT, FR9B or FR9J or FR9O, provides AT activity *in trans* to FR9E, FR9F, FR9G, FR9H, and FR9I and loads malonyl CoA extender unit to seven ACP domains of the PKS module-3 to module-9 (Figure 3A). Sequential elongations of the acyl-peptidyl-S-PCP intermediate by the PKS modules complete the biosynthesis of the FR901464 hybrid polyketide-peptide-polyketide carbon backbone. During this extension process, two tailoring steps including cyclization and  $\beta$ -methylation occur based on the PKS-bond intermediate. FR9Q, a cyclase-like enzyme may facilitate the nucleophilic

attack on C-3 of  $\alpha,\beta$ -unsaturated intermediate by the C-7 alcohol to provide the cyclic ether, yielding the first tetrahydropyran ring (Figure 3C). The  $\beta$ -methylation pathway is predicted as the following: (i) one of the three ATs, possibly FR9O, loads malonyl CoA to the holo ACP, FR9M; (ii) the KS<sup>s</sup> FR9N catalyzes decarboxylation reaction to yield acetyl-S-FR9M; (iii) the HCS FR9K catalyzes nucleophilic attack of the acetyl unit on the  $\beta$ -ketothioester linked to FR9H-ACP; (iv) dehydration and decarboxylation of the HMG-S-FR9H-ACP derivative are catalyzed by FR9L and ECH domain of FR9H to form the  $\Delta^3$  olefin isomer (Figure 3D). The chain termination of carbon backbone may process by a complex mechanism including B–V oxidation, cyclization, and decarboxylation. First, an oxygen atom is introduced into the C–C bond via a B–V reaction in the full-length acyl-S-ACP intermediate catalyzed by the OX domain of FR9H. Then, the mutated KS mediates the intermediate transfer to the last ACP. Next, dehydration, hydrolysis, decarboxylation, and further cyclization of the acyl-S-FR9I-ACP derivative occurs sequentially by DH and TE domain of FR9I to form the second pyran ring (Figure 3A). Finally, FR9R (P450 oxidase), FR9P (nonheme iron hydroxylase), and FR9S (N-acetyltransferase) could serve as candidate enzymes to catalyze the formation of C3–C18 epoxide, C-4 hydroxylation, and acetylation of the hydroxyl group at C-4', respectively, although the order of these tailoring steps remains to be determined experimentally.

## CONCLUSION

FR901464 is an antitumor natural product representing a new class of potent anticancer small molecules which target the spliceosome and inhibit both splicing and nuclear retention of pre-mRNA. We have identified the biosynthetic gene cluster and biochemically characterized the chain initiation step in FR901464 biosynthesis. The availability of the FR901464 biosynthetic gene cluster and proposed biosynthetic pathway provide an excellent opportunity to investigate the enzymatic mechanism and biosynthetic machinery. It adds another example of complex hybrid PKS/NRPS, HCS, and AT-less PKS, it also features a glyceryl derivative as PKS start unit and an oxidative B–V domain combining with TE for polyketide chain release. Additionally, the unprecedented characteristics include a rare *cis*-acting ER for enoyl reduction in AT-less PKS, the tandem active C in NRPS, several mutated KSs for polyketide chain intermediate transfer, two groups of consecutive ACPs, and different strategies to form two pyran moieties in one molecule, which will enrich the current knowledge of PKS biosynthesis. The work described here not only provides a foundation to investigate the biosynthetic mechanisms of a complex AT-less PKS system but also sets the stage for metabolic engineering the pathway for novel analogues to develop useful anticancer drugs or biological probes.

## ASSOCIATED CONTENT

**S Supporting Information.** Tables S1–S2, list of primers and summary of AT-less polyketide; Figures S1–S6, amino acid alignments of conserved regions of the PKS and NRPS domains, scheme of in-frame deletion mutants, identification of the genotype of the mutants, analysis of expressed proteins, ESI-MS analysis of ACPs, and structure of AT-less polyketide. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ■ ACKNOWLEDGMENT

We thank Prof. Victor De Lorenzo, Centro de Astrobiología (Instituto Nacional de Técnica Aeroespacial-CSIC), Spain, for providing the expression system in *Pseudomonas*; Prof. Jian-Hua Liu, Dr. Ru-Bing Liang, and You Wang, School of Life Science and Biotechnology, Shanghai Jiaotong University for providing the plasmid pRKaraRed and helpful suggestions on the genetic manipulation of *Pseudomonas*; Prof. Yi-Qiang Cheng, Department of Biological Sciences, University of Wisconsin-Milwaukee, for critical reading of the manuscript. This work was supported in part by grants from the National Basic Research Program of China (973 Program) 2010CB833200 and 2009CB118901, the National Natural Science Foundation of China (90913005, 20832009, and 20921091), the Chinese Academy of Science (KJCX2-YW-H08), and the Science and Technology Commission of Shanghai Municipality (08QH14031).

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