Enduracidin Analogues with Altered Halogenation Patterns Produced by Genetically Engineered Strains of *Streptomyces fungicidicus*

Xihou Yin,* Ying Chen, Ling Zhang, Yang Wang, and T. Mark Zabriskie*

Department of Pharmaceutical Sciences, College of Pharmacy, 203 Pharmacy Building, Oregon State University, Corvallis, Oregon 97331-3507

Received November 2, 2009

Enduracidins (**1**, **2**) and ramoplanin (**3**) are structurally and functionally closely related lipodepsipeptide antibiotics. They are active against multi-drug-resistant Gram-positive pathogens, including MRSA. Each peptide contains one chlorinated non-proteinogenic amino acid residue, Cl_2-Hpg or Cl-Hpg. To investigate the timing of halogenation and the importance of chlorination on bioactivity and bioavailability of enduracidin, and to probe the substrate specificity and portability of the ramoplanin halogenase, we constructed the mutant strain SfΔ30 in which the enduracidin halogenase gene *orf30* had been deleted and complemented it with the ramoplanin counterpart *orf20*. We also expressed *orf20* in the enduracidin wild-type producer. Metabolite analysis revealed SfΔ30 produced the novel analogues dideschloroenduracidins A (**4**) and B (**5**), while the recombinant strains SfΔ30R20 and SfR20 produced monodeschloroenduracidins A (**6**) and B (**7**) and a trichlorinated enduracidin (**8**), respectively. In addition, *orf30* self-complementation yielded the strain SfΔ30E30, which is capable of producing six peptides including **6** and **7**. MS/MS analysis positioned the single chlorine atom in **6** at Hpg^{13} and localized the third chlorine atom in **8** to Hpg^{11}. Biological evaluation of these enduracidin analogues indicated that all retained activity against *Staphylococcus aureus*. Our findings lay the foundation for further utilization of enduracidin and ramoplanin halogenases in combinatorial biosynthesis.

Downloaded via UNIV ILLINOIS URBANA-CHAMPAIGN on June 1, 2022 at 22:03:29 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

Enduracidins A (**1**) and B (**2**) are among 17 amino acid lipodepsipeptides discovered in the late 1960s from fermentations of the soil bacterium *Streptomyces fungicidicus* (ATCC21013).[1,2] The two peptides are analogues that differ by one carbon in the length of the attached lipid chain. Structurally, enduracidins are distinguished by a C_{12} or C_{13} 2*Z*,4*E* branched fatty acid moiety and the presence of numerous non-proteinogenic amino acid residues, such as enduracididine (End), 4-hydroxyphenylglycine (Hpg), 3,5-dichloro-4-hydroxyphenylglycine (Cl_2-Hpg), citrulline (Cit), and ornithine (Orn). Seven of the 17 amino acids have the D configuration, and six of the residues are Hpg or a chlorinated derivative. Enduracidin (for simplicity, the peptides will be referred to singularly) exhibits potent in vitro and in vivo antibacterial activity against a wide spectrum of primarily Gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA).[3−7] Minimal inhibitory concentrations (MICs) are as low as 0.05 μg/mL, and the effect is bactericidal.[4,6] Typical MICs for vancomycin toward sensitive strains of *S. aureus* range from 0.5 to 2 μg/mL. Enduracidin has been shown to be effective in humans for treating urinary tract and skin infections caused by MRSA, but not chronic bone infections.[8] No toxicity or side effects were reported.

The only peptide related to enduracidin that has been structurally characterized is ramoplanin (**3**, A-16686), reported in 1984 from the *Actinoplanes* strain ATCC33076.[9,10] The differences in the peptides are the shorter C_8 unsaturated lipid tail, an α-1,2-dimannosyl moiety appended to Hpg^{11}, and substitution of D-Orn and L-Leu for the D- and L-enduracididine residues at C-10 and C-15, respectively. Like enduracidin, ramoplanin exhibits potent bactericidal activity and was reported to be 4- to 8-fold more active than vancomycin versus 500 strains of Gram-positive species.[11] Ramoplanin was shown to be active against Gram-positive pathogens including vancomycin-resistant enterococci (VRE) and MRSA.[12,13] Ramoplanin has recently been in development for the treatment of *Clostridium difficile* infections.[14−16]

Halogenation occurs widely in the biosynthesis of more than 4000 natural products, and it is a common structural feature in many peptide antibiotics such as enduracidin and ramoplanin.[17] The steric and electronegative effects of chlorine can have significant consequences on bioactivity.[17−20] Therefore, halogenase genes have been targeted for combinatorial biosynthesis to create structural analogues of natural product antibiotics via genetic engineering.[18,21−25] In our previous report on the characterization of the enduracidin gene cluster, we found a single putative halogenase gene (*orf30*) and predicted it to be responsible for chlorination of the Hpg residue at C-13 of enduracidin. In this report, we demonstrate production of enduracidin analogues by genetic manipulation of the enduracidin and ramoplanin halogenases genes in *S. fungicidicus*.

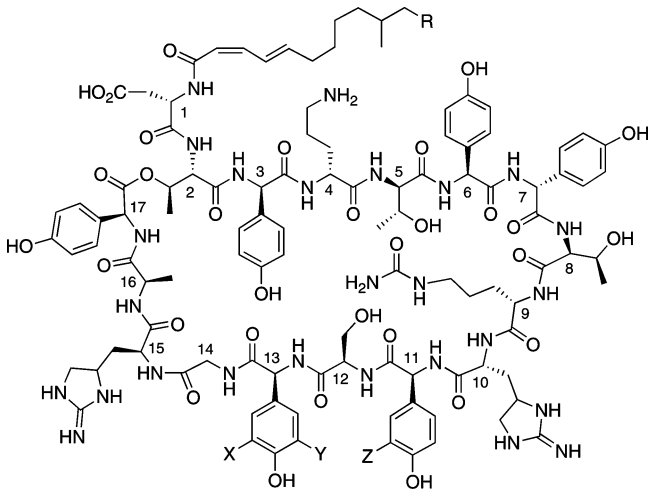
Results and Discussion

Generation of the In-Frame Deletion Mutant Strain SfΔ30. Sequence analysis of the enduracidin cluster revealed a single halogenase gene, *orf30*, which was predicted to introduce both chlorine atoms of the Cl_2-Hpg^{13} residue in enduracidin.[26] A halogenase-deficient mutant was constructed by in-frame deletion via double-crossover homologous recombination to decipher the timing of halogenation and the importance of this modification on antibiotic activity and to explore the substrate specificity of these enzymes for use in combinatorial biosynthesis. The gene replacement delivery plasmid pXY300Δ30 designed to delete *orf30* from the chromosome was constructed. After the in-frame deletion and error-free sequence of the insert were confirmed by restriction analysis and sequencing, pXY300Δ30 was conjugally introduced into the wild-type *S. fungicidicus*. The rest of the procedures for double-crossover homologous recombination were as described previously.[26] Thiostrepton-resistant exconjugants were isolated and passed through high-temperature (40 °C) selection. Independent markerless double-crossover mutants showing thiostrepton-sensitive phenotype were obtained. The *orf30* in-frame deletion mutants were further confirmed by PCR (Figure 1), sequencing, and Southern analysis (data not shown).

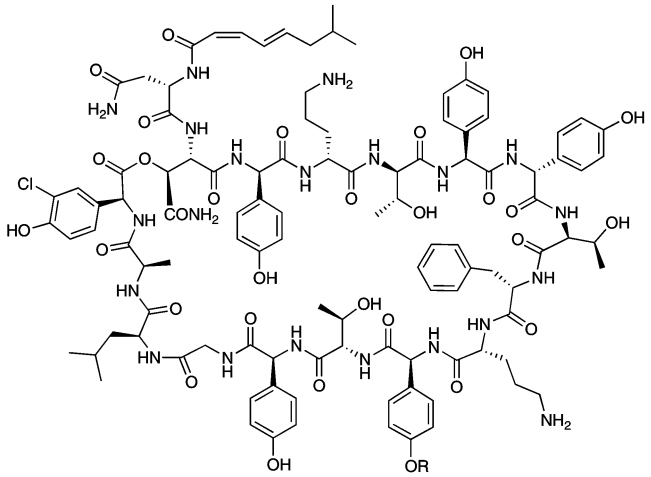
Production of Dideschloroenduracidins A and B. All media and growth conditions used for fermentation of the mutant strain SfΔ30 and methods for the metabolite preparation and analysis by

* To whom correspondence should be addressed. Tel: (541) 737-9842 or (541) 737-5774. Fax: (541) 737-3999. E-mail: Xihou.Yin@oregonstate.edu or Mark.Zabriskie@oregonstate.edu.

10.1021/np900710q © 2010 American Chemical Society and American Society of Pharmacognosy
Published on Web 03/30/2010



1: R = H; X = Y = Cl; Z = H
2: R = CH_3; X = Y = Cl; Z = H
4: R = X = Y = Z = H
5: R = CH_3; X = Y = Z = H
6: R = H; X = Cl; Y = Z = H
7: R = CH_3; X = Cl; Y = Z = H
8 R = H; X = Y = Z = Cl



3: R = α-1,2-dimannosyl

HPLC were the same as reported previously.[26] HPLC analysis of the metabolites produced by the mutant SfΔ30 revealed two new chromatographic peaks (Figure S1). These peaks were collected and analyzed by LC-ESIMS, which confirmed they were the dideschloroenduracidins A (**4**, $[M + 2H]^{2+} = m/z$ 1143.87, Figure 2A) and B (**5**, $[M + 2H]^{2+} = m/z$ 1151.00, Figure 2B). The production of **4** and **5** by SfΔ30 was also confirmed by MALDI-TOF MS analysis with the purified compounds (Figure 2C and D). This serves to support the hypothesis that Orf30 introduces both chlorine atoms on Hpg[13] of **1** and illustrates that prior chlorination of the free Hpg is not required for peptide assembly. Our results were similar to those observed when the disruption of the single halogenase gene in the balhimycin cluster, *bhaA*, led to complete loss of two chlorine atoms at residues 2 and 6 of this glycopeptide antibiotic.[22] Further, the results are consistent with the previous prediction that a single halogenase, ComH, is involved in the formation of the Cl_2-Hpg residues in complestatin.[27] Regarding the timing of halogenation, in the balhimycin system, complementation studies with β-hydroxytyrosine (Hty) and chlorinated β-hydroxytyrosine suggested that halogenation occurs after completion of Hty biosynthesis and during peptide assembly on the NRPS.[28] The formation of a chlorinated dipeptide when the balhimycin thioesterase was inserted behind module 2 to replace the C domain of module 3 in BpsA further supports that halogenation occurs on an NRPS-bound amino acid and not after completion of peptide formation.[29] The timing of halogenation of enduracidin is presumed to be similar to that of balhimycin.

Self-Complementation of the *orf30* In-Frame Deletion Mutant. To prove *orf30* is the only gene required for dichlorination of enduracidin, the construct pXY152aE30 was introduced into the in-frame deletion mutant SfΔ30. The resulting recombinant strain SfΔ30E30 carries an intact copy of *orf30* integrated into the chromosome, and its expression is controlled by the *ermE**p promoter.[30] The strain was fermented in production medium, and the metabolites were analyzed by MALDI-TOF MS. The results showed SfΔ30E30 produced six peptides: **1**, **2**, **4**, **5**, and monodeschloroenduracidins (**6** and **7**, Figure 3). The results unambiguously demonstrate that Orf30 alone is responsible for catalyzing the dichlorination observed in enduracidin. The poor efficiency of this self-complementation might be due to incomplete and/or less efficient processing of the substrate by the ectopic trans-complemented halogenase. The failure of complete restoration of chlorination of balhimycin with ectopic expression of the halogenase gene was suggested to be the outcome of the positional effect and/or different expression levels of *bhaA* in wild-type and recombinant strains.[22] It was previously noted that the wild-type *S. fungicidicus* produced two minor metabolites, enduracidins C and D, as the monochlorinated species.[31] These are presumably the same as **6** and **7** produced by the *orf30* self-complementation strain SfΔ30E30

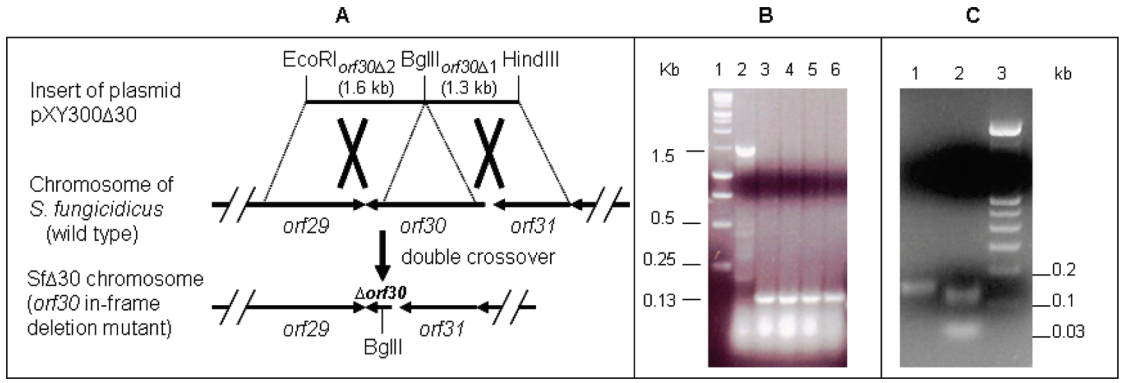


Figure 1. (A) Schematic presentation of the halogenase gene *orf30* in-frame deletion mutation. (B) PCR analysis confirming chromosomal in-frame deletion of *orf30* in the mutant strain SfΔ30 and BglII digestion of the gel-purified PCR products confirming the BglII site introduced into the chromosome of SfΔ30 (C). PCR products were separated on a 2% agarose gel. (B) Lane 1, molecular marker (Fermentas GeneRuler 1 kb DNA Ladder); lane 2, PCR product from wild-type *S. fungicidicus* (1.5 kb); lanes 3−6, PCR products from independent mutant colonies (0.13 kb). (C) Lane 1, gel-purified PCR product from SfΔ30; lane 2, gel-purified PCR product digested with BglII; lane 3, molecular marker (Fermentas GeneRuler 100 bp DNA Ladder).

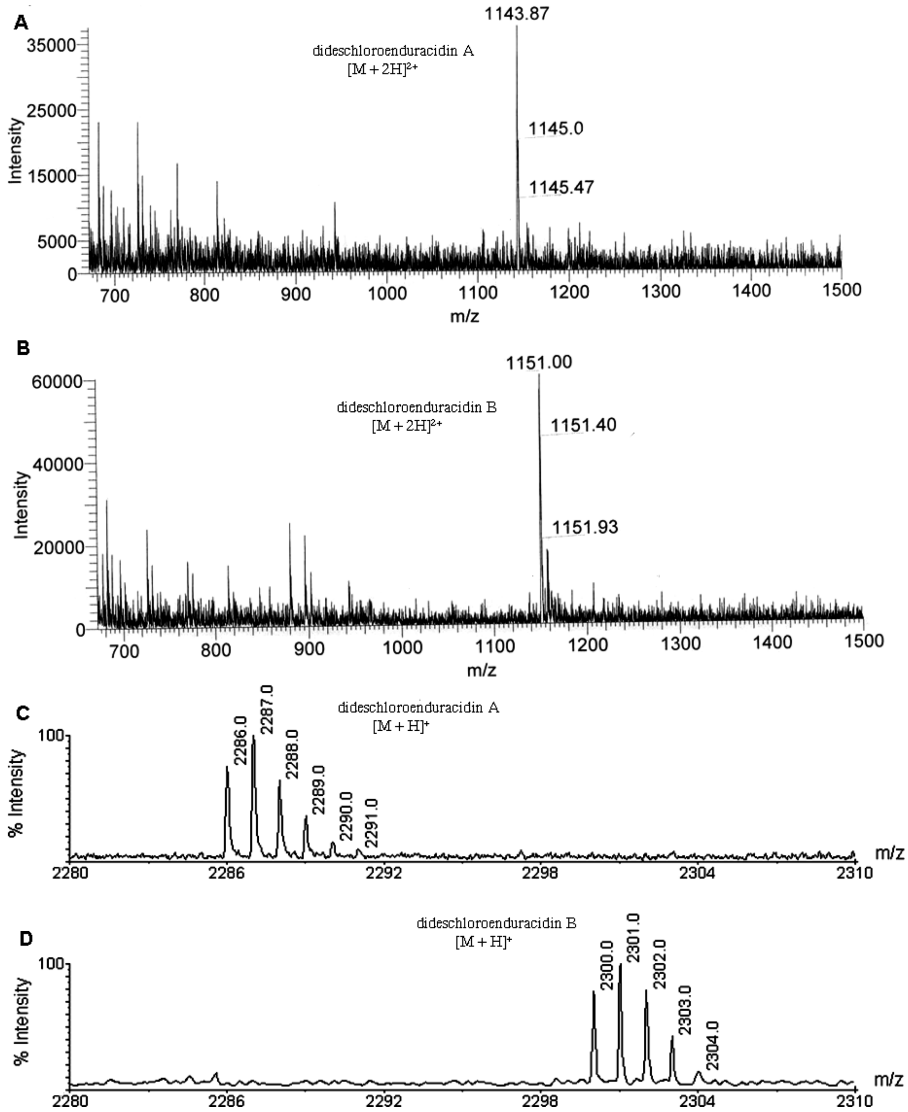


Figure 2. LC-ESIMS analysis of partially purified dideschloroenduracidins A (A) and B (B). MALDI-TOF MS analysis of the peaks corresponding to dideschloroenduracidins A (C) and B (D).

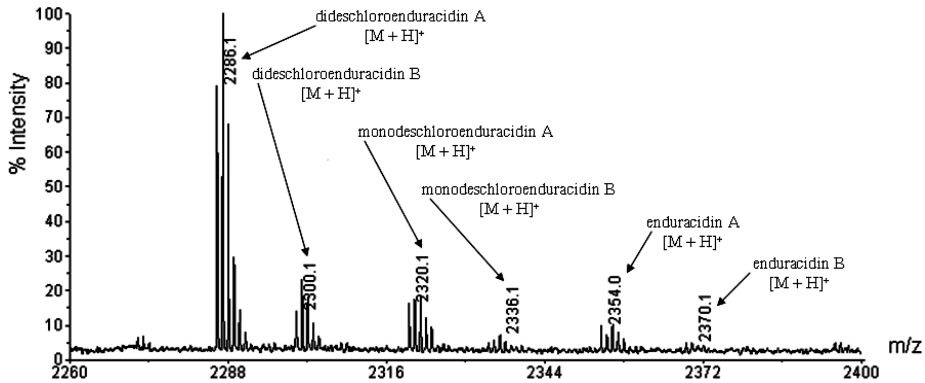


Figure 3. MALDI-TOF MS analysis of the metabolites produced by the fermentation of the recombinant strain SfΔ30E30.

because they are made by the same halogenase. The success of the *orf30* self-complementation motivated us to conduct the heterologous complementation of mutant SfΔ30 with the ramoplanin halogenase gene.

Constructing the Recombinant Strain SfΔ30R20. Sequence alignment of the enduracidin (Orf30) and ramoplanin (Orf20) halogenases showed an overall 62% amino acid identity (Figure S2). Depending on the factors governing substrate specificity, the ramoplanin halogenase may be able to complement the *orf30* in-frame deletion mutant SfΔ30 and restore production of the parent peptide or generate other enduracidin derivatives. To evaluate this hypothesis, the integrative plasmid pXY152aR20 was constructed by cloning the ramoplanin *orf20* into the integrative vector pSET152. pXY152aR20 was conjugally introduced into the mutant strain SfΔ30 to yield the recombinant strain SfΔ30R20. The expression of the ramoplanin *orf20* in the *orf30*-deficient mutant was driven by the strong constitutive promoter *ermE**p. Ectopic integration of pXY152aR20 into the chromosome of SfΔ30 was

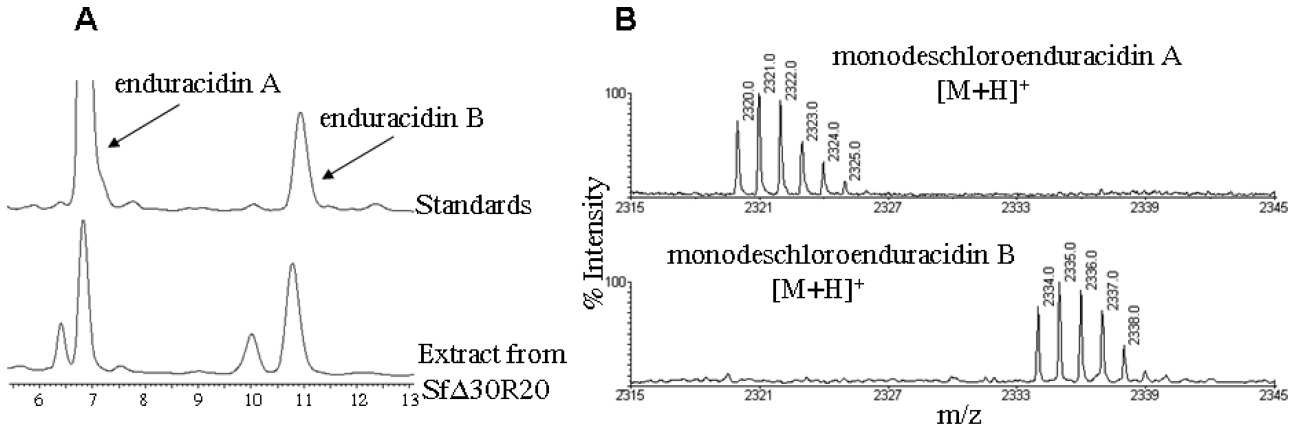


Figure 4. HPLC analysis of the crude extract from the fermentation of the recombinant strain SfΔ30R20 (A) and MALDI-TOF MS analysis of purified monodeschloroenduracidins A and B from SfΔ30R20 (B).

confirmed by Southern analysis (Figure S3) and resistance to apramycin (Am^R).

Production of Monodeschloroenduracidins A and B by Strain SfΔ30R20. SfΔ30R20 was fermented in the enduracidin production medium.[1] Metabolites were prepared from 8-day fermentation broth and mycelia and analyzed by HPLC and mass spectrometry. HPLC detected two minor and two major peaks in the metabolite extracts (Figure 4A). The major peaks have similar retention times to enduracidin A and B standards, while the two minor peaks clearly have shorter retention times than those of the standards. Analysis of the purified peaks by MALDI-TOF MS established that the two major peaks are monodeschloroenduracidins A and B, not enduracidins (Figure 4B), and the two minor peaks are dideschloroenduracidins (**4** and **5**). The production of monochlorinated species by SfΔ30R20 was also confirmed by LC-ESIMS analysis (Figure S4). In ramoplanin (**3**), a single chlorination occurs at Hpg residue 17. Production of monodeschloroenduracidins by the recombinant strain SfΔ30R20 suggested that the ramoplanin halogenase may be capable of conducting only a single chlorination at a specific amino acid residue. The observation that the restoration of neither the parent peptide enduracidins (**1** and **2**) nor a triply chlorinated species was detected in the fermentations of SfΔ30R20 appeared to be supportive of the above assumption.

Constructing Recombinant Strain SfR20 and Metabolite Analysis. Because we had entertained the possibility that the ramoplanin Orf20 may catalyze the chlorination of Hpg^{17} of enduracidin, we also transformed wild-type *S. fungicidicus* with the plasmid pXY152aR20. Ectopic integration of pXY152aR20 into the chromosome of *S. fungicidicus* was confirmed by Southern analysis (data not shown) and the Am^R phenotype. The resulting strain, SfR20, was fermented in enduracidin production medium, and the metabolite was analyzed by LC-ESIMS (Figure S5) and MALDI-TOF MS. Figure 5 shows that the predominant products were enduracidin A and a trichlorinated analogue (**8**).

Localization of the Chlorine Atom in Monodeschloroenduracidins Produced by SfΔ30R20. Because *orf20* is the only halogenase gene in the ramoplanin cluster, it is presumed responsible for the chlorination of Hpg^{17}. Due to the close structural similarity of enduracidin and ramoplanin, we thought it possible that chlorination by the ramoplanin Orf20 may occur at Hpg^{17}. However, the monodeschloroenduracidins detected in the self-complemented recombinant strain SfΔ30E30 would be expected to be chlorinated at residue 13, as in the dichloro species found in the wild-type organism. Therefore, it was necessary to independently identify the site of chlorination on the monochloro species produced by SfΔ30R20. To localize the position of the chlorine atom on the SfΔ30R20 peptide, the compound was subjected to tandem MS analysis. In the MS/MS spectrum of **1**, prominent ions are observed for fragment peptides End^{15}-Hpg^{17} (*m/z* 393.2), Ser^{12}-Hpg^{17} (*m/z*

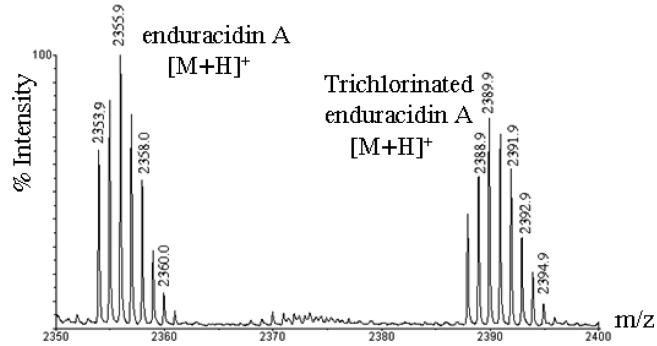
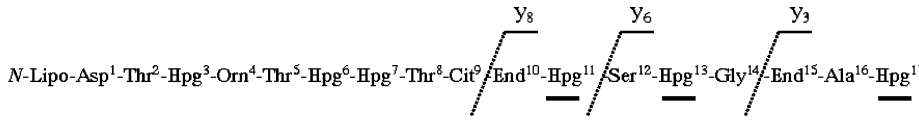


Figure 5. MALDI-TOF MS analysis of metabolites produced by recombinant strain SfR20.

754.2), and End^{10}-Hpg^{17} (*m/z* 1057.4) (Figure 6). Inspection of the MS/MS spectrum for the SfΔ30R20 monochloro peptide revealed that the ion at *m/z* 393.3 was still present, eliminating Hpg^{17} as chlorination site, but ions at *m/z* 1057.4 and 754.2 were absent. New fragment ions were seen at *m/z* 1023.4 and 720.2, corresponding to the addition of a single chlorine to Hpg^{13} in **6** and **7** (Figure 6).

The finding that Orf20 can halogenate Hpg^{13} raises the question of what factors govern substrate selection and what species is halogenated. The close structural relationship of enduracidin (**1** and **2**) and ramoplanin (**3**), including six Hpg residues, and the high sequence similarity of Orf20 and Orf30 along the entire length of the proteins, suggest that the primary determinant of halogenase substrate specificity may be dictated by local NRPS sequence. This would be consistent with the studies on balhimycin chlorination.[29] If the halogenase substrate was an advanced NRPS-bound species or a nascent peptide, one might expect that Orf20 would preferentially chlorinate Hpg^{17} over Hpg^{13}. Because the Orf20 is able to recognize and chlorinate Hpg^{13} of enduracidin, it would be very interesting to create a ramoplanin *orf20*-deficient mutant and determine if it can be complemented by enduracidin *orf30*. These complementation experiments would allow one to explore the potential halogenating capacity of Orf30 and to evaluate if it has the ability to catalyze single and/or dichlorination of residues Hpg^{17} and Hpg^{13}.

Localization of the Third Chlorine Atom on Trichlorinated Enduracidin. The presence of a trichlorinated peptide indicates that Orf20 must be capable of recognizing and chlorinating a residue other than Hpg^{13}, as was observed with the SfΔ30R20 strain. To localize the third chlorine atom, we again relied on tandem MS analysis. This was especially important because the metabolite was produced in low yield and we were unable to completely separate the trichloro analogue from enduracidin A. MS/MS allowed



Peptide	Fragment Y_8	Fragment Y_6	Fragment Y_3
End A (**1**)	1057.4	754.2	393.2
DideschloroEnd A (**4**)	989.4 = 1057.4 – 2Cl	686.2 = 754.2 – 2Cl	393.2
MonodeschloroEnd A (**6**)	1023.4 = 1057.4 – Cl	720.2 = 754.2 – Cl	393.2
ChloroEnd A (**8**)	1091.3 = 1057.4 + Cl	754.2	393.2

Figure 6. Localization of the chlorine atom on monodeschloroenduracidin A and chloroenduracidin A by MS/MS analysis.

us to work with partially purified peptides. In the MS/MS spectrum, prominent ions are observed for enduracidin A fragment peptides End^{15}-Hpg^{17} (*m/z* 393.2), Ser^{12}-Hpg^{17} (*m/z* 754.2), and End^{10}-Hpg^{17} (*m/z* 1057.4). Inspection of the MS/MS spectrum for the SfR20 trichloro peptide revealed that the ion at *m/z* 1057.4 was absent and a new fragment appeared at *m/z* 1091.3, corresponding to the addition of chlorine to the End^{10}-Hpg^{17} region. Because the ions at *m/z* 393.3 and 754.2 were still observed, the site of the third chlorination must be Hpg^{11} (Figure 6). Assuming no change in the basic halogenation mechanism of Orf20, chlorination is expected *ortho* to the phenolic hydroxy group in the analogues. Because there are no significant differences in antibacterial activities of the enduracidin analogues compared to the parent peptides, efforts to precisely localize the chlorine atoms were not pursued.

The introduction of a chlorine atom at Hpg^{11} of enduracidin by the ramoplanin halogenase was surprising. This unexpected outcome of the halogenation catalyzed by Orf20 further strengthened our assumption that local NRPS sequence plays an essential role in determining halogenase substrate specificity. This is in agreement with studies on balhimycin halogenation.[29] Through a complete set of halogenase complementation experiments, we have demonstrated the substrate flexibility and partial equivalency of the ramoplanin and enduracidin halogenases. These findings expand our knowledge about the capacity, substrate specificity, and portability of halogenases, and the knowledge gained here may be extended to create novel peptide antibiotics from other chlorinated natural product secondary metabolite gene clusters via combinatorial biosynthesis.

Biological Evaluation of Enduracidin Analogues. It is well established that the loss or replacement of the chlorine atom(s) from various natural products can have a pronounced affect on biological activities.[18−20,32] Hence, the enduracidin A analogues having varying degrees of chlorination were evaluated for in vitro antibacterial activity in an agar diffusion assay using *S. aureus* as an indicator organism. Due to the low yield and difficulty in separating the trichlorinated **8** from **1**, the extract containing compounds **1** and **8** was used in the bioassay. All the analogues tested retained activity against *S. aureus*. The monodeschloro compound **6** and the mixture of **1** and **8** showed no measurable difference in activity compared to **1**. The dideschloro compound **4** produced a zone of inhibition that was slightly less than that observed for **1** but was within typical experimental variation (Figure S6).

Experimental Section

Bacterial Strains, Plasmids, Fosmids, Media, and Culture Conditions. *Streptomyces fungicidicus* ATCC21013, *Actinoplanes* strain ATCC33076, and *Escherichia coli* S17-1 ATCC47055 were purchased from ATCC. *E. coli* XL1-Blue MR (Stratagene) and EPI300-T1^R (Epicenter) were routinely used as hosts for *E. coli* plasmids, fosmids, and *E. coli*−*Streptomyces* shuttle vectors. The pGEM-T easy cloning vector was from Promega. Plasmids pSET152 and pIJ773 were provided by Professor Keith Chater (JIC, Norwich, England). Plasmid pWHM860 harboring *ermE**p was provided by Professor Bradley Moore (UCSD, San Diego). ISP2 (Difco ISP Medium 2) and ISP4 and TSB (Bacto tryptic soy broth) were purchased from VWR. Media and culture conditions for *S. fungicidicus* were previously described.[1] All *E. coli* procedures were performed according to standard protocols.[33]

DNA Isolation and Manipulations. Isolation of chromosomal DNA from *S. fungicidicus* wild-type and the mutant strains and agarose gel electrophoresis were performed according to Kieser et al.[34] QIAprep Spin Miniprep kits (Qiagen) were used to prepare plasmids and fosmids from *E. coli* strains. Restriction endonucleases, DNA ligase, DNA polymerase, Klenow enzyme, and alkaline phosphatase were purchased from Roche, BioLabs, and Invitrogen and used according to the manufacturers' recommendations. Primers used for PCR and DNA sequencing were synthesized from Fisher. PCR products and restriction fragments from agarose gels were purified using QIAquick Gel Extraction kits from Qiagen.

Construction of Plasmid pXY300Δ30 for In-Frame Deletion of the *orf30* Gene. pXY300Δ30 was constructed by cloning two fragments that flank the chromosomal sequence destined for deletion into the *E. coli*−*Streptomyces* shuttle conjugal temperature-sensitive vector pXY300 containing the thiosptrepton resistance gene (*tsr*) for selection in *Streptomyces*.[35] An "upstream" 1.3 kb and a "downstream" 1.6 kb flanking sequence, designated *orf30*Δ1 and *orf30*Δ2, respectively, were generated by PCR using fosmid pXYF148 as the template.[26] Primers 30Δ1pf (5′-GTC**AAGCTT**GAGGAACTCGTGCTCG-3′, HindIII site is in bold) and 30Δ1pr (5′-CTG**AGATCT**ACTCATTCGGCCTC-3′, BglII site is in bold) were used to amplify fragment *orf30*Δ1; primers 30Δ2pf (5′-GCG**AGATCT**GGAGAGTACGCCGGCGA-3′; BglII site is in bold) and 30Δ2pr (5′-CTGACGGACGC**GAATTC**CCTTGC-3′; EcoRI site is in bold) were used to amplify fragment *orf30*Δ2. The location of these fragments within the enduracidin cluster is shown in Figure 1A. These two PCR fragments were appropriately restricted and simultaneously ligated with vector pXY300, prepared by digestion with *Eco*RI and *Hin*dIII, to yield plasmid pXY300Δ30. The in-frame deletion and error-free insert of pXY300Δ30 were confirmed by sequencing.

Construction of the Integrative Plasmid pXY152aR20 for Heterologous Complementation. To express the ramoplanin halogenase gene *orf20* in enduracidin halogenase-deficient mutant, we cloned *orf20* into an integrative plasmid pXY152a.[36] *orf20* was PCR-amplified by using a pair of primers (R20pf: 5′-GAGGA**CATATG**GCTGCTCAACCGGAAGAG-3′, NdeI site is in bold; R20pr: 5′-ATCT**GAATTCAGATCT**CAGTCGGCGGCGACCCACGT-3′, EcoRI and BglII sites are in bold) and the genomic DNA from ramoplanin producer *Actinoplanes* strain ATCC33076. The PCR product was then cloned into vector pGEM-T easy to afford plasmid pGEMTE-R20. Error-free *orf20* was confirmed by sequencing, then excised by digestion with NdeI and EcoRI and ligated into the similarly restricted vector pXY152a to yield the integrative plasmid pXY152aR20.

Construction of the Integrative Plasmid pXY152aE30 for *orf30* Self-Complementation. To self-complement the halogenase-deficient mutant, we cloned *orf30* into plasmid pXY152aR20 by substitution of

orf20.[26,37] *orf30* was PCR-amplified using a pair of primers (E30pf: 5′-TCAG**CATATG**CCGGGAGGCCGAATGAGTAC-3′, NdeI site is in bold; E30pr: 5′-TCGC**AGATCT**CAGGACGGATGCGGCTCCTC-3′, BglII site is in bold) and the genomic DNA from enduracidin producer *S. fungicidicus* ATCC21013. The PCR product was gel-purified and cloned into vector pGEM-T easy to generate plasmid pGEMTE-E30. Error-free *orf30* was confirmed by sequencing, excised by digestion with NdeI and BglII, and then ligated into the similarly restricted pXY152aR20 to yield the integrative plasmid pXY152aE30.

PCR Screening Protocol. Spores from independent mutant candidate colonies were inoculated in TSB liquid culture. After growth overnight, mycelia were harvested by centrifugation and washed twice with TE buffer (10 mM Tris, 1 mM EDTA), pH 8.0. Mycelia were resuspended in sterile H_2O and used as PCR template. PCR reaction mixture in a final volume of 100 μL contained 60 μL of mycelia, 150 pmol of each primer, 20 μL of 5X AccuPrime GC-rich buffer A (Invitrogen), and 1 μL of Polymix (added at 80 °C) from the Expand long template PCR system (Roche). PCR was performed as follows: 1 cycle at 95 °C for 3 min, 30 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 2 min. The reaction was terminated with one extension cycle at 72 °C for 10 min. PCR products were gel-purified and sequenced.

DNA Sequencing and Analysis. DNA sequencing was performed at Oregon State University Center for Genome Research and Biocomputing (CGRB) using the Amplitaq dye-terminator sequencing system (Perkin-Elmer) and Applied Biosystems automated DNA sequencers (models 373 and 377). Nucleotide sequences were determined for both strands. Sequence analysis was carried out using the VectorNTI (Invitrogen) software packages. Nucleotide and amino acid sequence similarity comparisons were carried out in public databases using the BLAST program.[38]

Southern Hybridization. Genomic DNA from *S. fungicidicus*, mutant SfΔ30, and recombinant strains SfR20, SfΔ30E30, and SfΔ30R20 was digested with appropriate restriction endonucleases, electrophoresed in 0.8% to 1% agarose gels, and transferred onto nylon membranes (Roche). Digoxigenin-labeled probes were used in Southern analyses. DNA probes were prepared using digoxigenin-labeled dUTP, and hybridization was revealed using a digoxigenin−DNA detection kit (Roche).

Production and Isolation of Metabolites. Fermentation conditions for the production of enduracidins from the wild-type strain, the mutant SfΔ30, and the recombinant strains SfΔ30R20 and SfR20 were as previously described.[1] Briefly, 60 g of fresh mycelia were washed with 120 mL of deionized H_2O, resuspended in 120 mL of MeOH, and sonicated with a microprobe tip for 1 min at 15 W (Microson ultrasonic homogenizer, model XL2000, Misonix Inc.). The suspension was shaken at 230 rpm at 18 °C for 3 h and then centrifuged at 2000*g* for 20 min. The supernatant was collected and evaporated to near dryness under reduced pressure, and resuspended in 10 mL of 90% MeOH. The solution was adjusted to pH 4.3 with 1 M HCl and centrifuged at 2000*g* for 20 min. The supernatant serves as the crude extracts for downstream analysis. Under the above culture and preparation conditions, wild-type *S. fungicidicus* produced an average of 8 mg/L of enduracidins. The production of dideschloroenduracidins and monodeschloroenduracidins by the corresponding mutants dropped to 4 mg/L. Production of trichlorinated enduracidins by the recombinant SfR20 decreased by approximately 10-fold, to an average yield of 0.8 mg per liter.

The crude extracts were further purified by passage through a Varian HF Mega Bond Elut-C18 column that was sequentially eluted with H_2O, 25%, 50%, and 100% CH_3CN, and 50% CH_3CN in 50 mM phosphate buffer, pH 4.3. Fractions were concentrated and analyzed by HPLC and bioassay. The fractions confirmed to contain the desired compounds were combined and further purified by semipreparative HPLC with a C18 column (Gemini 10 μM, 250 × 10 mm) from Phenomenex. Solvent A was H_2O with 0.1% TFA, and solvent B was CH_3CN with 0.1% TFA. The flow rate was 5 mL/min starting with 25% B and 75% A, increasing to 65% B over 30 min, and then held for a further 10 min. Separate 1 min fractions were collected over the program. Fractions were analyzed by HPLC, and those fractions containing the pure compounds were pooled for MS analysis and bioassay.

HPLC, LC-MS, and MS Analyses of Enduracidins. Enduracidin A and B standards were purchased from MP Biomedicals, LLC (Aurora, OH). Prior to HPLC analysis, samples were filtered through a 0.45 μm syringe filter. HPLC was performed using a Phenomenex Gemini C_{18} column, 5 μm, 4.6 × 150 mm, with isocratic elution in 30% CH_3CN and 70% 50 mM NaH_2PO_4, pH 4.5, at a flow rate of 1.0 mL/min. The UV region at 200−300 nm was scanned with a photodiode array detector, or 230 nm was monitored with a variable-wavelength detector. Two LC-MS systems were used to obtain the data reported here. The first is a ThermoFinnigan LCQ Advantage LC-MS system equipped with autosampler and photodiode array detector and controlled by the software Xcalibur 1.3 on a PC. The LC-MS analyses with this instrument were carried out at the same conditions mentioned above for HPLC except that the mobile phase for isocratic elution was replaced with 30% CH_3CN and 70% H_2O containing 0.1% TFA, and the positive mode electrospray ionization was used for MS detection. The second system is a ThermoFinnigan LTQ-FT Ultra mass spectrometer, equipped with an electrospray ionization source run in positive mode combined with a Waters CapLC system. LC-ESIMS analyses with this instrument were performed with a reversed-phase C_{18} 50 × 0.2 mm, 5 μm Michrom Magic column, using the mobile phase CH_3CN/H_2O with 0.1% formic acid and a linear gradient from 5% to 60% in 20 min, then to 90% in 10 min, at a flow rate of 4 μL/min.

MALDI-TOF MS analyses were performed using an Applied Biosystems ABI4700 TOF/TOF mass spectrometer in reflector mode with an accelerating voltage of 20 kV. Samples were mixed in a 1:4 ratio with α-cyano-4-hydroxycinnamic acid (HCCA) in 50% CH_3CN and 0.1% TFA. An aliquot of 0.5 μL of the sample solution was applied to the sample plate and air-dried.

Evaluation of Antibacterial Activity. *Staphylococcus aureus* ATCC29213 was used as an indicating microorganism in the bioassay and inoculated in LB broth. After growth at 37 °C overnight, 100 μL of the culture mixed with 5 mL of the top agar (mixture of equal volumes of nutrient agar and nutrient broth) was then overlaid onto a nutrient agar plate in which appropriately spaced wells were made by cutting out the agar plugs. Enduracidin standards and analogues were dissolved in 50% MeOH at a concentration of 20 μg/mL, and 100 μL of each solution was loaded into the wells. After incubating the plates at 37 °C for 16 h, the zones of inhibition were observed and compared, and the plates photographed.

Acknowledgment. This work was supported by NIH Grants R01GM69320 (M.Z.) and R01AI073784 (X.Y.), the OSU College of Pharmacy and General Research Fund (X.Y.), and the Medical Research Foundation of Oregon (X.Y.). Prof. P. Proteau is thanked for helpful discussions and critical reading of the manuscript. We thank Prof. K. Chater (JIC, Norwich, England) for providing plasmids pSET152 and pIJ773, and Prof. B. Moore (UCSD, San Diego) for providing plasmid pWHM860. Y. Wang is supported by the State Scholarship Fund through the China Scholarship Council of the Ministry of Education of the People's Republic of China. The Mass Spectrometry Facility at OSU is supported by NIEHS Grant P30 ES00210.

Supporting Information Available: HPLC analysis comparing wild-type *S. fungicidicus* metabolites with the extract from mutant SfΔ30, alignment of enduracidin and ramoplanin halogenases, southern hybridization confirming the recombinant strain SfΔ30R20, LC-MS data for monodeschloroenduracidins and trichlorinated enduracidins, as well as biological activity data are available. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Higashide, E.; Hatano, K.; Shibata, M.; Nakazawa, K. *J. Antibiot.* **1968**, *21*, 126–137.
(2) Asai, M.; Muroi, M.; Sugita, N.; Kawashima, H.; Mizuno, K. *J. Antibiot.* **1968**, *21*, 138–146.
(3) Goto, S.; Kuwahara, S.; Okubo, N.; Zenyoji, H. *J. Antibiot.* **1968**, *21*, 119–125.
(4) Tsuchiya, K.; Kondo, M.; Oishi, T.; Yamazaki, I. *J. Antibiot.* **1968**, *21*, 147–153.
(5) Kawakami, M.; Nagai, Y.; Fujii, T.; Mitsuhashi, S. *J. Antibiot.* **1971**, *24*, 583–586.
(6) Yourassowsky, E.; Monsieur, R. *Chemotherapy* **1972**, *17*, 182–187.
(7) Komatsuzawa, H.; Suzuki, J.; Sugai, M.; Miyake, Y.; Suginaka, H. *J. Antimicrob. Chemother.* **1994**, *33*, 1155–1163.
(8) Peromet, M.; Schoutens, E.; Yourassowsky, E. *Chemotherapy* **1973**, *19*, 53–61.
(9) Cavalleri, B.; Pagani, H.; Volpe, G.; Selva, E.; Parenti, F. *J. Antibiot.* **1984**, *37*, 309–317.

(10) Ciabatti, R.; Kettenring, J. K.; Winters, G.; Tuan, G.; Zerilli, L.; Cavalleri, B. *J. Antibiot.* **1989**, *42*, 254–267.
(11) Jones, R. N.; Barry, A. L. *Diagn. Microbiol. Infect. Dis.* **1989**, *12*, 279–282.
(12) Walker, S.; Chen, L.; Hu, Y.; Rew, Y.; Shin, D.; Boger, D. L. *Chem. Rev.* **2005**, *105*, 449–476.
(13) Fulco, P.; Wenzel, R. P. *Expert. Rev. Anti. Infect. Ther.* **2006**, *4*, 939–945.
(14) Monaghan, T.; Boswell, T.; Mahida, Y. R. *Postgrad. Med. J.* **2009**, *85*, 152–162.
(15) Bartlett, J. G. *Curr. Infect. Dis. Rep.* **2009**, *11*, 21–28.
(16) Hamburger, J. B.; Hoertz, A. J.; Lee, A.; Senturia, R. J.; McCafferty, D. G.; Loll, P. J. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 13759–13764.
(17) Gribble, G. W. *J. Chem. Educ.* **2004**, 1441–1449.
(18) Bister, B.; Bischoff, D.; Nicholson, G. J.; Stockert, S.; Wink, J.; Brunati, C.; Donadio, S.; Pelzer, S.; Wohlleben, W.; Sussmuth, R. D. *ChemBioChem* **2003**, *4*, 658–662.
(19) Eustaquio, A. S.; Pojer, F.; Noel, J. P.; Moore, B. S. *Nat. Chem. Biol.* **2008**, *4*, 69–74.
(20) Gerebtzoff, G.; Li-Blatter, X.; Fischer, H.; Frentzel, A.; Seelig, A. *ChemBioChem* **2004**, *5*, 676–684.
(21) Donadio, S.; Sosio, M. *Curr. Top. Med. Chem.* **2008**, *8*, 654–666.
(22) Puk, O.; Huber, P.; Bischoff, D.; Recktenwald, J.; Jung, G.; Sussmuth, R. D.; van Pee, K. H.; Wohlleben, W.; Pelzer, S. *Chem. Biol.* **2002**, *9*, 225–235.
(23) Weitnauer, G.; Muhlenweg, A.; Trefzer, A.; Hoffmeister, D.; Sussmuth, R. D.; Jung, G.; Welzel, K.; Vente, A.; Girreser, U.; Bechthold, A. *Chem. Biol.* **2001**, *8*, 569–581.
(24) Eustaquio, A. S.; Gust, B.; Li, S. M.; Pelzer, S.; Wohlleben, W.; Chater, K. F.; Heide, L. *Chem. Biol.* **2004**, *11*, 1561–1572.
(25) Eustaquio, A. S.; Gust, B.; Luft, T.; Li, S. M.; Chater, K. F.; Heide, L. *Chem. Biol.* **2003**, *10*, 279–288.
(26) Yin, X.; Zabriskie, T. M. *Microbiology* **2006**, *152*, 2969–2983.
(27) Chiu, H. T.; Hubbard, B. K.; Shah, A. N.; Eide, J.; Fredenburg, R. A.; Walsh, C. T.; Khosla, C. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 8548–8553.
(28) Puk, O.; Bischoff, D.; Kittel, C.; Pelzer, S.; Weist, S.; Stegmann, E.; Sussmuth, R. D.; Wohlleben, W. *J. Bacteriol.* **2004**, *186*, 6093–6100.
(29) Wohlleben, W.; Stegmann, E.; Sussmuth, R. D. *Methods Enzymol.* **2009**, *458*, 459–486.
(30) Bibb, M. J.; Janssen, G. R.; Ward, J. M. *Gene* **1985**, *38*, 215–226.
(31) Sugita, N.; Naito, K.; Asai, M.; Suzuki, T.; Higashide, E.; Mizuno, K. *J. Takeda Res. Lab.* **1972**, *31*, 313–330.
(32) Gerhard, U.; Mackay, J. P.; Maplestone, R. A.; Williams, D. H. *J. Am. Chem. Soc.* **1993**, *115*, 232–237.
(33) Sambrook, J., Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.
(34) Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation: Norwich, England, 2000.
(35) Yin, X.; O'Hare, T.; Gould, S. J.; Zabriskie, T. M. *Gene* **2003**, *312*, 215–224.
(36) Fei, X.; Yin, X.; Zhang, L.; Zabriskie, T. M. *J. Nat. Prod.* **2007**, *70*, 618–622.
(37) McCafferty, D. G.; Cudic, P.; Frankel, B. A.; Barkallah, S.; Kruger, R. G.; Li, W. *Biopolymers* **2002**, *66*, 261–284.
(38) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. *J. Mol. Biol.* **1990**, *215*, 403–410.

NP900710Q