A New Mode of Stereochemical Control Revealed by Analysis of the Biosynthesis of Dihydrogranaticin in *Streptomyces violaceoruber* Tü22

Takaaki Taguchi,[†] Yutaka Ebizuka,[†] David A. Hopwood,[‡] and Koji Ichinose*,[†]

Contribution from the Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, U.K.

Received April 10, 2001. Revised Manuscript Received July 10, 2001

Abstract: A class of *Streptomyces* aromatic polyketide antibiotics, the benzoisochromanequinones, all shows trans stereochemistry at C-3 and C-15 in the pyran ring. The opposite stereochemical control found in actinorhodin (3*S*, 15*R*, ACT) from *S. coelicolor* A3(2) and dihydrogranaticin (3*R*, 15*S*, DHGRA) from *S. violaceoruber* Tü22 was studied by functional expression of the potentially relevant ketoreductase genes, *act*III, *act*VI-ORF1, *gra*-ORF5, and *gra*-ORF6. A common bicyclic intermediate was postulated to undergo stereospecific reduction to provide either the 3-(*S*) or the 3-(*R*) configuration of an advanced intermediate, 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho[2,3-*c*]pyran-3-acetic acid (DNPA). Combinations of the four ketoreductase genes were coexpressed with the early biosynthetic genes encoding a type II minimal polyketide synthase, aromatase, and cyclase. *gra*-ORF6 was essential to produce (*R*)-DNPA in DHGRA biosynthesis. Out of the various recombinants carrying the relevant ketoreductases, the set of *gra*-ORF5 and -ORF6 under translational coupling (on pIK191) led to the most efficient production of (*R*)-DNPA as a single product, implying a possible unique cooperative function whereby *gra*-ORF6 might encode a "guiding" protein to control the regio- and stereochemical course of reduction at C-3 catalyzed by the *gra*-ORF5 protein. Updated BLAST-based database analysis suggested that the *gra*-ORF6 product, a putative short-chain dehydrogenase, has virtually no sequence homology with the *act*VI-ORF1 protein, which was previously shown to determine the 3-(*S*) configuration of DNPA in ACT biosynthesis. This demonstrates an example of opposite stereochemical control in antibiotic biosynthesis, providing a key branch point to afford diverse chiral metabolic pools.

Introduction

Recent progress in the understanding of the genetics of secondary metabolism has led to genetic engineering to generate many novel molecules with diverse structures and biological activities. Typical examples are seen in polyketide antibiotics, where there has been an increasing availability of genes encoding polyketide synthases (PKSs).[1] Two subtypes[2] of bacterial PKSs have been identified: type I (multifunctional large proteins with multiple modular domains), involved in macrolide biosynthesis; and type II (complexes of discrete monofunctional proteins), relevant to the aromatics. In vivo multistep synthesis through combinations of these proteins or of their constituent domains has succeeded in producing libraries of novel polyketides for macrolides[3] as well as aromatics:[4] this has led to the new approach of combinatorial biosynthesis of "unnatural natural products".

Further structural modification of early polyketide intermediates occurs by post-PKS modifying ('tailoring') steps.[2,5] They typically include oxidoreductions, dehydrations, rearrangements, and group transfers, which proceed in a regio- or stereospecific manner. For example, a chiral center involving a hydroxyl group is introduced by stereospecific reduction of the corresponding carbonyl functionality. Its different stereospecificities generally facilitate a key branch point in metabolic pathways. In plants, tropinone reductases (TR-I and TR-II) are involved in the biosynthesis of tropane alkaloids in *Hyoscyamus niger*, and convert tropinone into tropine (3α-hydroxytropane) and pseudotropine (3β-hydroxytropane), respectively, under strict stereochemical control. The respective enzymes are both short-chain dehydrogenases with significant overall amino acid sequence similarity.[6] Determination of the crystal structure revealed[7] that the opposite specificities are determined by enzymes with a conserved overall folding, by changing the amino acids in the substrate binding site to orient the substrate, tropinone, in the catalytic cavity.

* To whom correspondence should be addressed. Phone: +81-3-5841-4742. Fax: +81-3-5841-4744. E-mail: ichinose@mol.f.u-tokyo.ac.jp.
[†] The University of Tokyo.
[‡] John Innes Centre.

(1) Hutchinson, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3336−3338.
(2) Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465−2497.
(3) (a) Xue, Q.; Ashley, G.; Hutchinson, C. R.; Santi, D. V. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11740−11745. (b) McDaniel, R.; Thamchaipenet, A.; Gustafsson, C.; Fu, H.; Betlach, M.; Ashley, G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1846−1851.
(4) (a) McDaniel, R.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *Nature* **1995**, *375*, 549−554. (b) Shen, Y.; Yoon, P.; Yu, T.-W.; Floss, H. G.; Hopwood, D. A.; Moore, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3622−3627.
(5) Ichinose, K.; Taguchi, T.; Ebizuka, Y.; Hopwood, D. A. *Actinomycetologica* **1998**, *12*, 99−109.
(6) Nakajima, K.; Hashimoto, T.; Yamada Y. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9591−9595.
(7) Nakajima, K.; Yamashita, A.; Akama, H.; Nakatsu, T.; Kato, H.; Hashimoto, T.; Oda, J.; Yamada Y. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4876−4881.

10.1021/ja015981+ CCC: $20.00
Published on Web 10/18/2001

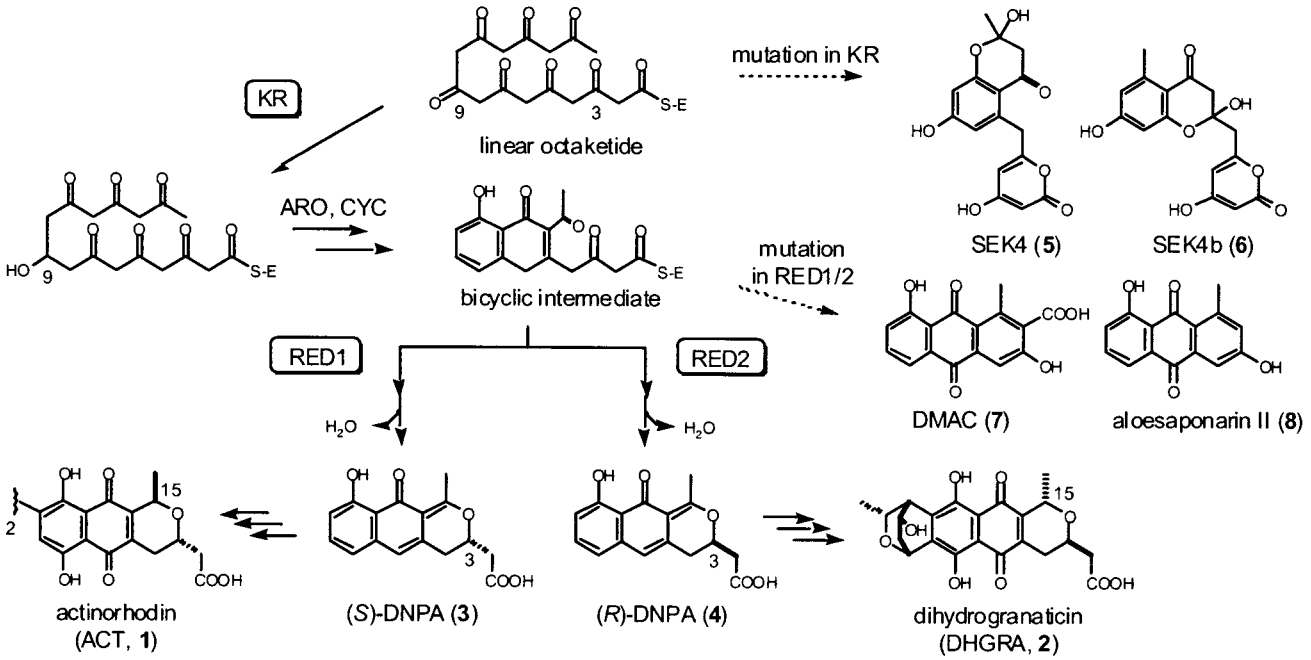

Figure 1. Proposed biosynthetic pathways of actinorhodin and dihydrogranaticin. A linear octaketide produced by the minimal PKS is subjected to subsequent steps catalyzed by KR (ketoreductase for C-9), ARO (aromatase), CYC (cyclase), and RED1/2 (stereospecific reductase for C-3). RED1/2 control stereospecific pyran ring formation, providing either (3*S*, 15*R*) or (3*R*, 15*S*) configuration. Mutation of KR or RED1/2 leads to production of the shunt products, SEK4/SEK4b[22] or DMAC/aloesaponarin II,[12] respectively. The intermediates up to the bicyclic intermediate are tentatively shown as enzyme-bound (CO−S−E.). Numbering of carbon atoms is based on the biosynthetic origin.

Here, we deal with opposite stereospecificities in antibiotic biosynthesis. Benzoisochromanequinone (BIQ) antibiotics[2,5] are aromatic polyketides produced by *Streptomyces* spp. The pyran rings of naturally occurring BIQs all show trans configuration in respect of their C-3 and C-15 chiral centers, either (3*S*, 15*R*) represented by actinorhodin (ACT, **1**) from *Streptomyces coelicolor* A3(2) or (3*R*, 15*S*) as found in dihydrogranaticin (DHGRA, **2**) from *Streptomyces violaceoruber* Tü22 (Figure 1). A linear octaketide produced by the type II minimal PKS is modified by the following reactions to produce the common bicyclic intermediate:[8] reduction at C-9 by a ketoreductase (KR, defined hereafter as functioning at C-9), aromatization of the first ring by an aromatase (ARO), and second ring formation by a cyclase (CYC). All the encoding genes are part of the respective biosynthetic gene cluster: the *act* cluster for ACT and the *gra* cluster for DHGRA. We proposed[8] the next step to be a metabolic branch point in the BIQs, that is, stereospecific reduction at C-3 of the bicyclic intermediate. In ACT biosynthesis, *act*VI-ORF1 was proven[9] to encode a dedicated reductase (RED1) to establish the (*S*) configuration at C-3 and to afford the first chiral intermediate, 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho[2,3-*c*]pyran-3-(*S*)-acetic acid ((*S*)-DNPA, **3**). An obvious expectation was the existence of an *act*VI-ORF1 homologue, encoding a reductase (RED2) with the opposite stereospecificity to produce (*R*)-DNPA (**4**), in the *gra* cluster. However, there is no apparent candidate among the ORFs characterized[10] in the entire cluster. The *gra* cluster instead was found to contain five ORFs, *gra*-ORF5, -6, -17, -22, and -26, likely to encode ketoreductases. We then demonstrated[11] that *gra*-ORF5 and -ORF6 restore blue pigmentation characteristic of the ACT chromophore to the *S. coelicolor* B22 mutant, which is functionally deficient in *act*VI-ORF1, indicating the production of ACT-like products with possible pyran ring formation under unnatural stereochemical control at C-3. The present study investigates this possibility and provides an example of opposite stereospecificity in antibiotic biosynthesis controlled by ketoreductases with virtually no sequence similarity.

Experimental Section

Optical rotations were determined on a Jasco P-1010 digital polarimeter. CD spectra of DNPAs were measured on a Jasco J-600 spectropolarimeter. LC/MS spectra were recorded on a Thermo quest LCQ equipped with a Hewlett-Packard HP1100 series LC system.

Bacterial Strains and DNA Manipulations. The host strain for all the plasmids was *S. coelicolor* A3(2) CH999,[12] which is an *act* cluster-deficient strain (*proA1, argA1, SCP1*$^-$, *SCP2*$^-$, *redE60*, Δ*act*). General DNA manipulations were according to standard procedures.[13] *Streptomyces* manipulations were as described.[14]

Plasmids. Seven plasmids were constructed based on the *Streptomyces* expression vector, pRM5,[12] according to the method described for pIJ5660.[9] A summary of the constructs is in Table 2. To make pIK177, the *Sph*I site in the *act*III gene of pRM5 was used to generate a frameshift mutation by Klenow treatment. The expression cassettes for *gra*-ORF5, *gra*-ORF6, and *gra*-ORF5+6 (a tandem set with translational coupling) were derived from pIK115, pIK165, and pIK119,[11] respectively. An *Sph*I site was engineered on each end of

(8) Fernández-Moreno, M. A.; Martínez, E.; Caballero, J. L.; Ichinose, K.; Hopwood, D. A.; Malpartida, F. *J. Biol. Chem.* **1994**, *269*, 24854−24863.

(9) Ichinose, K.; Surti, C.; Taguchi, T.; Malpartida, F.; Booker-Milburn, K. I.; Stephenson, G. R.; Ebizuka, Y.; Hopwood, D. A. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 395−400.

(10) Ichinose, K.; Bedford, D. J.; Tornus, D.; Bechthold, A.; Bibb, M. J.; Revill, W. P.; Floss, H. G.; Hopwood, D. A. *Chem. Biol.* **1998**, *5*, 647−659.

(11) Ichinose, K.; Taguchi, T.; Bedford, D. J.; Ebizuka, Y.; Hopwood, D. A. *J. Bacteriol.* **2001**, *183*, 3247−3250.

(12) McDaniel, R.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *Science* **1993**, *262*, 1546−1550.

(13) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning. A laboratory manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1986.

(14) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces genetics*; The John Innes Foundation: Norwich, UK, 2000.

Table 1. BLAST Analysis of Ketoreductase Genes in This Study

gene designation	proteins (aa)	similarity[a] (%)			Putative conserved domain detected	refs
		*act*VI-ORF1	*gra*-ORF5	*gra*-ORF6		
*act*III	261	no	79	47	short-chain alcohol dehydrogenase	19
*act*VI-ORF1	307		no	no	3-hydroxyacyl-CoA dehydrogenase	8
gra-ORF5	272			49	short-chain alcohol dehydrogenase	10, 20
gra-ORF6	249				short-chain alcohol dehydrogenase	10, 20

[a] Data from BLAST2 SEQUENCES; no, no significant similarity was found.

the respective cassettes, followed by introduction into either pRM5 or pIK177 to give the plasmids, pIK186−pIK191.

Conditions for Culture and HPLC Analysis. Culture conditions for *Streptomyces* recombinant strains were as described.[15] The supernatant (100 μL) of each culture was subjected directly to reversed-phase HPLC analysis on a Tosoh 8020 system under the following conditions: column, TSK gel ODS-80T$_M$ (4.6 mm × 15 cm, Tosoh); column temperature, 40 °C; gradient elution, solvent A (0.5% AcOH in acetonitrile) and solvent B (0.5% AcOH in deionized H_2O), gradient profile (0−5 min, 35% A; 5−30 min, 35−95% A; 30−35 min, 95% A; 35−40 min, 95−35% A); flow rate, 1.0 mL/min; photodiode array detector (PD-8020. Tosoh), 250−500 nm.

LC/APCIMS Analysis. The metabolites of CH999/pIK177 and CH999/pIK190 were analyzed by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS) under the same chromatographic conditions except for gradient profile. The gradient profile was as follows: 0−35 min, 10−95% A; 35−40 min, 95% A; 40−45 min, 95−10% A.

Isolation of DNPA. Spores of CH999/pIJ5660, CH999/pIK187, CH999/pIK188, or CH999/pIK191 were inoculated to seed medium (10 mL), and four cultures for each recombinant were shaken on a rotary shaker (220 rpm) at 30 °C, for 2 days. A 2-mL sample of the seed culture was transferred into 100 mL of production medium in each 500-mL Erlenmeyer flask. Twenty production cultures were shaken on a rotary shaker (200 rpm) at 28 °C, for 4 days. After centrifugation of the culture, the supernatant was extracted with chloroform and the solvent was removed in vacuo. The crude extract was subjected to silica gel column chromatography and eluted with chloroform/EtOAc (9:1). Fractions containing DNPA were combined, and the solvent was removed in vacuo. DNPA was purified further by preparative HPLC using the Tosoh 8010 system under the following conditions: column, TSK gel ODS-80T$_M$ (7.8 mm × 30 cm, Tosoh); column temperature, 40 °C; eluent, 40% aqueous acetonitrile containing 0.5% AcOH; flow rate, 2.5 mL/min; detector, 254 nm. The yields (mg/L) of DNPAs from the recombinants derived from the different constructs were pIK187, 9.3; pIK188, 14; pIK191, 37; and pIJ5660, 18.

Optical Rotations and CD Spectra of DNPAs. $[\alpha]_D$ in acetonitrile: pIK187, −221.6° (*c* 0.21); pIK188, −213.9° (*c* 0.88); pIK191, −204.7° (*c* 0.84); pIJ5660, +265.4° (*c* 0.84). The concentration of each sample in acetonitrile for CD measurement was as follows: pIK187, 0.21 g/L; pIK188, 0.22 g/L; pIK191, 0.22 g/L; pIJ5660, 0.21 g/L.

HPLC Analysis Using a Chiral Column. Pure DNPAs were subjected to chiral HPLC analysis on a Tosoh 8020 system under the following conditions: chiral column, TSK gel Enantio-OVM (4.6 mm × 15 cm, Tosoh); column temperature, 33 °C; eluent, 50 mM $AcONH_4$ (pH 4.2)/acetonitrile (85:15); flow rate, 1.5 mL/min; detector, 434 nm.

Computer Analysis of DNA and Protein Sequences. Sequences were analyzed with the PSI-BLAST[16] and BLAST 2 SEQUENCES[17] programs provided by the National Center for Biotechnology Information, NIH. A World Wide Web (www) version (http://ncbi.nlm.hih.gov/Blast) was applied using the default parameters therein.

Results

Database Analysis of *act* and *gra* Ketoreductase Genes. The present study focused on the two reduction steps at C-3 and C-9 in BIQ biosynthesis (Figure 1). Homologous proteins representing the reductase for C-9, KR, are encoded in numerous gene clusters for aromatic polyketide antibiotics; all of them resemble typical short-chain alcohol dehydrogenases (SCADs).[2,18] The translated products of the encoding genes, *act*III[19] for ACT and *gra*-ORF5 for DHGRA,[20] together with the putative ketoreductase genes, *act*VI-ORF1 and *gra*-ORF6, were analyzed by an interactive www version of the updated BLAST programs, PSI-BLAST[16] and BLAST2 SEQUENCES.[17]

The results are summarized in Table 1. The *gra*-ORF6 product was recognized as a short-chain dehydrogenase with significant similarity (47/49%) to the KR genes, whereas the deduced product of *act*VI-ORF1 was assigned as a dehydrogenase with more specific function as a 3-hydroxyacyl-CoA dehydrogenase. There was no significant similarity (as defined by the BLAST2 SEQUENCES program) between *gra*-ORF6 and *act*VI-ORF1.

Effect of Adding a Ketoreductase to a Set of PKS, ARO, and CYC Genes. To clarify the function of *gra*-ORF5 and -ORF6 relevant to the configuration at C-3 in DHGRA biosynthesis, seven plasmids were constructed based on the expression vector, pRM5, and introduced into the host strain, *S. coelicolor* CH999, which lacks the entire *act* gene cluster.[12] pRM5 provides the genes encoding the enzymes required to produce the bicyclic intermediate (Figure 1), which undergoes spontaneous cyclization to produce two shunt products, 3.8-dihydroxy-1-methyl-anthraquinone-carboxylic acid (DMAC, **7**), and aloesaponarin II (**8**). Addition of *act*VI-ORF1 to the pRM5 system, in pIJ5660, was previously demonstrated[9] to produce **3**,[21] indicating that *act*VI-ORF1 encodes a dedicated reductase for C-3. Earlier work[22] demonstrated that loss of KR resulted in production of two shunt products, SEK4 (**5**) and SEK4b (**6**), suggesting its influence on the regiospecificity of a subsequent aldol-type cyclization leading to the bicyclic intermediate. Therefore, a KR-deficient version of pRM5, pIK177, was used as a host for the addition experiments. Production of **5** and **6** (minor) by CH999/pIK177 was confirmed by LC/APCIMS analysis (data not shown).

Of the newly created recombinants, CH999/pIK189 produced **7** and **8**, clearly proving that *gra*-ORF5 encodes a KR that is functionally identical with *act*III. Three recombinants carrying this or the *act*III KR gene, as well as *gra*-ORF6, CH999/pIK187,

(15) Taguchi, T.; Itou, K.; Ebizuka, Y.; Malpartida, F.; Hopwood, D. A.; Surti, C. M.; Booker-Milburn, K. I.; Stephenson, G. R.; Ichinose, K. *J. Antibiot.* **2000**, *53*, 144−152.

(16) Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *25*, 3389−3402.

(17) Tatusova, T. A.; Madden, T. L. *FEMS Microbiol. Lett.* **1999**, *174*, 247−250.

(18) Persson, B., Krook, M.; Jörnvall., H. *Eur. J. Biochem.* **1991**, *200*, 537−543.

(19) Hallam, S. E.; Malpartida, F.; Hopwood, D. A. *Gene* **1998**, *74*, 305−320.

(20) Sherman, D. H.; Malpartida, F.; Bibb, M. J.; Kieser, H. M.; Bibb, M. J.; Hopwood, D. A. *EMBO J.* **1989**, *8*, 2717−2725.

(21) Cole S. P.; Rudd, B. A. M.; Hopwood, D. A.; Chang, C.-J.; Floss, H. G. *J. Antibiot.* **1987**, *40*, 340−347.

(22) (a) Fu, H.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *J. Am. Chem. Soc.* **1994**, *116*, 4166−4170. (b) Carreras, C. W.; Piper, R.; Khosla, C. *J. Am. Chem. Soc.* **1996**, *118*, 5158−5159. (c) McDaniel, R.; Ebert-Khosla, S.; Fu, H.; Hopwood, D. A.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11542−11546.

Table 2. Effect of Adding Reductases to Minimal PKS, ARO, and CYC[a]

constructs	additional genes	product(s)	comments	refs
pRM5	*act*III	**7**, **8**	C-9 reduction to produce the bicyclic system	12
pIKI86	*act*III, *gra*-ORF5	**7**, **8**	*gra*-ORF5 and *act*III function as a reductase for C-9 (KR)	*b*
pIKI87	*act*III, *gra*-ORF6	**4**, **7**, **8**	gra-ORF6 provides reduction at C-3 (RED2)	*b*
pIKI88	*act*III, *gra*-ORF5+6	**4**	same as above, and more efficiently	*b*
pIKI77		**5**	loss of KR causes loss of control for the subsequent cyclizations	22; *b*
pIKI89	*gra*-ORF5	**7**, **8**	*gra*-ORF5 substitutes for *act*III	*b*
pIKI90	*gra*-ORF6	**5**	*gra*-ORF6 product is not a KR	*b*
pIKI91	*gra*-ORF5+6	**4**	*gra*-ORF5+6 provide KR and RED2 most efficiently	*b*
pIJ5660	*act*III, *act*VI-ORF1	**3**	*act*VI-ORF1 encodes a dedicated reductase for C-3 (RED1)	9

[a] The genetic arrangement of *gra*-ORF5 and *gra*-ORF6 shows potential translational coupling (indicated by "+"). An independent expression cassette for *gra*-ORF6 was engineered as described[11] and used for pIK187 and pIK190. Major products whose structures are in Figure 1 are listed (see Experimental Section for other data on DNPA). [b] This work.

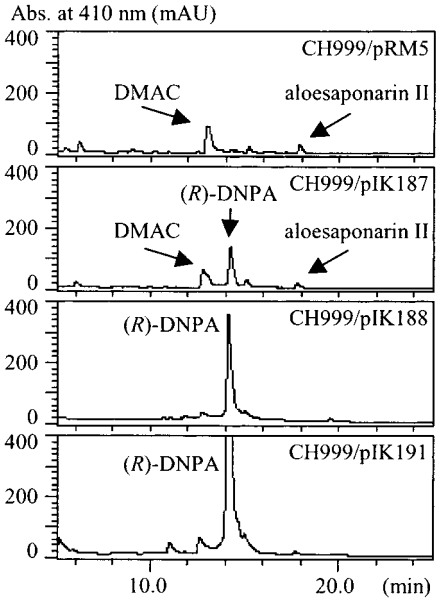

Figure 2. HPLC chromatograms of the metabolites of recombinant strains, CH999/pRM5, CH999/pIK187, CH999/pIK188, and CH999/pIK191.

CH999/pIK188, or CH999/pIK191, produced DNPA, demonstrating the requirement of *gra*-ORF6 for reduction at C-3 in the absence of *act*VI-ORF1. HPLC profiles (Figure 2) of the culture medium revealed apparent differences among the recombinants. pIK191 carries the natural genetic arrangement of the *gra* cluster, where *gra*-ORF6 is likely to be translationally coupled with *gra*-ORF5,[20] and gave the most efficient production of DNPA. Addition of an extra copy of a KR gene (*act*III) to *gra*-ORF5+6 (pIK188) gave a slightly less efficient production. A heterologous combination of *gra*-ORF6 and *act*III (pIK187) resulted in a significantly lower yield of DNPA, together with substantial production of the shunt products, DMAC and aloesaponarin II. Expression of *gra*-ORF6 along with the earlier enzymes, but in the absence of a KR (pIK190), had no effect on the production profile of the recombinants, suggesting that *gra*-ORF6 alone *cannot* substitute for a KR. The results are summarized in Table 2.

CD Spectra and Chiral HPLC Analysis of DNPAs. The CD spectrum was measured for (*R*)-DNPAs purified from the recombinants. All of the spectra were essentially identical, and each gave a curve that was the mirror image of that of (*S*)-DNPA from CH999/pIJ5660 (Figure 3A). Chiral HPLC analysis suggested that both (*S*)- and (*R*)-DNPA were optically pure, unambiguously demonstrating the strict opposite stereospecificities determined by *act*VI-ORF1 for ACT and *gra*-ORF6 for DHGRA, respectively.

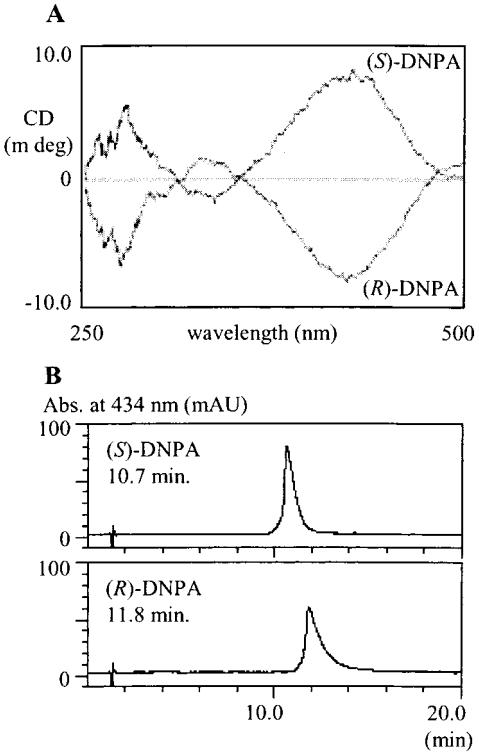

Figure 3. Superimposed CD spectra (A) and chiral HPLC profiles (B) of DNPAs from the recombinant strains. Data for (*S*)-DNPA from CH999/pIJ5660 and (*R*)-DNPA from CH999/pIK191 are shown; those of (*R*)-DNPA from the strains carrying pIK187 and pIK188 were identical.

Discussion

The present study clearly demonstrated that *gra*-ORF6 is essential for determination of the (*R*) configuration at C-3 in DHGRA biosynthesis. This gene shows medium similarity (~45%) with a family of *act*III-type reductase (KR) genes, most of which have been identified in the biosynthetic gene clusters of actinomycete aromatic polyketides.[2] Recent interactive database analysis has been successfully applied to fold recognition[23] and three-dimensional modeling[24] of a target protein. An attempt was made to identify any recognizable similarity of protein structure among the ketoreductase genes in this study, but no significantly new information was obtained (Table 1).

Functional assignment of *act*VI-ORF1 was predictable, because the deduced product is an apparent homologue of 3-hydroxyacyl-CoA dehydrogenase whose substrate motif is

(23) (a) Panchenko, A. R.; Marchler-Bauer, A.; Bryant, S. H. *J. Biol. Chem.* **2000**, *296*, 1319−1331. (b) Aravind, L.; Koonin, E. V. *J. Mol. Biol.* **1999**, *287*, 1023−1040.

(24) Joens, D. T. *J. Mol. Biol.* **1999**, *292*, 195−202.

identified in the structure of the bicyclic intermediate.[8] Beyond a putative cofactor binding motif, the *gra*-ORF6 protein has virtually no sequence or motif similarity with the deduced product of *act*VI-ORF1. A possibility is that both gene products are dedicated stereospecific reductases (RED1/2, Figure 1) with opposite stereospecificities. If so, this is in sharp contrast to the case of the two reductases, TR-I (273 amino acids) and -II (259 amino acids), mentioned earlier,[6,7] which are very similar in sequence. They showed high overall identity (64%) and belong to a family of SCADs characteristic of a common N-terminal cofactor-binding domain.[8] Three highly conserved glycine residues (shown in italics) in the domain are found in all the reductases in this study: *act*III, ^{13}*G*ATS*GIG*^{19}; *act*VI-ORF1, ^{8}*GAG*TI*G*^{13}; *gra*-ORF5, ^{24}*GATSGIG*^{30}; *gra*-ORF6, ^{15}*G*ASSS*GIG*^{21}, suggesting, at least, a similar way of cofactor binding.

Possible involvement of such dedicated ketoreductases was recently demonstrated by the studies[25] on the 2-deoxygenation step in deoxyhexose formation in antibiotic biosynthesis, where the opposite stereospecificity at the C-3 ketoreduction of a hexose moiety is conferred by the unrelated ketoreductases.[26] Another example is known for the opposite isomer-specific enzymes, constituting two distinct protein families: two forms of lactate dehydrogenase (LDH). Although L-isomer-specific enzymes possess similar N-terminal NAD-binding domains, this is not the case for D-lactate dehydrogenase. L-LDH (321 amino acids) and D-LDH (332 amino acids) from *Lactobacillus plantarum* showed[27] ^{14}*GDGAVG*^{19} and ^{152}*GTGRIG*^{157}. LDH is rather a long dehydrogenase outside the limits of the category of a typical SCAD (250−300 amino acids), indicating a marked difference from the opposite stereospecificities controlled by the two proteins with unrelated primary sequences described here.

Particularly noteworthy are differences in the efficiency of (*R*)-DNPA production in the various recombinants. The reconstituted set of *gra*-ORF5+6, together with the earlier biosynthetic genes, on pIK191, led to production of (*R*)-DNPA as a single product most efficiently. It is intriguing to speculate that the two gene products might cooperatively function as a KR as well as RED2. The presence of an extra copy of a KR gene, *act*III (pIK188), might interfere with this cooperation (62% reduction in yield), and the heterologous combination of *gra*-ORF6 and *act*III (pIK 187) gave an even more negative effect, with substantial production of shunt products, DMAC and aloesaponarin II. The earlier result[11] that *gra*-ORF5+6 (*but not gra*-ORF6 alone) restored blue pigmentation in the *S. coelicolor* B22 mutant showed that *gra*-ORF5+6 function as RED2. Such a cooperative function postulated here is known for the type II PKS components. Type II minimal PKSs contain a set of three protein subunits: the two β-ketoacylACP synthase subunits, KSα, KSβ (the latter also referred to as the chain length factor[12] or the chain initiation factor[28]), and an acyl carrier protein (ACP). In vitro expression of these components revealed that *act* KSα and KSβ were actively coexpressed from the natural genetic arrangement of translational coupling.[29] From this we may speculate that *gra*-ORF6 might encode a "guiding" protein to control the regio- and stereochemical course of reduction at C-3 catalyzed by the *gra*-ORF5 protein (or artificially the *act*III protein). The reductases acting at C-9 as KRs, here represented by the *act*III and *gra*-ORF5 gene products, would exert stereochemical control, as in the classical case of the fatty acid synthase KR. Its outcome cannot be readily determined because the resulting OH group is subsequently removed by dehydration. However, since the stereochemistry at C-3 is opposite in ACT and DHGRA, it is evident that in one pathway the stereochemistry at C-3 would be the same as at C-9, while in the other it would have opposite stereochemistry. Perhaps the latter is the case for ACT, necessitating a separate, dedicated enzyme, RED1 (the *act*VI-ORF1 product), for C-3 reduction. For DHGRA, C-3 could be reduced with the correct stereochemistry by the KR (the product of *gra*-ORF5 or, artificially, of *act*III), but this enzyme would require "guidance" to C-3 (as opposed to its standard role at C-9) by an additional protein: this control of regiospecificity would be the role of the *gra*-ORF6 product.

Recently, we demonstrated[30] that the *act*VI-ORF1 product is indeed a dedicated reductase for C-3 by feeding a series of synthetic analogues of the bicyclic intermediate into CH999/pIJ5675 in which *act*VI-ORF1 is expressed, to give enantioselective reduction. Testing is in progress to see if the *gra* reductase genes encode a similar reducing activity for such substrates.

Acknowledgment. We thank Kevin I. Booker-Milburn and G. Richard Stephenson for helpful discussions. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) "Genome Biology" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (12206030), to K.I. Further financial support was obtained from the John Innes Foundation to D.A.H. and the Japan Society for the Promotion of Science (Research for the Future Program, Grant JSPS-RFTF96I00302 to Y.E.; Japan-UK Bilateral Research Program to K.I.). We thank Dr. R. J. Cox, University of Bristol, for a generous gift of SEK4/4b.

Supporting Information Available: CD spectra of DNPAs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org. See any current masthead page for ordering information and Web access instructions.

JA015981+

(25) (a) Draeger, G.; Park, S.-H.; Floss, H. G. *J. Am. Chem. Soc.* **1999**, *121*, 2611−2612. (b) Chen, H.; Agnihotri, G.; Guo, Z.; Que, N. L. S., Chen, X. H.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 8124−8125.

(26) Sequence analysis based on the BLAST2 SEQUENCES program suggested that the relevant ketoreductases encoded by *gra*-ORF26 in *S. violaceoruber* and *Tyl*C1 in *Streptomyces fradiae* showed no significant homology.

(27) Taguchi, H.; Ohta, T. *J. Biol. Chem*. **1991**, *266*, 12588−12594.

(28) Bisang, C.; Long, P. F.; Cortes, J.; Westcott, J.; Crosby, J.; Matharu, A.-L.; Cox, R. J.; Simpson, T. J.; Staunton, J.; Leadley, P. F. *Nature* **1999**, *401*, 502−505.

(29) Matharu, A.-L.; Cox, R. J.; Crosby, J.; Byrom, K. J.; Simpson, T. J. *Chem. Biol.* **1998**, *5*, 699−711.

(30) Anson, C. E.; Bibb, M. J.; Booker-Milburn, K. I.; Clissold, C.; Haley P. J.; Hopwood, D. A.; Ichinose, K.; Revill, W. P.; Stephenson, G. R.; Surti, C. M. *Angew. Chem., Int. Ed*. **2000**, *39*, 224−227.