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Biosynthesis of the Aminocyclitol Subunit of Hygromycin A in *Streptomyces hygroscopicus* NRRL 2388

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SUMMARY

The antibacterial activity of hygromycin A (HA) arises from protein synthesis inhibition and is dependent upon a methylenedioxy bridged-aminocyclitol moiety. Selective gene deletions and chemical complementation in *Streptomyces hygroscopicus* NRRL 2388 showed that the *hyg18* and *hyg25* gene products, proposed to generate a *myo*-inositol intermediate, are dispensable for HA biosynthesis but contribute to antibiotic yields. Hyg8 and Hyg17, proposed to introduce the amine functionality, are essential for HA biosynthesis. Hyg6 is a methyltransferase acting on the aminocyclitol, and a Δ*hyg6* mutant produces desmethylenehygromycin A. Deletion of *hyg7*, a metallo-dependant hydrolase homolog gene, resulted in methoxyhygromycin A production, demonstrating that the corresponding gene product is responsible for the proposed oxidative cyclization step of methylenedioxy bridge formation. The methyl/methylene group is not required for in vitro protein synthesis inhibition but is essential for activity against *Escherichia coli*.

INTRODUCTION

Aminocyclitols, which are characterized by the presence of a cyclohexane moiety with hydroxyl and amino or guanidino substituents, are found in a large class of natural products with broad-ranging biological properties. The aminocyclitol-aminoglycosides have long been known for their antibacterial activities and have found applications as antibiotics in clinical use (streptomycin, gentamicin, and kanamycin), veterinary medicine (spectinomycin), and agriculture (kasugamycin and validamycin A) (Flatt and Mahmud, 2007; Mahmud, 2009). The C_7N aminocyclitol-containing compound, acarbose, has α-glucosidase inhibitory activity, potentially useful in the treatment of type II insulin-independent diabetes mellitus (Mahmud, 2003). Structure-activity relationship studies have established the importance of the aminocyclitol moiety for the biological activity of many of these natural products. Genetic and biochemical studies have also revealed the complexity and diversity of the biosynthetic pathways that produce these critical aminocyclitol moieties (Flatt and Mahmud, 2007; Mahmud, 2009).

Hygromycin A (HA, compound **1**) (Figure 1) is a secondary metabolite produced by the soil bacterium *Streptomyces hygroscopicus* NRRL 2388 (Mann et al., 1953; Pittenger et al., 1953). HA is structurally distinguished by the presence of three distinct moieties, 5-dehydro-α-L-fucofuranose (subunit A), (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid (subunit B), and the aminocyclitol, 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol (subunit C). The mechanism of action of HA as a bacterial ribosomal peptidyl transferase inhibitor, and also its hemagglutination inactivation, antitreponemal, and immunosuppressant properties have been well-elucidated (Guerrero and Modolell, 1980; Nakagawa et al., 1987; Omura et al., 1987; Uyeda et al., 2001; Yoshida et al., 1986). Herbicidal properties have been identified for HA and methoxyhygromycin A (**2**) (Figure 1), a shunt product or pathway intermediate obtained in the course of HA biosynthesis (Kim et al., 1990; Lee et al., 2003). Structure-activity relationship studies have revealed that subunit C is indispensable for HA's antibacterial activity (Hayashi et al., 1997).

A plausible pathway for HA biosynthesis has been proposed, based on isotope-labeled precursor incorporation studies (Habib et al., 2003). Analyses of labeling patterns of the resulting HA have shown that (1) subunit A originates from glucose-6-phosphate via a mannose intermediate, (2) the central subunit B is derived from 4-hydroxybenzoic acid and propionic acid in a polyketide-like manner, and (3) *myo*-inositol and methionine are the precursors for subunit C. It has further been suggested that the glycoside bond (which links subunits A and B) and the amide bond (linking subunits B and C) are formed after each of the subunits is assembled. The results from the biosynthetic incorporation studies provided a strategy for identification, and subsequent cloning and sequencing of the HA biosynthetic gene cluster of *S. hygroscopicus* (GenBank DQ314862) (Palaniappan et al., 2006). Analysis of this 31.5 kb gene cluster led to the identification of 29 open reading frames (ORFs).

Several of the gene products were assigned putative roles in biosynthesis of the aminocyclitol subunit of HA. In the proposed pathway, glucose-6-phosphate is first converted to *myo*-inositol-1-phosphate (MIP) by a *myo*-inositol-1-phosphate synthase encoded by *hyg18*. MIP is then dephosphorylated to form *myo*-inositol by the *hyg25* gene product, a putative *myo*-inositol phosphatase. Analogous steps are also encountered in the biosynthesis of the aminocyclitol moiety of

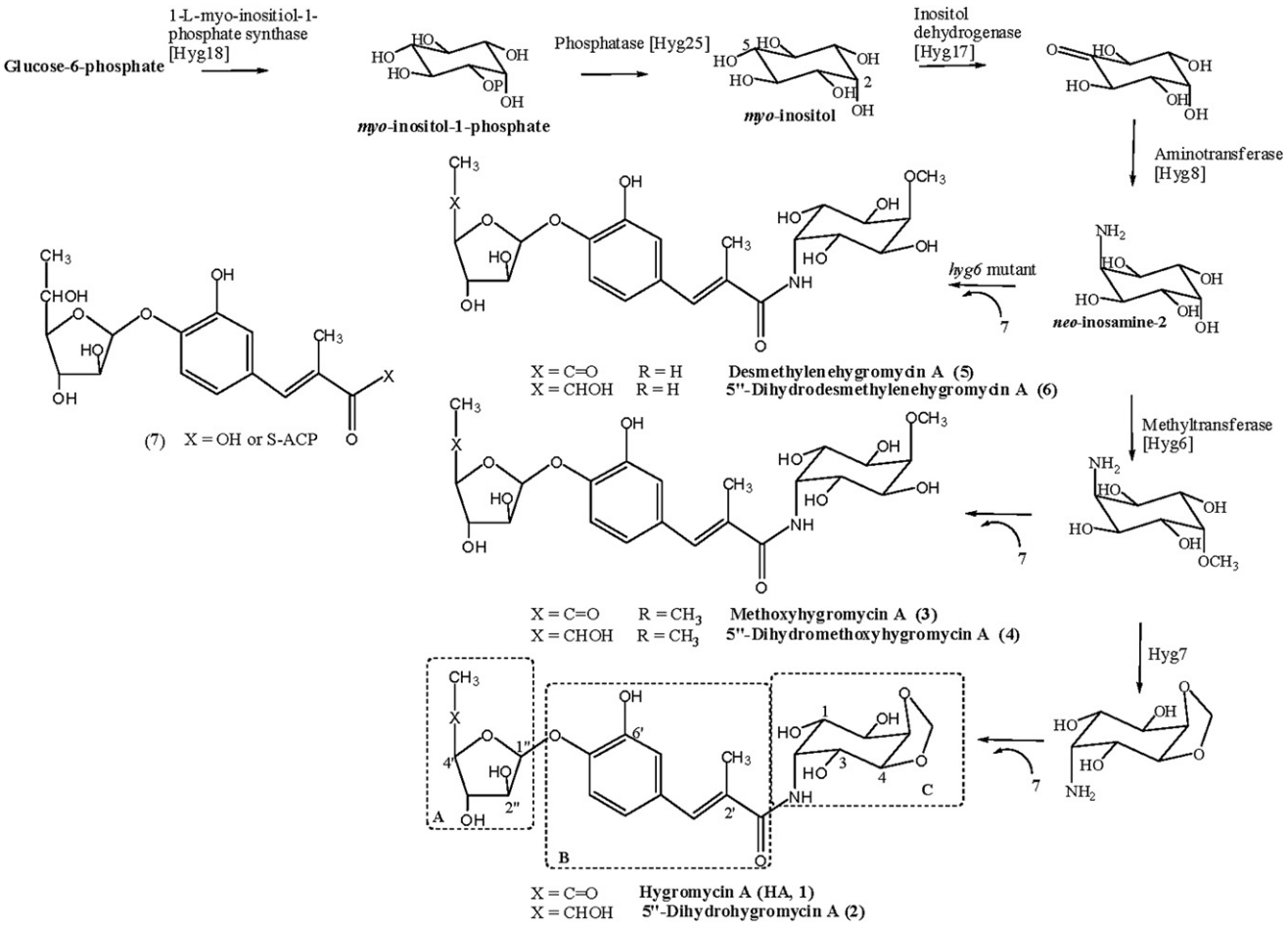

Figure 1. Relationship between Hygromycin A Analogs and the Biosynthetic Pathway Leading to the Aminocyclitol Moiety

The three structural moieties, 5-dehydro-α-L-fucofuranose (subunit A), (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid (subunit B), and 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol (subunit C) are indicated.

streptomycin, bluensomycin, spectinomycin, fortimicin, and the D-inositol of kasugamycin (Flatt and Mahmud, 2007). The subsequent oxidation and transamination reactions in the above compounds occur at the C2 hydroxyl of *myo*-inositol to form *scyllo*-inosamine, whereas in the case of HA biosynthesis the labeling studies are consistent with them occurring at the C5 hydroxyl, leading to the unique *neo*-inosamine-2 product. An inositol dehydrogenase (encoded by *hyg17*) and a putative aminotransferase (encoded by *hyg8*) were proposed to catalyze these steps, respectively. A distinct structural feature of HA is the methylenedioxy bridge between C4 and C5 of the C subunit, shown to be derived from *S*-adenosylmethionine (SAM). It has been proposed that the bridge formation involves methylation of the C5 hydroxyl group of *neo*-inosamine-2 (which is equivalent to C1 of *myo*-inositol) by a SAM-dependent methyltransferase, followed by cyclization. The *hyg6* and *hyg29* genes in the HA gene cluster are methyltransferase homologs, and it was not possible to determine from sequence analysis which of the two encodes the putative *O*-methyltransferase acting on *neo*-inosamine-2. No candidate gene for the subsequent cyclization step was identified and sequence analysis gave no insight into the timing of the various steps.

We report herein a verification of the proposed roles of these *hyg* genes in biosynthesis of the aminocyclitol subunit through a series of targeted gene disruption experiments and chemical complementation studies. A biologically active new desmethylene analog of HA has been purified and characterized from a Δ*hyg6* mutant, demonstrating that Hyg6 is the C5 *O*-methyltransferase that introduces the methyl group on the aminocyclitol and that introduction of the amine group can occur without this step. The analyses have also shown that the *hyg7* gene product is required for the cyclization step that generates the methylenedioxy bridge. The selective production of hygromycin analogs by the Δ*hyg6* and Δ*hyg7* mutants has provided an opportunity to probe the importance of a methyl or methylene group on the aminocyclitol ring for both in vitro protein synthesis inhibitory activity and antibacterial activity.

RESULTS

Hygromycin Production by Mutant Strains

The HA biosynthetic gene cluster harbors seven genes that are predicted to be functional in the biosynthesis of the 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol moiety (subunit C)

Table 1. Results of Feeding Antibiotic Precursors to Different *hyg* Mutant Strains

	Antibiotic Production			Production with *myo*-inositol Supplementation			Production with Subunit C Supplementation		
Strain	HA (mg/l)	3 (mg/l)	5 (mg/l)	HA (mg/l)	3 (mg/l)	5 (mg/l)	HA (mg/l)	3 (mg/l)	5 (mg/l)
Wild type	1190 ± 120			1090 ± 40					
Δ*hyg18* (*myo*-inositol-1-phosphate synthase)	588 ± 21			1090 ± 15					
Δ*hyg25* (*myo*-inositol-1-phosphatase)	961 ± 84			956 ± 74					
Δ*hyg17* (*myo*-inositol dehydrogenase)	147 ± 20			204 ± 41			630 ± 12		
Δ*hyg8* (Aminotransferase)	—	—					N/D		
Δ*hyg8*+Δ*hyg7*							3 ± 1		
Δ*hyg6* (Methyltransferase)	—	—	530 ± 60			435 ± 30	180 ± 90		690 ± 270
Δ*hyg29* (Methyltransferase)	485 ± 21								
Δ*hyg7*		287 ± 19			411 ± 19		671 ± 40	386 ± 33	

N/D, not detectable.

(Palaniappan et al., 2006). A polymerase chain reaction (PCR)-targeted gene replacement strategy for in vivo functional analyses of these genes was used (Gust et al., 2003) and the fermentation broths of the corresponding mutants were examined for the presence of HA or its analogs. The mutants were also chemically complemented with either subunit C (the putative final product of the aminocyclitol pathway) or the putative *myo*-inositol intermediate, and the effect on product ratios and yields were determined (Table 1).

The involvement of *myo*-inositol-1-phosphate synthase and *myo*-inositol phosphatase to generate *myo*-inositol from glucose-6-phosphate has been reported from a host of diverse sources such as higher plants and animals, parasites, fungi, green algae, bacteria, and archaea (Majumder et al., 2003). The *hyg18* gene product is proposed to be a *myo*-inositol-1-phosphate synthase and the Δ*hyg18* mutant produced HA, **3,** and their corresponding C5″-reduced analogs **2** and **4** (Figure 1) at approximately 50% of the levels observed for the wild-type strain (Table 1). A small amount (~23 mg/l) of (E)-3-(3-hydroxy-4-O-α-fucofuranosylphenyl)-2-methylacrylic acid (**7**) (Figure 1) was also observed. This shunt product is not observed in the wild-type but has been identified previously in the SCH30 (short-chain dehydrogenase, Δ*hyg26*) mutant (Palaniappan et al., 2006). Chemical complementation of the Δ*hyg18* mutant with *myo*-inositol led to almost complete restoration of HA and loss of production of the **7** shunt product. In contrast, no increase in HA yields was observed with addition of *myo*-inositol to the wild-type strain (suggesting that *myo*-inositol is limiting in the Δ*hyg18* mutant but not in the wild-type). The *hyg25* gene product is predicted to be a *myo*-inositol phosphatase, and a Δ*hyg25* mutant was observed to produce HA in amounts comparable to that in the wild-type, both in the absence and presence of *myo*-inositol.

Blocking the aminocyclitol pathway at steps subsequent to the proposed *myo*-inositol intermediate had more pronounced effects on antibiotic production. Disruption of *hyg17*, which presumably encodes a *myo*-inositol dehydrogenase for C5 oxidation of *myo*-inositol, resulted in an 8-fold decrease in HA production (Table 1). Consistent with predictions, chemical complementation of the Δ*hyg17* mutant with *myo*-inositol did not lead to restoration of HA production. Chemical complementation of this mutant with subunit C led to a 4-fold increase in HA production levels, to approximately 50% of that seen in the wild-type. This experiment provided clear evidence that Hyg17 operates at a step in the aminocyclitol pathway after formation of the *myo*-inositol and that the final subunit C can be incorporated intact into HA. The Δ*hyg17* strain was also grown in the presence of valienamine, the C_7N aminocyclitol moiety used in biosynthesis of the antifungal antibiotic validamycin A (Mahmud et al., 2007). Liquid chromatography mass spectrometry (LC-MS) analyses of the fermentation broth showed the presence of an ionic species with a $[M+H]^+$ of 496 amu, the expected mass resulting from valienamine incorporation in place of the normal biosynthetic aminocyclitol moiety. A limited supply of valienamine precluded efforts to purify the proposed new HA analog for either structural elucidation or bioactivity assays. Further mutasynthesis experiments in Δ*hyg17* using amino sugars such as glucosamine, galactosamine, and mannosamine did not result in production of any detectable new HA products.

The *hyg8* gene is proposed to encode a putative class III pyridoxal-phosphate-dependent aminotransferase, catalyzing transamination of C5-oxidized *myo*-inositol to produce *neo*-inosamine-2 (Palaniappan et al., 2006). A complete loss of HA production was observed in the Δ*hyg8* mutant and could not be restored by chemical complementation with subunit C. An additional mutation, where *hyg7* and *hyg8* were both deleted, also abolished HA production completely. In this case, however, very low levels of HA production were observed with subunit C addition. As described below, a Δ*hyg7* mutant gives dramatically different results, and the loss of HA production and poor chemical complementation with subunit C appear to be linked to loss of *hyg8*.

The *hyg6* and *hyg29* genes show homology to methyltransferases and analysis of their predicted amino acid sequences did not indicate which of them was likely required for the proposed SAM-dependant methylation of *neo*-inosamine-2. A Δ*hyg29* mutant was generated, and high-performance liquid

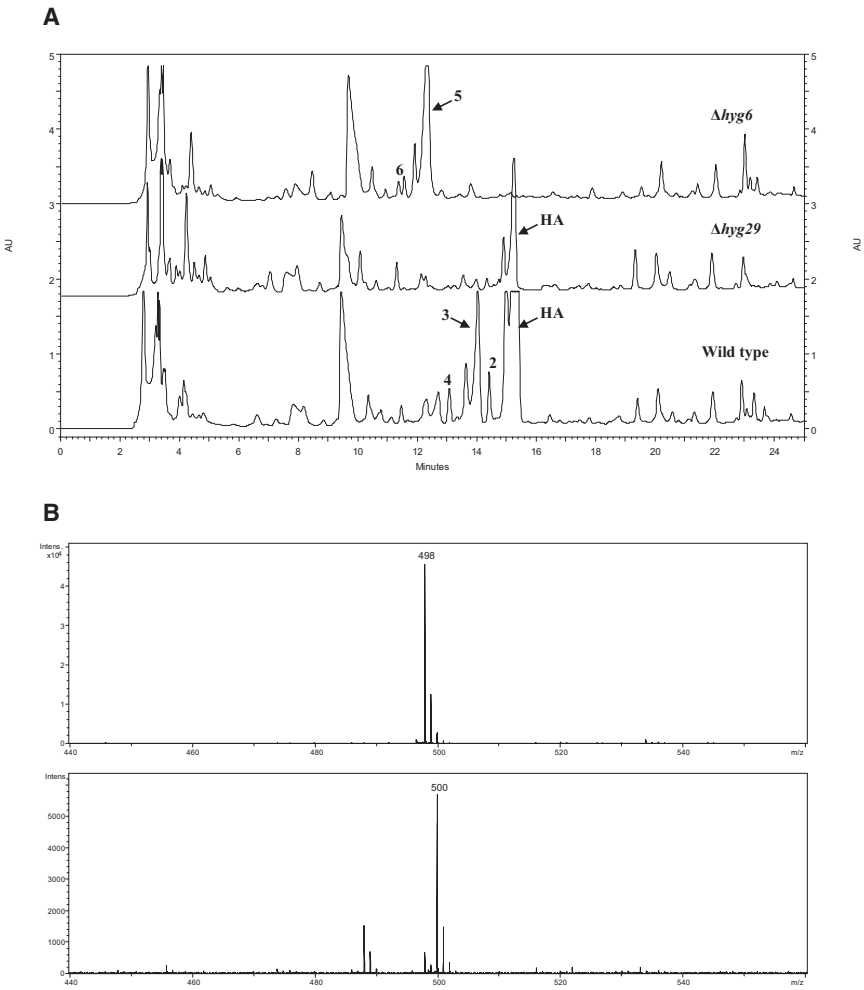

Figure 2. Effect of Methyltransferase gene (*hyg6*, *hyg29*) Disruptions on Hygromycin A Production

(A) Reverse-phase HPLC analysis of fermentation broths of wild-type, Δ*hyg29*, and Δ*hyg6* strains. Disruption of *hyg29* caused a decrease in hygromycin A production. Disruption of *hyg6* resulted in the generation of new metabolites with shorter retention times than hygromycin A.

(B) Mass spectrometric analysis (negative mode) of Δ*hyg6* fermentation broth. Two new species were identified with $[M\text{-}H]^-$ values of 498 and 500 corresponding to desmethylenehygromycin A (**5**) and 5″-dihydrodesmethylenehygromycin A (**6**), respectively.

chromatography (HPLC) and MS analyses of the fermentation broth of this strain revealed the same products (HA and **3**) at approximately 60% of those seen in the wild-type. In contrast, the production of HA was completely abolished in a Δ*hyg6* strain, and HPLC analysis of the fermentation broth of this mutant revealed a dominant new peak and an additional minor peak with shorter retention times than HA under the standard reverse-phase HPLC conditions (Figure 2). The major peak showed a mass in negative mode of m/z = 498 $[M - H]^-$ and the minor peak showed a mass of m/z = 500 $[M - H]^-$. An m/z of 499 is consistent with the mass of HA that is missing the methylene group bridging *O*-4 and *O*-5. An m/z of 501 observed for the smaller peak corresponds to a reduced derivative of the major peak. The MS analyses were thus consistent with production of desmethylene HA analogs (**5, 6**) (Figure 1) by the Δ*hyg6* strain. The production titers of **5** were ~400 mg/l. When subunit C was provided to this strain, low levels of HA could be observed although the levels of **5** were not significantly altered.

A unique feature of the aminocyclitol moiety of HA is the C4-C5 methylenedioxy bridge, which is essential for optimal biological activity (Hecker et al., 1992). Sequence analyses of the 29 ORFs of the HA gene cluster did not reveal a putative candidate gene product for the cyclization step of bridge formation. We had hypothesized that the *hyg7* gene product, which is homologous to D-aminoacylases/amidohydrolases, mediates formation of the amide bond between subunits B and C, and that a Δ*hyg7* mutant would accumulate **7.** However, as shown in Figure 3A, the Δ*hyg7* strain produced **3** and trace levels of **4**. Chemical complementation with subunit C, but not *myo*-inositol, led to a restoration of HA production (55% of that seen in the wild-type strain) (Figure 3A). A series of chemical complementation experiments of this strain with subunit C in the presence of either radiolabeled [2-^{3}H] *myo*-inositol or [carboxyl-^{14}C] 4-hydroxybenzoic acid was also carried out (Figure 3B). These analyses demonstrated that while HA production was restored by supplementation with subunit C, radiolabeled *myo*-inositol incorporation was only observed into **3**. A similar study with radiolabeled 4-hydroxybenzoic acid resulted, predictably, in its incorporation into both HA and **3**. Taken together, these observations clearly indicate that cyclization of the C5 methoxy group, resulting in formation of the methylenedioxy bridge, is dependant upon the *hyg7* gene product.

Structural Elucidation of Desmethylene HA Analogs

The two new compounds isolated from the Δ*hyg6* strain were purified by semipreparative HPLC and their structures were elucidated by nuclear magnetic resonance (NMR). The most obvious change observed in the ^{1}H-NMR spectra of **5** and **6** was the absence of either the three-proton singlet at ~3.5 ppm (for the *O*-5 methyl group of **3**), or two one-proton singlets at ~5.18 ppm and 4.83 ppm (for the methylene group bridging O-4 and O-5 of HA).

The differences in the ^{1}H-NMR spectra between **5** and **6** involve the fucofuranose moiety. The H-6″ signal for **5** is a singlet at 1.99 ppm (overlapping with protons from α–CH_3), whereas the H-6″ signal for **6** is a doublet at 1.20 ppm. The H-4″ signal is also shifted from ~4.4 ppm in **5** to 3.74 ppm in **6** (shifts are approximate for H-4″ in **5** because this signal is obscured by other signals at the same chemical shift). There is also a new signal

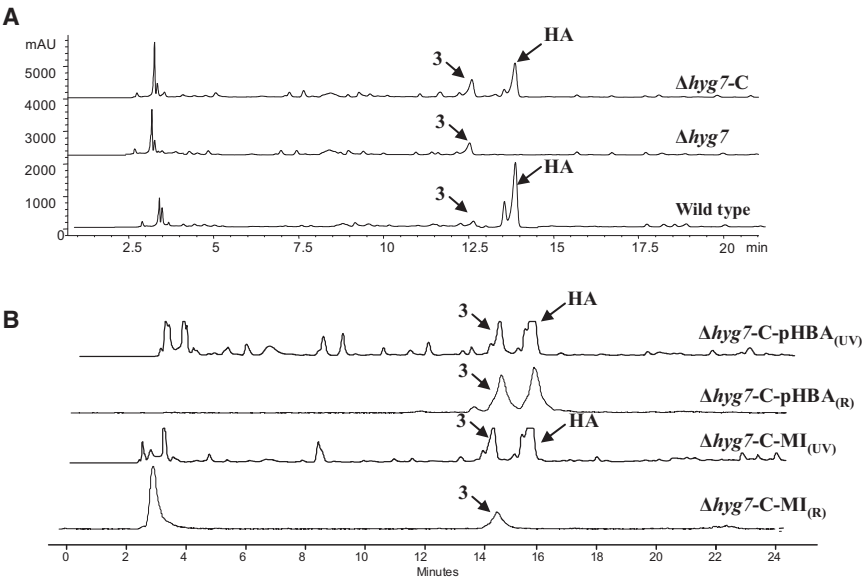

Figure 3. Effect of *hyg7* Gene Disruption on Hygromycin A Production

(A) Reverse-phase HPLC analysis of Δ*hyg7* fermentation broth. The Δ*hyg7* strain produced only compound **3**. Restoration of HA production was observed upon subunit C supplementation as seen in the HPLC chromatogram "Δ*hyg7*-C."

(B) HPLC analysis of Δ*hyg7* fermentation supplemented with C subunit in the presence of [2-^{3}H] *myo*-inositol or [carboxyl-^{14}C] 4-hydroxybenzoic acid. Δ*hyg7*-C-MI$_{(UV)}$ and Δ*hyg7*-C-pHBA$_{(UV)}$ are UV traces of fermentation broth of Δ*hyg7* supplemented with subunit C in the presence of [2-^{3}H] *myo*-inositol and [carboxyl-^{14}C] 4-hydroxybenzoic acid, respectively. Δ*hyg7*-C-MI$_{(R)}$ and Δ*hyg7*-C-pHBA$_{(R)}$ represent the radiolabeled traces recorded by Beta-Ram (R) radioactivity quantization system for their respective UV traces. This analysis clearly indicated the role of *hyg7* in the cyclization step of methylenedioxy bridge formation.

for H-5″ of **6** that appears at approximately 3.79 ppm, which is obscured by other signals.

Activity of Desmethylenehygromycin A

The antibacterial activity of **5** was determined using Δ*tolC E. coli* as test organism and compared with that of HA, **3**, and **7** to determine the importance of the methyl/methylene group on the cyclitol. The MIC$_{90}$ of HA for Δ*tolC E. coli* was determined to be 10 μg/ml (Table 2). Compounds **3** and **5** had much less activity, with the MIC$_{90}$ for both being 150 μg/ml. Compound **7** lacked inhibitory activity even at a concentration of 250 μg/ml. The compounds were also assessed for their ability to inhibit the synthesis of green fluorescent protein (GFP) using an *E. coli* in vitro coupled transcription-translation system (Dinos et al., 2004; Szaflarski et al., 2008). HA was shown to be highly active in vitro, having an IC$_{50}$ and IC$_{90}$ of 0.18 μM and 0.25 μM, respectively, whereas compound **7** was inactive exhibiting no effect on translation at 75 μM. In contrast to their poor MIC values, both **3** and **5** displayed significant activity as in vitro transcription-translation inhibitors, although their IC$_{50/90}$ values were higher than those of HA (Table 2, Figure 4).

Resistance of Δ*hyg29* Mutant Strain to HA

In order to verify whether the *hyg29* gene contributed to self-resistance of *S. hygroscopicus*, spores of the wild-type and Δ*hyg29* strains were grown on agar plates with different HA concentrations. The MIC$_{95}$ of HA for the wild-type was found to be 400 μg/ml. The mutant also showed high level of self-resistance, with an MIC$_{95}$ value of 300 μg/ml.

Table 2. Comparison of MIC$_{90}$ and IC$_{50/90}$ Data for Hygromycin A and Analogs

	HA	3	5	7
MIC$_{90}$ (μg/ml)	10	150	150	>250
IC$_{50}$ (μM)	0.18	0.5	0.32	>75
IC$_{90}$ (μM)	0.25	2	1	>75

DISCUSSION

Myo-inositol represents a common intermediate in the majority of pathways that generate aminocyclitol components of aminoglycoside antibiotics (Flatt and Mahmud, 2007; Mahmud, 2009). Branch points from this intermediate then give rise to the various products. In the proposed biosynthetic pathway (Figure 1B) that generates the aminocyclitol moiety of hygromycin A, this branching step is oxidation of the C5 hydroxyl of myo-inositol to form *neo*-inosose (Habib et al., 2003). The steps that precede *myo*-inositol are well known, reasonably widespread in organisms, and involve the sequential action of a MIP synthase (catalyzing formation of *myo*-inositol- phosphate from glucose 6-phosphate) and a MIP phosphatase. In actinomycetes these are essential activities, required for the biosynthesis of the essential metabolite mycothiol (the major cellular thiol and redox co-catalyst). For this reason, actinobacterial genomes mostly harbor more than one gene, and sometimes have several genes with either L-*myo*-inositol-1-phosphate synthase or inositol monophosphatase signatures (Wehmeier and Piepersberg, 2009). Thus for many aminocyclitol pathways, it appears that genes encoding these two enzymes are not necessarily located within the corresponding biosynthetic gene cluster. In the streptomycin producer *S. griseus*, a MIP synthase has been purified and it has been shown that the corresponding gene is not present in the streptomycin biosynthetic gene cluster (Flatt and Mahmud, 2007; Pittner et al., 1979; Sipos and Szabo, 1989). The MIP synthase is also lacking in the biosynthetic gene clusters of bluensomycin and spectinomycin (Flatt and Mahmud, 2007), although this enzymatic activity is presumably required for formation of these natural products. The partial loss of HA production with deletion of *hyg18* and restoration with *myo*-inositol are consistent with (1) Hyg18 encoding a putative MIP synthase required for efficient production of MIP and (2) the presence of an additional MIP synthase that can catalyze this reaction in *Streptomyces hygroscopicus*. A MIP phosphatase gene is present in the streptomycin (*strO*) and spectinomycin

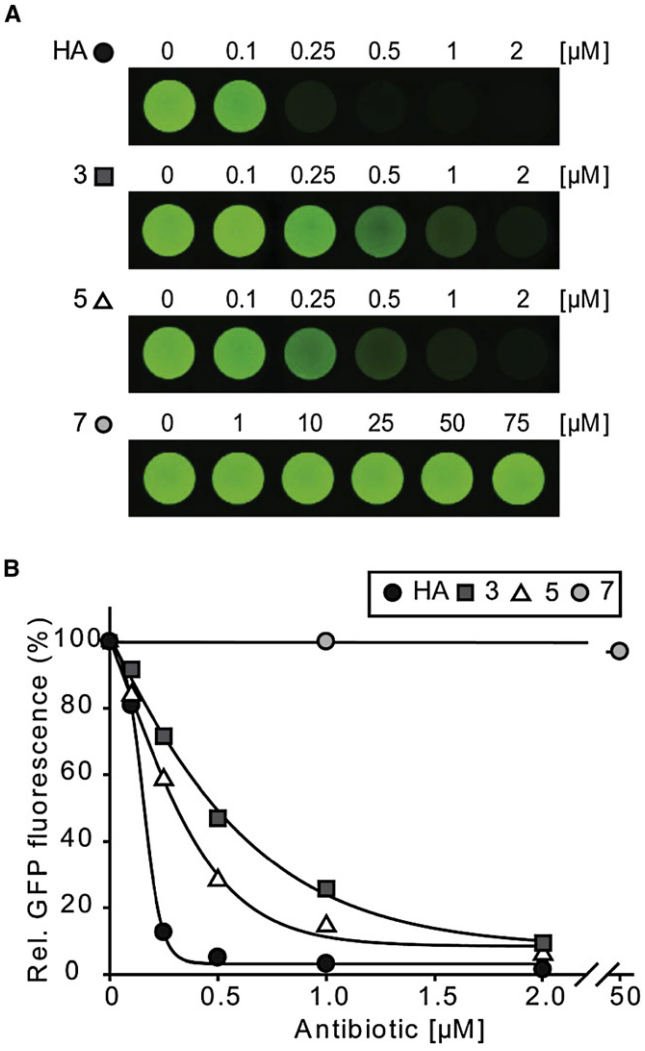

Figure 4. Effect of Hygromycin A and Derivatives on In Vitro Transcription-Translation

(A) Detection of template-dependent synthesis of GFP using fluorescence in the absence or presence of increasing concentrations (μM) of the antibiotic hygromycin A (HA), methoxyhygromycin A (**3**), desmethylenehygromycin A (**5**), or 5″-dihydroAB subunit (**7**) (see Figure 1 for structures).

(B) Quantitation of (A). GFP fluorescence is given as a percentage where 100% is defined as the fluorescence detected in the absence of the antibiotic.

(*speA*) gene clusters. The function of SpeA has also been biochemically confirmed (Ahlert et al., 1997; You-Young et al., 2003), although it has not been determined if these genes are essential for biosynthesis of the antibiotics. The *hyg25* gene product has conserved domains of the haloacid dehalogenase like hydrolase superfamily, which includes phosphatases (although there is no significant sequence similarity between Hyg25, and either known MIP phosphatases, StrO or SpeA). The wild-type production levels of HA by the *hyg25* mutant do not provide evidence that Hyg25 is a MIP phosphatase. However, because this is the only putative phosphatase in the HA biosynthetic gene cluster, the evidence indicates that an enzyme not encoded by the *hyg* gene cluster can catalyze dephosphorylation of MIP.

Steps subsequent to *myo*-inositol intermediate involve introduction of the amine functionality (at the C-5 hydroxyl) and introduction of the methyl group (at the C2 hydroxyl group). Although the exact order of these steps in the normal biosynthetic process is unclear, the production of desmethylenehygromycin A by Δ*hyg6* demonstrates that the amine functionality can be introduced without the methyl group. In such a pathway, the C-5 hydroxyl group would be oxidized to form *neo*-inosose, with a subsequent transamination to form *neo*-inosamine-2 (Figure 1B). The data with Δ*hyg17* strain (an almost a 90% reduction in HA yields relative to wild-type strain, and a 4-fold increase in these yields with supplementation with subunit C, but not *myo*-inositol) support the proposed role of this gene product as an *myo*-inositol dehydrogenase. The continued production of low levels of HA in this strain suggests that another enzyme or enzymes present in *S. hygroscopicus* are able to catalyze C5 oxidation of *myo*-inositol. The data with the Δ*hyg8* mutant (complete loss of HA production and modest HA production on supplementation with the subunit) support the proposed role of the gene product as the pyridoxal-phosphate-dependent aminotransferase that introduces the amine functionality. The poor yields of HA in the chemical complementation experiments with subunit C are puzzling, given how effective this moiety is at restoring HA production in other mutants. The HA shunt product **7,** which would be expected to form in the absence of adequate levels of the aminocyclitol moiety, was not observed in Δ*hyg8.* A polar effect from replacement of *hyg8* with the apramycin resistance marker may account for these observations (the current lack of a genetic complementation system did not permit this possibility to be tested). Another possible explanation is that the intermediate *neo*-inosamine-2 may have a key regulatory role. In principle, only Δ*hyg8* is unable to generate this intermediate.

The production of desmethylene analogs **5** and **6** by the Δ*hyg6* strain provide conclusive evidence that the C4-C5 methylene group in HA (and the methyl group in **3)** is carried out by an *O*-methylation activity of Hyg6 and not by Hyg29, the second methyltransferase homolog in the HA gene cluster (Figure 1). The high titers of **5** and **6** also demonstrate that introduction of the amine functionality in *myo*-inositol is not dependant upon methylation. The MIC analyses showed that the antibacterial activity of **5** against Δ*tolC E. coli* was 15 times less than that of HA. Interestingly, the MIC value of **5** was the same as that of **3**, which has a C5-OCH_3 group instead of the methylenedioxy bridge. The lower antibacterial activity of **3** has been reported previously (Chida et al., 1990; Yoshida et al., 1986). HA analogs in which the aminocyclitol is replaced by aminocyclohexadiols and aminocyclohexatriols have also shown markedly reduced biological activity (Hecker et al., 1992). The MIC data on the new shunt product **5** finalize these analyses, demonstrating that for maximal antibiotic activity subunit C must be an aminocyclitol and that this must be modified through formation of the methylenedioxy bridge. An unexpected observation is that removal of the methylenedioxy ring does not lead to a loss in the in vitro protein synthesis inhibition activity. Compounds **3** and **5**, where the methylenedioxy ring is disrupted, both retained potent protein synthesis inhibitory activity despite showing poor MIC values (Figure 4, Table 2). In contrast compound **7**, which lacks the aminocyclitol ring, is totally inactive. In fact, the IC_{50}

of these compounds (0.3–0.5 μM) is comparable to that of the macrolide antibiotic tylosin (~0.4 μM) in the same in vitro transcription-translation assay (A.L.S. and D.N.W., unpublished data). These data suggest that the aminocyclitol ring is indeed essential for translation inhibition activity, whereas an intact methylenedioxy ring is not. It appears that the loss in biological activity due to methylenedioxy ring disruption stems from another factor, potentially a reduced uptake of the drug into the cell rather than abolishing the ribosome binding ability of the compound.

Inactivation of the second methyltransferase homolog, *hyg29*, resulted in a decrease in the yield of HA, although the antibiotic production profile and self-resistance were not affected. Because HA binds to the ribosomes, rRNA methylation by Hyg29 may provide a self-resistance mechanism in *S. hygroscopicus*. There is as yet no evidence to support such a role for *hyg29* and, moreover, ribosomal self-resistance is not always observed for ribosome-targeting antibiotics (e.g., oleandomycin) (Cundliffe, 1989). If the *hyg29* gene product is indeed an rRNA methylase, the high level of HA resistance of the Δ*hyg29* mutant suggests that in the absence of target-site modification by *hyg29*, self-resistance is possibly being conferred by the other resistance determinants in the HA gene cluster, namely a HA inactivating *O*-phosphotransferase (Dhote et al., 2008), and putative efflux pumps encoded by *hyg19* and *hyg28*.

The final step in the proposed pathway for formation of the C-subunit of HA is an oxidative cyclization to provide the methylenedioxy bridge. No gene product likely responsible for catalyzing this step was identified in the initial analyses of the *hyg* biosynthetic gene cluster (Palaniappan et al., 2006). The role of the *hyg7* gene product was unknown. A more detailed analysis has revealed clear homology of Hyg7 with aminoacylases, which catalyze hydrolytic deacetylation of *N*-acetyl-D-amino acids. Furthermore, Hyg7 is a member of the metallo-dependant hydrolase superfamily. Enzymes in this class have hydrolytic activities and typically have two, or less commonly one, metal ion-binding sites (Lai et al., 2004). Our analysis of Hyg7 revealed the four highly conserved residues (a Cys, Asp, and two His) that comprise a single metal-binding site and suggest a role in a hydrolytic process. Nonetheless, the selective production of methoxyhygromycin A (**3**) by Δ*hyg7* and the restoration of HA upon supplementation with subunit C clearly demonstrate that Hyg7 is required for the oxidative cyclization process which forms the methylenedioxy bridge of HA. Natural products with a methylenedioxy bridge have been identified more commonly in plants, and involve an initial methylation with subsequent action by a cytochrome p450 enzyme complex (Ikezawa et al., 2003). There are a limited number of examples of secondary metabolites from actinomycetes possessing methylenedioxy bridge. A 1,3-dioxine ring is found in dioxapyrrolomycin from *Streptomyces fumanus*, simaomicin from *Actinomadura madurae*, and the streptovaricins from *Streptomyces spectabilis* (Charan et al., 2006; Carter et al., 1989; Staley and Rinehart, 1991). FR-900109 from *S. prunicolor*, the dioxolides from *S. tendae*, and pseudoverticin from *S. pseudoverticillus* possess a 1,3-dioxolane ring similar to that seen in HA (Blum et al., 1996; Cui et al., 2007; Koda et al., 1983). Deuterium labeling studies in dioxapyrrolomycin by Charan et al. indicated an oxidative cyclization mechanism for methylenedioxy bridge formation similar to that reported for the plant metabolite berberine (Bjorklund et al., 1995; Charan et al., 2006). The *pyr20* gene product in the biosynthetic gene cluster of pyrrolomycin has resemblance to cytochrome P450 enzymes (and not to Hyg7) and has been proposed to potentially carry out the above function (Zhang and Parry, 2007). However, to the best of our knowledge there have been no genetic or biochemical characterization of the processes that lead to methylenedioxy bridge formation in bacterial natural product biosynthesis. Our results with *hyg7* represent the first example of in vivo characterization of a functional methylenedioxy bridge-forming gene of bacterial origin. Furthermore, the data indicate that a member of the metallo-dependent hydrolase superfamily, rather than a cytochrome p450 enzyme, is involved. The role of Hyg7 in this process, and the need for other enzymes or cofactors, remains to be biochemically determined.

The ability of subunit C to restore HA biosynthesis in most of the mutants described herein, demonstrates that it is an effective substrate for the enzyme that catalyzes amide bond formation. It is thus possible that the normal HA biosynthetic process involves formation of a complete C subunit prior to formation of the amide bond. Formation of **3** and **5**, and the apparent incorporation of valienamine, suggest relaxed substrate specificity of the corresponding coupling enzyme (Figure 1B). However, the data do not preclude the possibility that the final steps of C-subunit biosynthesis (methylation and formation of the methylenedioxy bridge) occur after amide bond formation.

SIGNIFICANCE

The roles of key *hyg* gene products in formation of the structurally unusual, methylene-bridged aminocyclitol of HA have been elucidated. Deletion of key genes in the pathway leads in some cases to accumulation of intermediates in the aminocyclitol biosynthetic process. As such the genetic tools to access novel HAs in *Streptomyces hygroscopicus* and to generate various aminocyclitols for combinatorial biosynthetic processes (generating new structurally diverse compounds) have been identified. The methylenedioxy bridge in HA has been shown not to be required for in vivo versus translation inhibition activity, but is required for biological uptake. These discoveries may help in the continued evaluation of HA-based structures for development of novel antibacterials. The discovery of the need for Hyg7 in the oxidative cyclization that yields the methylene-bridged aminocyclitol suggests a new enzymatic paradigm for formation of these unusual structural moieties.

EXPERIMENTAL PROCEDURES

Chemicals, Bacterial Strains, Growth Conditions, and General Procedures

Hygromycin A and 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol (subunit C) were kindly supplied by Pfizer Inc. [2-^{3}H] *myo*-inositol (20.0 Ci/mmol) was purchased from Moravek Biochemicals. [carboxyl-^{14}C]-4-hydroxybenzoic acid was obtained from American Radiolabeled Chemicals Inc. All other antibiotics and chemicals were purchased from Sigma Aldrich. The *E. coli* Δ*tol*C strain, deficient in the outer membrane protein TolC, was procured from the *E. coli* Genetic Stock Center at Yale University. All *E. coli* strains were grown following standard protocols (Sambrook and Russell, 2001). *S. hygroscopicus* wild-type and mutant strains were maintained using media

Table 3. PCR Primers Used for Gene Disruptions

Gene	Putative Function	Primer Name	Primer Sequence (5′-3′)
hyg6	Methyltransferase	*hyg6*_Forw	CGCCCCTCGACCGCGAAGACCTTCTGGGGGCAGCGGATG*ATTCCGGGGATCCGTCGACC*
		*hyg6*_Rev	ATGGTGGTCCGGCCTCCTCGTGTCGTCTGCGTGCCACTG*TGTAGGCTGGAGCTGCTTC*
hyg7	D-aminoacylase	*hyg7*_Forw	GACGACACGAGGAGGCCGGACCACCATGCATGACCTGAT*ATTCCGGGGATCCGTCGACC*
		*hyg7*_Rev	TCGCGCGCCCCGCCGGTGCGGCGGGCCGCCTCGCGGTCA*TGTAGGCTGGAGCTGCTTC*
hyg8	Aminotransferase	*hyg8*_Forw	CTGTCCGAGAAGGACTACGTCATCGAGCGGGACCGGCTG*ATTCCGGGGATCCGTCGACC*
		*hyg8*_Rev	GGTTTCCGCGAAGAACGCGTCCTGCATCGCCCGTGACCG*TGTAGGCTGGAGCTGCTTC*
hyg17	*myo*-inositol dehydrogenase	*hyg17*_Forw	TTCCGCCGTCGTGCGGGCCGCGGGGGTGAGCCGGTGAGC*ATTCCGGGGATCCGTCGACC*
		*hyg17*_Rev	GTCATCCCCTCGCGCCGCCGGCCAGGACGCCACCGGTCA*TGTAGGCTGGAGCTGCTTC*
hyg18	*myo*-inositol-1-phosphate synthase	*hyg18*_Forw	GGCCTGCGGCCCGGAGATTTCGCGAAGGGAAGAACCATG*ATTCCGGGGATCCGTCGACC*
		*hyg18*_Rev	CACGGGTCGGATCTCGGTTCGGAAACGCCCGGCGGCTCA*TGTAGGCTGGAGCTGCTTC*
hyg25	*myo*-inositol-1-phosphatase	*hyg25*_Forw	CACGCATGTCCGATACGACCGAATCGGGGTTGAGTGATG*ATTCCGGGGATCCGTCGACC*
		*hyg25*_Rev	GCGTCAGCGAATTCTGAGCCGCGCAACCGTCCGCGGTCA*TGTAGGCTGGAGCTGCTTC*
hyg29	Methyltransferase	*hyg29*_Forw	GCCGCCCGACCCGAGGCGCCCGGAGACGCCGCCGCGATG*ATTCCGGGGATCCGTCGACC*
		*hyg29*_Rev	GCCGTCCGGCCTCTGTCCACCAGCTGTGCGCCGCCTCCA*TGTAGGCTGGAGCTGCTTC*

The 39-nucleotide homologous region flanking the targeted gene is indicated in normal font. The italicized primer region is homologous to pIJ773.

and culture conditions as described earlier (Habib et al., 2003; Palaniappan et al., 2006). For qualitative analysis of the fermentation broths, the mycelium was removed by centrifugation and the supernatant was examined by HPLC and LC-MS as described previously (Palaniappan et al., 2006).

Targeted Disruption of the Aminocyclitol Biosynthetic Genes

The individual *hyg* genes were individually replaced with apramycin resistance cassette using the PCR-targeted *Streptomyces* gene replacement method (Gust et al., 2003; Palaniappan et al., 2006). The primer sequences used to amplify the resistance cassette from pIJ773 plasmid are listed in Table 3. All of the genes, except *hyg29*, were first disrupted in cosmid 17E3. For *hyg29* disruption, cosmid 15A10 was used. The recombinant cosmids were subsequently transferred to *S. hygroscopicus* wild-type by conjugation and the exoconjugants resulting from homologous recombination were selected based on resistance to apramycin. The genotype of the mutant strains was confirmed by PCR amplification using an appropriate set of outer primers (Table 3), and by sequencing the PCR product.

In the studies on *hyg8*, the apramycin resistance gene was removed from the *hyg8* mutant cosmid 17E3 using FLP recombinase. This cosmid with the 81 bp scar in place of *hyg8* sequence was then used to for replacement of *hyg7* with the apramycin resistance gene. The resulting 17E3 cosmid derivative was introduced into the wild-type *S. hygroscopicus* to provide the desired *hyg7+hyg8* deletion mutant.

Chemical Complementation Studies

The wild-type and appropriate mutant strains were grown in 5 ml production medium and treated with an amino sugar (the HA C subunit, glucosamine, galactosamine, or valienamine,) after 24 hr of fermentation to a final concentration of 5 mM. [2-^{3}H] *myo*-inositol and [carboxyl-^{14}C] 4-hydroxybenzoic acid were added to a final concentration of 0.4 μCi/ml. The supernatant was analyzed by HPLC and LC-MS after 6 days of fermentation (Palaniappan et al., 2006). Fermentation broths from the radiolabeled precursor feeding studies were analyzed by Beckman HPLC system linked to Beta-Ram (R) radioactivity quantization system (IN/US Systems, Inc.).

Quantitative Analysis of Antibiotic Production

Quantitative analyses of production of HA and its analogs were determined from triplicate cultures of the wild-type and mutant strains. Pure HA was used to generate a standard curve of peak area versus amount in μg within a 5 to 50 μg range. Then 50 μl filtered fermentation broth from each culture was used for HPLC analyses. The amounts of HA and related products in each injection sample were determined from their individual peak areas using the standard curve as reference and used to determine total production in the fermentations.

Purification and Characterization of Desmethylenehygromycin A (5) and 5″-Dihydrodesmethylenehygromycin A (6)

The desmethylene HA analogs (**5**, **6**) (Figure 1) produced by the Δ*hyg6* strain were extracted from culture filtrate and purified as described earlier (Palaniappan et al., 2006; Habib et al., 2003). The two products were characterized by MS and NMR techniques. ^{1}H-NMR spectra were recorded on a Bruker AMX-400 NMR. Two-dimensional correlated spectography correlation spectra were recorded on a Bruker AMX-600 NMR spectrometer. Coupling constants (*J*) were expressed in Hertz. Abbreviations for multiplicities are: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

Hygromycin A (1)

^{1}H-NMR (400 MHz, D_2O) δ 7.10 (1H, d, *J* = 8.0, H-9′), 7.01 (1H, s, H-3′), 6.88 – 6.91 (2H, m, H-5′, H-8′), 5.69 (1H, d, *J* = 4.4, H-1″), 5.18, 4.83 (2H, -OCH_2O-), 4.50 – 4.48 (1H, m, H-2), 4.40 – 4.18 (5H, m, H-2″, H-4″, H-4, H-5, H-6), 4.08 (1H, t, *J* = 2.8, H-3″), 3.95 (1H, dd, *J* = 5.2, H-1), 3.82 (1H, dd, *J* = 3.6, H-3), 2.16 (3H, s, H-6″), 2.02 (3H, s, α-CH_3).

Desmethylenehygromycin A (5)

^{1}H-NMR (400 MHz, D_2O) δ 7.14 (1H, d, *J* = 8.4, H-9′), 6.99 (1H, s, H-3′), 6.94 (1H, s, H-5′), 6.93 (1H, d, *J* = 8.4, H-8′), 5.72 (1H, d, *J* = 4.4, H-1″), 4.74 – 4.62 (1H, m, H-2), 4.41 – 4.40 (2H, m, H-3″, H-4″), 4.30 – 4.22 (1H, m, H-2″), 4.02 (1H, t, *J* = 2.8, H-5), 3.95 – 3.92 (2H, m, H-1, H-3), 3.72 (2H, dd, *J* = 10.0, 2.8, H-4, H-6), 1.99 (6H, s, α-CH_3, H-6″).

5″-Dihydrodesmethylenehygromycin A (6)

^{1}H-NMR (D_2O, 400 MHz) δ 7.17 (1H,d, *J* = 8.2, H-9′), 7.08 (1H, s, H-3′), 7.01 (1H, s, H-5′), 6.99 (1H, d, *J* = 8.2, H-8′), 5.69 (1H, d, *J* = 4.4, H-1″), ~4.7 (1H, partially obscured by solvent peak, H-2), 4.33-4.29 (2H, m, H-2″, H-3″), 4.11 (1H, t, *J* = 2.8, H-5), 4.03 (2H, dd, *J* = 10.0, 4.2, H-1, H-3), 3.87-3.80 (3H, m, H-4, H-6, H-5″), 3.74 (1H, t, *J* = 6.0, H-4″) 2.08 (3H, s, α-CH_3), 1.13 (3H, d, *J* = 6.8, H-6″).

MIC_{90} of HA and Analogs for Δ*tolC E. coli*

Five microliters of an overnight culture of Δ*tolC E. coli* were added to 195 μl final volume of fresh LB supplemented with HA or its analogs (Figure 1). The tubes were grown with shaking for 2 hr at 37°C and the absorbance at 600 nm was measured. The MIC_{90} was defined as the lowest concentration of the antibiotic at which 90% of growth was inhibited compared to a control *E. coli* culture grown in the absence of any antibiotic.

Coupled Transcription-Translation Assay

All coupled transcription-translation experiments were performed using an *E. coli* lysate-based system in the presence and absence of antibiotics as described previously (Dinos et al., 2004; Szaflarski et al., 2008). Reactions were transferred into 96-well microtiter plates and the GFP fluorescence was measured with a Typhoon Scanner 9400 (Amersham Bioscience) using

a Typhoon blue laser module (Amersham Bioscience). Images were then quantified using ImageQuantTL (GE Healthcare) and represented graphically using SigmaPlot (Systat Software, Inc.).

Hygromycin Sensitivity of Δ*hyg29* strain

The MIC_{95} of HA for the Δ*hyg29* mutant and the wild-type strain were determined by the agar plate dilution method (Dhote et al., 2008). Briefly, spores were plated on ISP2 agar plates containing varying amounts of HA, incubated at 30°C for 48 hr, and scored for growth. The MIC_{95} was defined as the lowest concentration of HA that prevented visible growth of 95% or more of the colony forming units on the agar plate.

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