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Production of Hygromycin A Analogs in *Streptomyces hygroscopicus* NRRL 2388 through Identification and Manipulation of the Biosynthetic Gene Cluster

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Summary

Hygromycin A, an antibiotic produced by *Streptomyces hygroscopicus* NRRL 2388, offers a distinct carbon skeleton structure for development of antibacterial agents targeting the bacterial ribosomal peptidyl transferase. A 31.5 kb genomic DNA region covering the hygromycin A biosynthetic gene cluster has been identified, cloned, and sequenced. The hygromycin gene cluster has 29 ORFs which can be assigned to hygromycin A resistance as well as regulation and biosynthesis of the three key moieties of hygromycin A (5-dehydro-α-L-fucofuranose, (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid, and 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol. The predicted Hyg26 protein has sequence homology to short-chain alcohol dehydrogenases and is assigned to the final step in production of the 5-dehydro-α-L-fucofuranose, catalyzing the reduction of α-L-fucofuranose. A *hyg26* mutant strain was generated and shown to produce no hygromycin A but 5″-dihydrohygromycin A, 5″-dihydromethoxyhygromycin A, and a 5″-dihydrohygromycin A product lacking the aminocyclitol moiety. To the best of our knowledge, these shunt metabolites of biosynthetic pathway intermediates have not previously been identified. They provide insight into the ordering of the multiple unusual steps which compromise the convergent hygromycin A biosynthetic pathway.

Introduction

Hygromycin A (compound **1**) (Figure 1) is an antibiotic first isolated from the fermentation broth of several strains of *Streptomyces hygroscopicus* in 1953 [1]. A second structurally unrelated antibiotic, the aminoglycoside hygromycin B, was later isolated from *S. hygroscopicus* [2]. Early studies demonstrated that hygromycin A had a relatively broad spectrum of activity against gram-positive and -negative bacteria [1, 3]. Almost three decades later, Guerrero and Modolell demonstrated that the mode of action was inhibition of the ribosomal peptidyl transferase activity. Initial studies also demonstrated that hygromycin A (1) blocked the binding of either chloramphenicol or lincomycin to the ribosomes [4] and (2) bound more tightly than chloramphenical. More recent footprinting experiments have shown that macrolides only block binding of hygromycin A to the ribosome if they contain a mycarose unit [5]. Crystallographic evidence indicates that in such macrolides, the C5-disaccharide group extends from the polypeptide exit channel into peptidyl transferase center [6]. Hygromycin A is not a macrolide and thus offers a distinct carbon skeleton and binding mode to other antibiotics that target the bacterial ribosome. As such, it represents a promising starting point for generating new antibiotics to treat infections with drug-resistant pathogens.

Hygromycin A has also been reported to have additional activities and potential applications. It exhibits hemagglutination inactivation activity and high antitreponemal activity [7, 8], leading to the possible application of hygromycin A-related compounds for the treatment of swine dysentery, a severe mucohemorrhagic disease thought to be caused by *Serpulina* (*Treponema*) *hyodysenteriae* [8, 9]. Hygromycin A has also been reported to possess an immunosuppressant activity in the mixed poor lymphocyte reaction but does not work via suppression of interleukin 2 production [10]. Most recently, methoxyhygromycin A (compound **2**) (Figure 1), an analog of hygromycin A produced in the same fermentation broth, has been shown to have herbicidal activity and has led to the suggestion that it could be developed as a biological agent for weed control [11].

Semisynthetic programs based on hygromycin A and its attractive biological properties have been reported [12–14]. This work has led to the synthesis of over 100 analogs and determination of their activity, both in terms of MICs for *Serpulina hyodysenteriae* and their ability to inhibit protein synthesis in an *E. coli* cell extract. The resulting structure activity relationship (SAR) has revealed that the unusual aminocyclitol moiety is an important component for the antibacterial activity, while the 5-dehydro-α-L-fucofuranose moiety is not essential and can be replaced with hydrophobic allyl group. Reduction in the antibacterial activity is also observed with replacement in methyl group of central (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moiety with propyl, allyl, or hygrogen [14, 15]. For the most part, these structural analogs were prepared by a semisynthetic method with hygromycin A as a starting point. A total synthesis of hygromycin A and C2″epi-hygromycin A has also been reported [12, 16]. Several multistep syntheses of the 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol moiety have also been reported [17]. The most recent synthesis of this was enantioselective and accomplished in 14 steps with an overall 12% yield [18].

We have sought a complementary approach of deciphering the biosynthetic process with a long-term aim of using this as an economical means for generating hygromycin analogs for further development. In our preliminary work, we determined the biosynthetic origins of the three unusual and structurally distinct moieties of hygromycin A. Mannose was shown to provide the 5-dehydro-α-L-fucofuranose moiety, 4-hydroxybenzoic acid and propionic acid the central (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moiety, and glucose and

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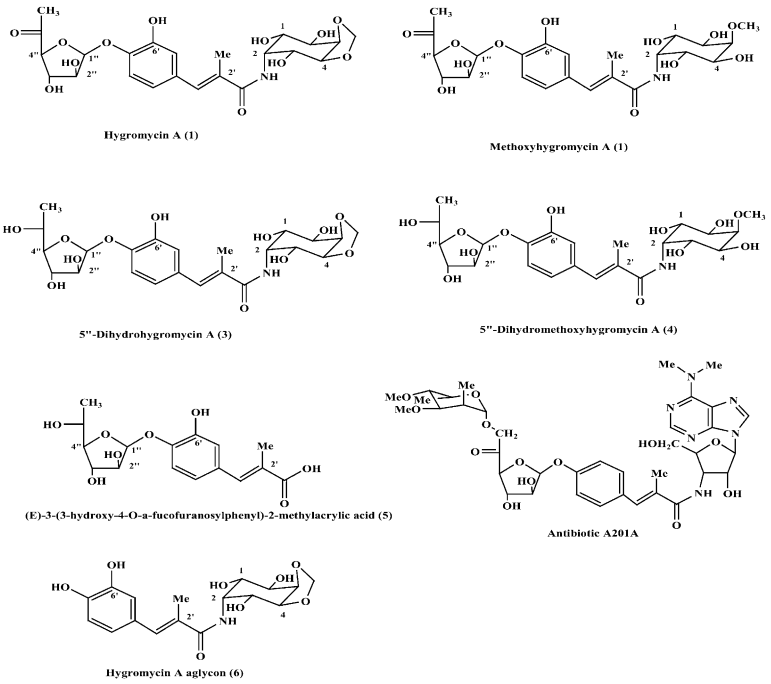

Figure 1. Structures of Hygromycin A, Hygromycin A Analogs, and Related Compounds

methionine the 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol moiety [19]. A convergent biosynthetic pathway was proposed (Figure 2) from these observations, and we now report how this formed the basis for a PCR approach that has led to the identification of the hygromycin A biosynthetic gene cluster of *S. hygroscopicus* NRRL 2388. The gene cluster has been cloned, sequenced, and analyzed, and putative assignments for the majority of corresponding gene products in the biosynthesis of the three structural moieties of hygromycin A have been proposed. Furthermore, manipulation of the gene cluster has been shown to give rise to hygromycin A analogs and shunt products that have not previously been identified. These findings provide important insights into the order of the biosynthetic steps and are a promising step toward the long-term objective of this work.

Results and Discussion

Cloning and Sequencing of the Hygromycin A Biosynthetic Gene Cluster

Previous studies on hygromycin A have suggested a convergent biosynthesis from 5-dehydro-α-L-fucofuranose, (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid, and 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol [19]. The 5-dehydro-α-L-fucofuranose moiety was shown to be derived from mannose, and a pathway proceeding from a nucleoside diphosphate (NDP) activated mannose, through NDP-4-keto-6-deoxymannose and

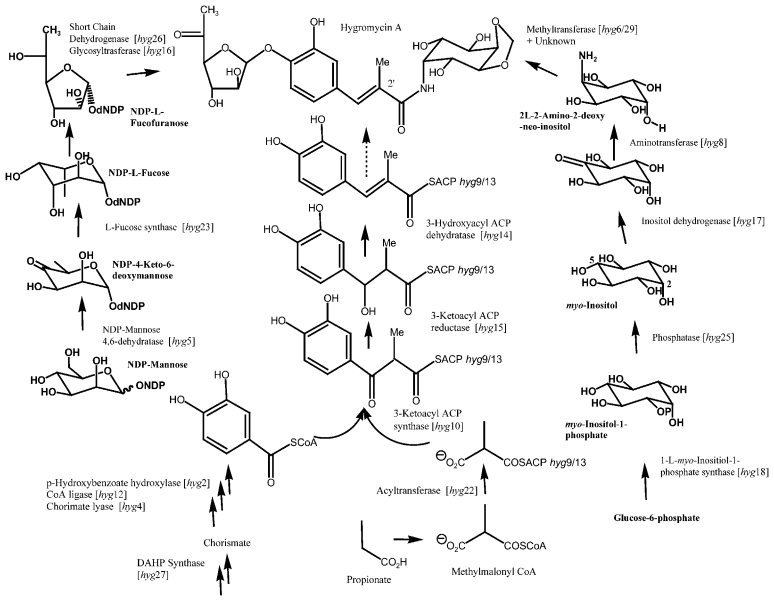

Figure 2. Proposed Role of *hyg* Gene Products in Hygromycin A Biosynthesis in *S. hygroscopicus* NRRL 2388

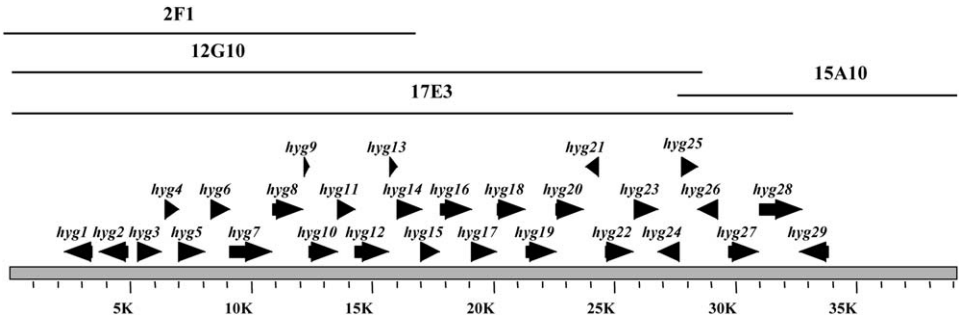

Figure 3. Organization of the Hygromycin A Biosynthetic Gene Cluster in *S. hygroscopicus* NRRL 2388

Each arrow indicates the direction of transcription of each predicted open reading frame. The overlapping cosmid clones namely 2F1, 12G10, 17E3, and 15A10 covering the entire gene cluster is also depicted. The entire putative hygromycin A biosynthetic gene cluster was obtained by shotgun sequencing 17E3 cosmid and primer walking 7 kb of cosmid 15A10 cosmid clone. The sequence of the entire gene cluster and the proposed function for individual ORFs is summarized in Table 1, and nucleotide sequence submitted to GenBank with accession number DQ314862.

NDP-L-fucose, was proposed. Genes encoding proteins with homology to GDP-D-mannose-4,6-dehydratase (MDH) have been identified in the nystatin, candicidin, and other antibiotic biosynthetic gene clusters [20–22]. Moreover, the partially reported antibiotic A201A gene cluster by A. Jimenez and coworker (GenBank accession number X84374), which bears significant structural similarities to hygromycin A (Figure 1), also contains a gene encoding a putative MDH. A BLAST search for similar genes in two reported genome sequences from *S. coelicolor* A3(2) [23] and *S. avermitilis* MA-4680 [24] failed to produce sequences encoding primary metabolic proteins with significant homology. These observations suggested a gene encoding a putative MDH as a target for identification of the hygromycin A biosynthetic gene cluster of *S. hygroscopicus*.

Accordingly, a pair of degenerate primers based on the highly conserved motifs observed by creating an alignment of predicited MDHs was used to amplify a portion of *hyg5* from genomic DNA of the hygromycin A producer *S. hygroscipoicus* NRRL 2388. Sequencing confirmed that the PCR product encoded a protein with homology to putative MDH enzymes. The partial *hyg5* fragment was then used to screen a cosmid library of *S. hygrocospicus* NRRL 2388 to identify hygromycin A biosynthetic gene cluster. Cosmid clone 2F1 was identified through screening the first 600 cosmid clones and was sequenced. Sequence analysis suggested that only a portion (15 kb) of the predicted biosynthetic gene cluster was present. A PCR fragment of *hyg14* was obtained with this cosmid clone as a template and used to further screen the library, resulting in the identification of three more overlapping cosmid clones, 12G10, 17E3, and 15A10, respectively. The entire putative hygromycin A biosynthetic gene cluster was obtained by shotgun sequencing 17E3 cosmid and primer walking 7 kb of cosmid 15A10 (Figure 3).

A contiguous 40 kb DNA region encoding the putative hygromycin biosynthetic gene cluster was thus sequenced adequately on both strands with these three cosmids (2F1, 17E3, and 15A10) and analyzed for putative open reading frames (ORFs) with DS Gene software (Accelrys) and Frame program [25]. A total of 29 ORFs, designated *hyg1–29*, were aligned with homologous sequences of GenBank by using BLAST programs [26]. The predicted functions of these *hyg* gene products and their corresponding homologs are listed in Table 1. Genes adjacent to *hyg1* encoded a hypothetical protein and a putative glucose dehydrogenase, respectively, while those adjacent to *hyg29* encoded a putative pyruvate dehydrogenase and transcriptional regulator. These genes flanking the 29 *hyg* ORFs were highly homologous to genes identified in the genome sequences of *S. coelicolor* [23] and *S. avermitilis* [24], could not readily be assigned a dedicated function in the biosynthesis of hygromycin A, and likely are involved in other cellular processes.

Gene Products Putatively Required for 5-Dehydro-α-L-Fucofuranose Biosynthesis

Analysis of the *hyg* cluster indicated the presence of several putative genes believed to be involved in the proposed biosynthetic pathway to the 5-dehydro-α-L-fucofuranose moiety from NDP-mannose (Figure 2) [19]. The *hyg5* gene used to identify the biosynthetic gene cluster encodes a putative MDH that would catalyze the first step in this process, the conversion of an activated NDP-mannose into NDP-4-keto-6-deoxymannose. The subsequent step was proposed to be a conversion to NDP-L-fucose (involving epimerization at the C-3 and C-5 positions of the hexose ring and an NADPH-dependent reduction at the C-4 position) and is likely catalyzed by the *hyg23* gene product, which has clear homology (>60% identity) to L-fucose synthetases. The proposed next step, conversion from the pyranose to the furanose form, was proposed [19] to follow a similar mechanism to that established for the UDP-galactopyranomutase [27, 28]. None of the *hyg* genes encoded proteins with sequence homology to this protein and no clear candidate for this step can be identified. The *hyg20* gene product encodes proteins with homology to transglucosylases in databases and may catalyze this step. Interestingly, Hyg20 has homology to Ata16 protein, which is encoded by the antibiotic A201A gene cluster. The structure of antibiotic A201A suggests a pyranose-furanose ring might also be required in the biosynthetic pathway. The final step in the predicted pathway to 5-dehydrofucofuranose is an

Table 1. Gene Annotation of *hyg* Gene Cluster and Comparison with Homologous Proteins in the Public Database

Gene	Amino Acids	Proposed Function	Homologous Protein in Database	Identity/Similarity	Accession Number
hyg1	375	AfsR regulatory protein	afsR (*S. coelicolor*)	37/52	BAA14186
hyg2	399	p-hydroxybenzoate hydroxylase	4-hydroxybenzoate hydroxylase (*B. japonicum*)	51/66	NP773060
hyg3	323	Regulatory protein	putative regulatory protein (*S. griseus*)	42/56	CAA68515
hyg4	173	Chorismate lyase	Chorismate pyruvate lyase (*Methanopyrus kandleri*)	28/44	NP613406
hyg5	352	Mannose dehydratase	GDP-D-mannose dehydratase (*Kineococcus radiotolerans*)	67/77	ZP00227152
hyg6	249	Methyltransferase	Ata11 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	31/42	CAD62205
hyg7	579	Unknown	hypothetical protein (*M. avium*)	51/67	NP963248
hyg8	411	Aminotransferase	aminotransferase (*Prochlorococcus marinus*)	33/52	NP893936
hyg9	83	ACP	AtaPKS2 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	34/55	CAD62191
hyg10	378	β-ketoacyl synthase 1	AtaPKS3 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	54/64	CAD62192
hyg11	252	Unknown	Ata9PKS4 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	48/59	CAD62193
hyg12	451	CoA-ligase	Ata18 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	48/61	CAD62194
hyg13	96	ACP	Ata19 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	43/64	CAD62195
hyg14	347	3-hydroxyacyl ACP dehydratase	Ata2 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	43/59	CAD62196
hyg15	256	3-ketoacyl ACP reductase	Ata4 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	54/67	CAD62198
hyg16	431	Glycosyltranferase	Ata5 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	63/74	CAD62199
hyg17	330	*myo*-inositol dehydrogenase	hypothetical protein (*M. avium*)	41/56	NP962413
hyg18	363	*myo*-inositol-1-phosphate synthase	Myo-inositol-1-phosphate synthase (*Thermobifida fusca*)	78/87	ZP00291606
hyg19	416	Transmembrane protein	Ata9 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	50/63	CAD62203
hyg20	370	Transglucosylase	Ata16 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	60/71	CAD62189
hyg21	183	Phosphotranseferase	Ard2 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	56/66	CAD62197
hyg22	391	Acyltransferase	AtaPKS1 protein (*Saccharothrix mutabilis* subsp. *capreolus*)	45/52	CAD27643
hyg23	325	Fucose synthase	EpiA (*M. avium*)	62/74	NP960166
hyg24	287	Unknown	hypothetical protein (*M. avium*)	43/58	NP962413
hyg25	213	*myo*-inositol-1-phosphatase	stsD (*S. griseus*)	44/59	CAA70013
hyg26	271	Short chain dehydrogenase	hypothetical protein (*M. avium*)	53/70	NP961344
hyg27	401	DAHPS synthase	DAHP synthase (*Actinosynnema pretiosum*)	62/71	AAC13561
hyg28	570	ABC transporter	Putative ABC transporter (*S. avermitilis*)	78/86	NP822846
hyg29	378	Methyltransferase	carboxylmethyltransferase (*Thermobifida fusca*)	45/59	ZP00293268

oxidation and is assigned to the *hyg26* gene product that has homology to a family of short-chain dehydrogenases (Table 1). As described below, subsequent genetic experiments have confirmed this assignment. A glycosyltransferase encoded by *hyg*16 is expected to be responsible for formation of the glycosidic linkage between the 5-dihydro-α-L-fucofuranose and the 4-hydroxy group of the (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moiety (the juncture in the biosynthetic process in which this link is formed has yet to be determined). A similar glycosidic linkage is required in the biosynthesis of antibiotic A201A and may be carried out by the Ata5 protein, which has 63% identity to the Hyg16 (Table 1).

Gene Products Putatively Required for (*E*)-3-(3,4-Dihydroxyphenyl)-2-Methylacrylic Acid Biosynthesis

Based on the results from previous biosynthetic studies with ^{13}C labeled precursors, we had proposed that this central (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moiety was derived from a condensation of methylmalonyl-ACP with 3,4-dihydroxybenzoyl CoA or 4-hydroxybenzoyl CoA [19]. The 3-keto group of the resulting 3-(3,4 dihydroxyphenyl)-3-oxopropanoyl CoA (ACP) would then be processed by reduction and dehydration steps in a manner similar to that observed for polyketide biosynthetic processes (Figure 2). The gene products of *hyg9–15*, *hyg27*, *hyg4*, and *hyg22* can be assigned

putative roles in this process. As antibiotic A201A has the same structural moiety as hygromycin A, with the exception of the 3-hydroxy substituent, similar enzymes are anticipated to be required for their biosynthesis. At the time this project was initiated, a partial sequence of the antibiotic A201A gene sequence had been reported. Analysis showed that *ard1* and *ard2* were resistance determinants, while *ataP3*, *ataP5*, *ataP4*, and *ataP7* were likely involved in formation of the N^6,N^6-dimethyl-3′-amino-3′-deoxyadenosine moiety, which is not present in hygromycin A (the full sequence of the ataPKS1 gene was also reported). Additional sequence information from this cluster has now been deposited and is consistent with our prediction; clear homologs of *hyg9–15* and *hyg22* are observed (Table 1).

The pathway to this moiety is proposed to start with 4-hydroxybenzoic acid, derived from chorismate via the enzyme chorismate lyase. A putative chorismate lyase is encoded by *hyg4* (Table 1). The chorismate required for this enzyme reaction presumably is generated by the shikimate pathway. The committed step of this pathway, which generates aromatic amino acids and other primary metabolites in plants and bacteria [29], is catalyzed by 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase. DAHP synthase is subject to feedback inhibition by aromatic acids by an allosteric mechanism [30]. Genes encoding homologs of DAHP synthase are often found in antibiotic biosynthetic gene clusters where a shikimate metabolite is used as a biosynthetic precursor. Presumably, these homologs are not inhibited by aromatic amino acids and help ensure an adequate supply of shikimate/chorismate during secondary metabolism [31]. Consistent with these observations, a gene (*hyg27*) encoding a DAHP synthase homolog is noted in the hygromycin biosynthetic gene cluster. The *hyg2* gene product encodes a protein with homology to 4-hydroxybenzoate hydroxylase and is assigned a role in the formation of 3,4-dihydroxybenzoic acid. It is not clear if the hydroxylation occurs directly on 4-hydroxybenzoic acid or at a later juncture in the biosynthesis of the (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moiety. This hydroxylation is not required in antibiotic A201A biosynthesis, and our analysis revealed that there is no Hyg2 homolog encoded by the corresponding biosynthetic gene cluster.

In the following step, the 3,4-dihydroxybenzoic acid (or 4-hydroxybenzoic acid if the hydroxylation occurs at a later step in the pathway) is presumably activated by conversion to a thioester. The *hyg12* gene product is assigned to this step based on its homology to a family of CoA-ligases (Table 1). This enzyme may catalyze formation of either a coenzyme A or an acyl carrier protein (ACP) thioester; the predicted sequences of the *hyg9* and *hyg13* gene products are ACP homologs. One of these ACPs is presumably required for making methylmalonyl ACP for condensation with the activated 3,4-dihydroxybenzoyl thioester. The other ACP is also likely involved in one of the subsequent steps. The sequence analysis revealed that *hyg22* encodes a putative acyl transferase (AT) with some low sequence similarity to AT domains in type I modular PKSs and may catalyze this methylmalonyl CoA-methylmalonyl ACP interconversion. The presumed AT ataPKS1 encoded by the antibiotic A201A biosynthetic gene cluster has sequence homology to both Hyg22 and to AT domains in modular type I polyketide synthase (PKS) and likely has a similar role. Sequence analysis of both Hyg21 and the ataPKS1 revealed conserved sequence motifs characteristic of methylmalonyl-CoA-specific rather than malonyl CoA-specific ATs [32–34]. In contrast, *hyg10* (and the homolog from the antibiotic A201A cluster) encodes a β-ketoacyl synthase (KS) with low homology to discrete KS proteins in type II PKS, but not KS domains in modular type I PKSs. It is presumed that this KS protein, whose sequence is quite distinct from other known KS proteins, is required for catalyzing the decarboxylative condensation reaction with methylmalonyl ACP. A multiple sequence alignment of the predicted Hyg10 and AtaPKS3 with the type II PKS KS proteins revealed the presence of the two highly conserved active site histidines [35], which are presumably required for catalyzing the methylmalonyl ACP decarboxylation. However, in both Hyg10 and AtaPKS3, a serine residue is observed in place of the highly conserved nucleophilic cysteine residue (required for formation of the acyl thioester intermediate in the catalytic cycle of these enzymes). Although serine acts as the key active site nucleophile in ATs for generating enzyme bound acyl ester intermediates [35, 36], we are unaware of such a role for this residue catalyzing C-C bond formation in a β-ketoacyl synthase. A naturally occurring KS domain containing serine at the active site in place of cysteine has been reported in the loading module of the pimaricin PKS cluster of *S. natalensis* [37] but most likely catalyzes a decarboxylation (C-C bond cleavage) in the same way as KS-Q domains [38]. The *hyg11* gene product exhibited 48% identity to *ataPKS4* of antibiotic A201A gene cluster and very low amino acid identity to several putative type II KS proteins in the database. The highly conserved catalytic triad of KS proteins and domains was not observed in either Hyg11 or A201A, and no clear role can be assigned based on the sequence analysis.

The 3-(3,4-dihydrophenyl)-3-hydroxy-2-methyl-propionyl ACP proposed to be generated by Hyg10 may be converted to (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylyl ACP by the action of a 3-hydroxylacyl ACP dehydratase (Hyg14) and 3-ketoacyl ACP reductase (Hyg15) (Figure 2). Similar roles can be envisioned for the *ata2* and *ata4* gene products (with 59% and 67% homology to Hyg14 and Hyg15, respectively), from antibiotic A201A gene cluster.

Overall, these analyses reveal the presence of a set of homologous genes in the both hygromycin A and A201A gene clusters, which can be assigned to the synthesis of the (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid (hygromycin) and (*E*)-3-(4-hydroxyphenyl)-2-methylacrylic acid (antibiotic A201A) moiety. Many aspects of this proposed process appear unusual, including the use of a discrete AT to load methylmalonyl CoA and a KS protein with an active site serine residue. Nonetheless, the exact role of these proteins and the specific ordering of the biosynthetic steps remains to be determined.

Gene Products Putatively Required for 2L-2-Amino-2-Deoxy-4,5-O-Methylene-Neo-Inositol Biosynthesis

Previously, we had shown that the aminocyclitol portion of hygromycin A was derived from glucose and that the

incorporation pattern for D-[1,2-$^{13}C_2$]glucose was consistent with a proposed pathway leading from glucose 6-phosphate through a *myo*-inositol intermediate (Figure 2). The first two steps of this pathway would require a *myo*-inositol-1-phosphate synthase (*hyg18* gene product) and *myo*-inositol phosphatase (*hyg25* gene product). We have previously noted that these two steps are also required in the biosynthetic pathway that generates the *scyllo*-inosamine-derived moiety of streptomycin moiety. StsD a putative *myo*-inositol phosphatase involved in the streptomycin biosynthetic pathway has 44% identity to Hyg25 (Table 1). The pathways diverge after *myo*-inositol, with a proposed oxidation of C5 in the case of hygromycin A. A putative inositol dehydrogenase (encoded by *hyg17*) is likely responsible for catalyzing this step. A subsequent transamination catalyzed by the *hyg8* gene product is then proposed to provide 2L-2-amino-2-deoxy-*neo*-inositol [19]. The Hyg8 sequence shows homology to class III pyridoxal-phosphate-dependent aminotransferases and low homology to L-glutamine:*scyllo*-inosose aminotransferase StsC, which catalyzes a similar reaction in streptomycin biosynthesis [39].

One of the intriguing features of the aminocyclitol portion of hygromycin A is the presence of a C-4 and C-5 methylene bridge. At which stage in the aminocyclitol biosynthetic process the methylene bridge is formed remains to be determined. In a previous biosynthetic study, we have shown labeling of this methylene carbon of hygromycin by L-[*methyl*-^{13}C]methionine, consistent with a pathway utilizing *S*-adenosylmethionine and a methyl transferase (*hyg6* or *hyg29* gene product). The structure of methoxyhygromycin (Figure 1) suggests that the methyl group may be transferred onto the C5 hydroxy group and that the penultimate intermediate in this pathway would be 2L-2-amino-2-deoxy-5-*O*-methyl-*neo*-inositol (Figure 2). Formation of the final 2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol would require an oxidation and represents a highly unusual conversion in sugar biochemistry. Examination of the hygromycin biosynthetic gene cluster does not reveal a clear candidate protein for catalysis of this step. If formation of the methylene bridge occurs before formation of the amide bond of hygromycin A, then methoxyhygromycin (2), which is reportedly less active than hygromycin [7] and observed in all fermentations of the *S. hygroscopicus* strain, may represent a shunt metabolite. Alternatively, methoxyhygromycin may be the penultimate intermediate with methylene bridge formation representing the last step in the biosynthetic pathway.

Resistance Genes

The presence of one or more resistance genes within actinomycete biosynthetic gene clusters is well documented [40]. Analysis of the *hyg* gene cluster revealed several candidates genes whose predicted products, a methyltransferase (Hyg6 or Hyg29), a transmembrane protein (Hyg19), a phosphotransferase (Hyg21), and an ABC transporter (Hyg28), are likely to be involved in providing resistance to hygromycin A.

It has been shown that antibacterial activity of hygromycin A involves inhibition of the ribosomal peptidyl transferase, with a binding site on the large ribosomal subunit closely related to that observed for chloramphenicol and lincomycin [4]. We speculate that methylation of the nucleotide at the binding site of the *S. hygroscopicus* ribosome by a methyltransferase would provide resistance to hygromycin A. Ribosomal methylation is a well-established mechanism of resistance to this class of antibiotics, and genes encoding the appropriate methyl transferases are often located within the antibiotic biosynthetic gene cluster [41–43].

The *hyg21* gene product is predicted to be a phosphotransferase and has high amino acid sequence similarity with *ard2* gene product from the A201A gene cluster (66% homology). It has been shown that Ard2 protein catalyzes an ATP-dependent phosphorylation of the C2 hydroxyl group in the furanose moiety of antibiotic A201A, thereby inactivating the aminonucleoside antibiotic [44]. Hyg21 may provide resistance to hygromycin A by a similar mechanism. It has previously been established that hygromycin A is a substrate of Acr A/B efflux pump in *E. coli* and that this is the major cause for its ineffectiveness against enteric gram-negative bacteria such as *E. coli* and *Salmonella* where this efflux pump is widespread [14]. Furthermore, Hyg19 and Hyg28 have amino acid sequence homology to Ata9 (65% similarity) and Ard1 (86% similarity), and the ability of Ard1, a member of the ABC transporter superfamily, to provide resistance to antibiotic A201A has previously been established [45]. Therefore, we propose that the Hyg19 (a transmembrane protein) and Hyg28 (an ABC transporter) may provide an additional layer of resistance to *S. hygroscopicus* by catalyzing hygroymcin A efflux.

Regulatory Genes

Comparison of *hyg* genes with the databases revealed two genes that likely regulate hygroymcin A biosynthesis. The *hyg1* gene encodes a protein with 52% sequence similarity to the AfsR transcriptional regulatory protein of *S. coelicolor* A3(2). A clear helix-turn-helix motif can be identified at the N-terminal region of Hyg1, which is proposed to be a transcriptional activator for the pathway. A similar role is envisioned for Hyg3, which has amino acid sequence homology to the StrR, a pathway-specific activator that regulates streptomycin biosynthetis in *S. griseus* [46].

Production of 5″-Dihydrohygromycin Analogs

A genetic experiment was used to confirm that the *hyg* biosynthetic gene cluster is responsible for hygromycin A biosynthesis. Allelic replacement of the *hyg26* gene in *S. hygroscopicus* by *aac(3)IV* resistance marker (conferring apramycin resistance) and *oriT* led to the SCH30 mutant. As described above, Hyg26 has homology to a family of short-chain dehydrogenases and is proposed to catalyze the final oxidative step in the predicted pathway to 5-dehydrofucofuranose moiety of hygromycin A.

As shown in Figure 4B, HPLC analyses of *S. hygroscopicus* NRRL 2388 reveals the clear presence of hygromycin A (1) and methoxyhygromycin A (2) (the 5″ epimeric forms of these two compounds appear as smaller shoulder peaks at a slightly earlier retention time). Production levels under the fermentation conditions used were typically 350 mg/l methoxyhygromycin A and 880 mg/l hygromycin A. In contrast, the SCH30 mutant generates no detectable levels of hygromycin A or

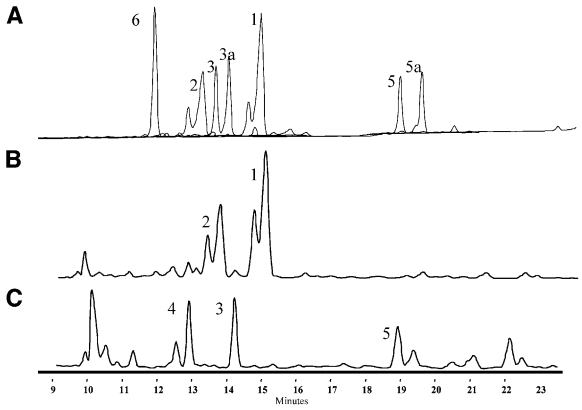

Figure 4. HPLC Analyses of the Authentic Hygromycin A and Its Analogs

(A) Overlay of standards generated by reduction and hydrolysis of pure hygromycin A standard.

(B) Fermentation broth of wild-type strain.

(C) Fermentation broth of the SCH30 mutant. (1, hygromycin A; 2, methoxyhygromycin A; 3 and 3a, diastereomeric mixture of 5″-dihydrohygromycin A; 4, 5″-dihydromethoxyhygromycin A; 5 and 5a, diastereomeric mixture of (E)-3-(3-hydroxy-4-O-α-fucofuranosyl phenyl)-2-methylacrylic acid; and 6, hygromycin A aglycone.)

methoxyhygromycin A but generates three new peaks (**3**, **4**, and **5** in Figure 4C). The three new fermentation products were purified and characterized and shown to be 5″-dihydrohygromycin A (**3**), 5″-dihydromethoxyhygromycin A (**4**), and (*E*)-3-(3-hydroxy-4-*O*-α-fucofuranosylphenyl)-2-methylacrylic acid (**5**).

5″-dihydrohygromycin A (**3**) was produced in the SCH30 mutant with fermentation titers of approximately 180 mg/l. MS analyses revealed an m/z of 514 $(M + H)^+$ for **3** (Figure 5B), 2 Da larger than the 512 $(M + H)^+$ observed for hygromycin A and consistent with the proposed structure. Careful LC-MS analyses of the hygromycin A products from the wild-type strain revealed that **3** may also be produced albeit at low levels (less than 1% of hygromycin A). A nonstereoselective reduction of hygromycin A with sodium borohydride provided diastereomers of 5″-dihydrohygromycin A (peaks **3** and **3a** in Figure 4A). LC-MS analyses revealed an m/z of 514 $(M + H)^+$ for both of these. A coinjection of this diastereomeric mixture with **3** isolated from the SCH30 mutant revealed that it coeluted with peak **3** and not **3a**. This observation is consistent with the proposed structure for this product and demonstrates that only one diastereomeric product is made in this mutant. Proton and ^{13}C NMR analyses were also consistent with this structure. A comparative COSY NMR analyses of **3** and hygromycin A revealed the appearance of a new three-proton doublet (~1.2 ppm) for the C-6″ methyl group and a decrease in the integration for the peak at ~2.1 from six protons to three protons. This doublet is consistent with an upfield shift for methyl group at C-6″ of **3** due to the presence of a proton at C-5″. The H-5″ resonance appears as a pentet at 3.79 ppm, which couples to the methyl doublet at ~1.2 ppm (H-6″) as well as another peak at ~3.6 ppm (H-4″). The H-4″ resonance (a doublet in hygromycin A) also changes in **3**, where it is now a doublet of doublets (coupled to both H-5″ and H-3″) and is shifted upfield (C-5″ is less electron withdrawing). The ^{13}C NMR showed the disappearance of the ketone peak (C-5″) at 210.1 ppm and appearance of a new peak at 69.29 ppm, which corresponds to the new alcohol carbon peak (C-5″). Small changes in the resonances of the other furanose carbons were also observed. The HMQC (heteronuclear multiple quantum coherence) spectrum analysis was also consistent with structure of **3**. It showed that the new carbon signal at 69.29 ppm (C-5″) displays a cross peak at 3.79 ppm (H-5″) in the ^1H dimension.

5″-dihydromethoxyhygromycin A (**4**) was produced in the SCH30 mutant with fermentation titers of approximately 140 mg/l. MS analyses revealed an m/z of 516 $(M + H)^+$ for **4** (Figure 5B), 4 Da larger than the 512 $(M + H)^+$ observed for hygromycin A and consistent with the proposed structure. Careful LC-MS analyses of the hygromycin A products from the wild-type strain revealed that **4** may also be produced albeit at very low levels (less than 1% of hygromycin A). Two peaks (each with an m/z of 516 $[M + H]^+$ by LC-MS analyses), one of which coelutes with **4**, were obtained by a nonstereoselective reduction of methoxyhygromycin A by using $NaBH_4$. Proton NMR analyses of the purified 5″-dihydromethoxyhygromycin A (**4**) revealed a methoxy singlet at 3.52 ppm, absent in **3** and hygromycin but present in methoxyhygromycin [7, 11]. The proton resonances for the α-L-fucofuranose moiety of **4** were the same as those observed for **3**, including the presence of an H-5″ proton coupled to both the H-6″ methyl protons and H-3″ proton. Analysis of the ^{13}C NMR of dihydromethoxyhygromycin A showed the disappearance of the ketone peak (C-5″) at 210.1 ppm and appearance of a new peak at 69.31 ppm, which corresponds to the new alcohol carbon peak (C-5″). Small changes in the resonances of the other furanose carbons were also observed. The HMQC (heteronuclear multiple quantum coherence) spectrum analysis was also consistent with structure of **4**, with the new carbon signal at 69.31 ppm (C-5″) displaying a cross peak at 3.79 ppm (H-5″) in ^1H dimension. Chemical reduction of methoxyhygromycin A with sodium borohydride provided a diasteromeric mixture of dihydromethoxyhygromycin A (**4**). MS and ^1H NMR analysis were consistent with this being a diastereomeric mixture of 5″-dihydromethoxyhygromycin A (**4**). This mixture was coinjected in the HPLC with a sample of naturally produced dihydromethoxyhygromycin A from the mutant and one of the peaks of the diasteromeric mixture coeluted with that of the natural one. The ^{13}C NMR spectrum of the chemically obtained dihydromethoxyhygromycin A was exactly similar to that obtained from the mutant, confirming its structure.

The final product isolated from the SCH30 mutant was (*E*)-3-(3-hydroxy-4-*O*-α-fucofuranosylphenyl)-2-methylacrylic acid (**5**), which was produced at levels of approximately 125 mg/l. This peak was not observed in fermentations of the wild-type strain. LC-MS revealed an m/z of 363 $(M + Na)^+$ for **5** (Figure 5B), consistent with the proposed structure. Proton and ^{13}C NMR analyses revealed all of the resonances associated with the α-L-fucofuranose and (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moieties of both **3** and **4**, but not those of the aminocyclitol moiety, supporting the structural assignment for this compound. Further support was provided by generating a diastereomeric mixtrure of **5**, by

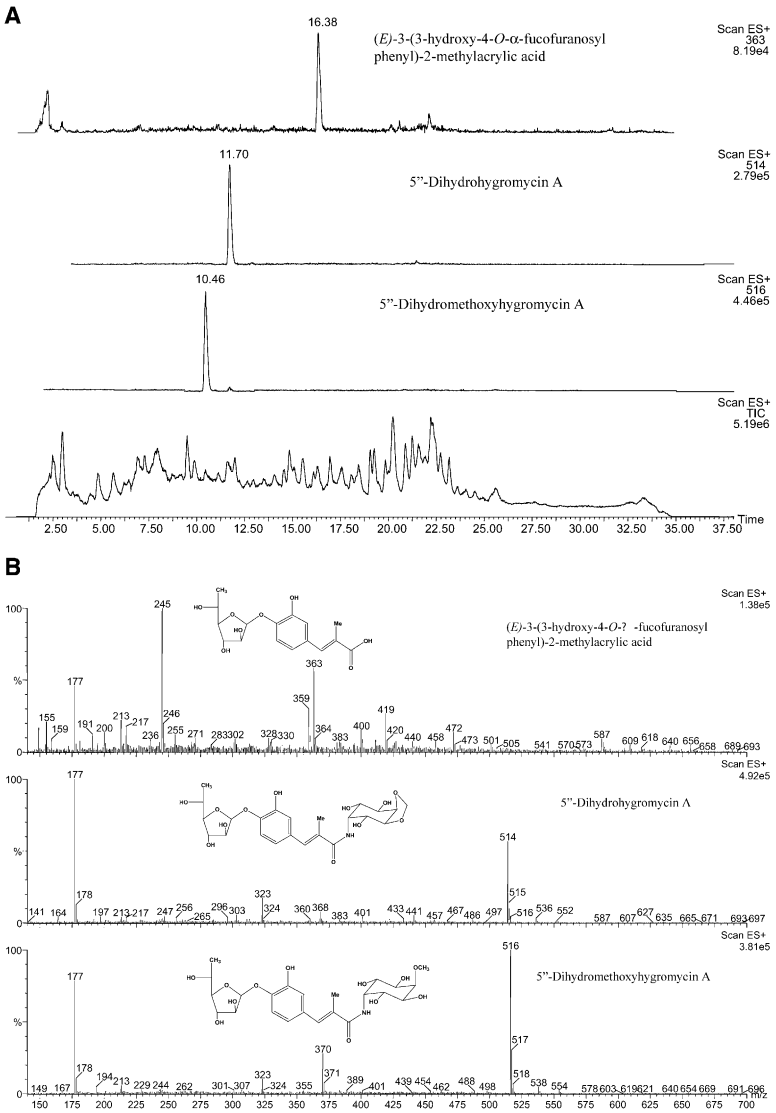

Figure 5. LC-MS Analyses of the Hygromycin A Analogs from SCH30 Mutant

(A) LC-MS analysis of the fermentation broth of the SCH30 mutant. Panels in ascending order represent the total ion chromatogram and single-ion monitoring for an m/z of 516, 514, and 363.

(B) Mass spectral analyses of hygromycin A analogs produced by the SCH30 mutant.

nonstereoselective reduction of hygromycin A followed by base hydrolysis. Two products were formed in this process (**5** and **5a** in Figure 4A), one of which coeluted with the purified material obtained from the SCH30 strain.

Acid hydrolysis of a mixture of **3** and **3a**, provided the hygromycin aglycone lacking the furanose moiety (**6**), (*E*)-3-(3,4-dihydroxyphenyl)-2-methyl-*N*-(2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo*-inositol)acrylamide) (Figures 1 and 4A). LC-MS analyses failed to show any detectable level of this compound from fermentations of either the wild-type strain or the SCH30 mutant.

Implication for Hygromycin A Biosynthesis

The analysis of hygromycin products made by strain SCH30 provides compelling evidence that the last step in production of the 5-dehydro-α-L-fucofuranose moiety is an oxidation of α-L-fucofuranose catalyzed by the *hyg26* gene product (Figure 6). It is not possible to determine from these analyses of the SCH30 mutant if this oxidation step occurs before or after formation of the glycosidic bond with the (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylic acid moiety of hygromycin A. Nonetheless, studies in which either **3** or **4** was fed to a *hyg23* mutant (this mutant is presumably blocked in formation of the L-fucose and does not produce any detectable hygromycin products) (N.P., S.A., and K.A.R., unpublished data) does not lead to hygromycin production. This suggests that both **3** and **4** are shunt products, and not pathway intermediates. While not conclusive, this observation suggests that the Hyg26 catalyzed oxidation may occur at an earlier stage in the biosynthetic process (Figure 6).

The production of significant levels of **5** in the SCH30 mutant suggests that the glycosidic linkage in hygromycin A can be formed in the absence of the amide linkage. In fermentations of the wild-type strain, the SCH 30, and the *hyg23* mutant (blocked in formation of NDP-activated L-fucose), a hygromycin A analog lacking the dehydrofucofuranose moiety (**6**) was not observed. These observations suggest that the glycosidic bond of hygromycin may both precede and be a prerequisite for formation of the amide linkage (Figure 6). It is not possible to determine from these analyses if there is a specific

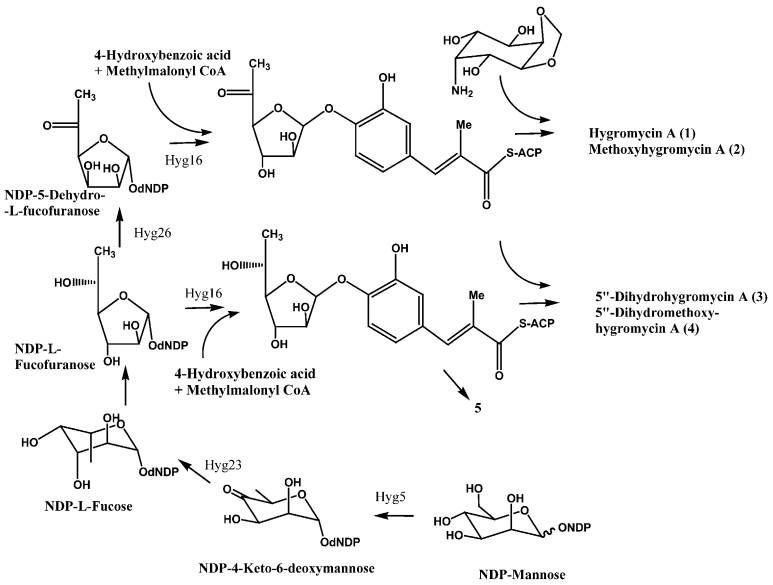

Figure 6. Proposed Working Hypothesis of Hygromycin A Biosynthesis and Role of the *hyg26* Gene Product

step in the conversion of 4-hydroxybenzoic acid to the (*E*)-3-(3,4-dihydroxyphenyl)-2-methylacrylyl thioester where this glycosidic bond is formed.

The combined yields of **3** and **4** in the SCH30 (320 mg/l) are approximately 25% of levels of **1** and **2** produced by the wild-type strain, suggesting that the enzymes catalyzing the amide and/or glycosidic bond formation have some degree of substrate flexibility. Nonetheless, the significant yields of **5** indicate that amide bond formation is perhaps slower by using a pathway intermediate with a fucofuranose moiety, possibly leading to formation of this shunt metabolite through a hydrolytic process.

Significance

This work describes a new and unusual biosynthetic gene cluster that makes hygromycin A and methoxyhygromycin A, which are structurally unusual aminocyclitol antibiotics produced by *Streptomyces hygroscopicus* NRRL 2388. This is the first reported gene cluster for a member of this class of important compounds. Among the notable features of the hygromycin A biosynthesis is the convergent nature of the process and the ability to generate a highly unusual aminocyclitol moiety with a methylene bridge that is essential for biological activity. We have established a genetic system for manipulating the hygromycin biosynthetic gene cluster in *S. hygroscopicus* and have shown that this can be used to provide significant yields of new hygromycin A analogs in a stereoselective and cost-effective manner. Hygromycin A analogs may serve as useful starting points for producing molecules with potential clinical or agricultural applications.

Experimental Procedures

Bacterial Strains, Media, and Culture Conditions

S. hygroscopicus NRRL 2388 and the SCH30 mutant were maintained and grown on ISP2 medium (0.4% yeast extract, 1.0% malt extract, 0.4% dextrose, 2.0% agar at pH 7.2), while the mannitol soy flour (MS) media [47] was used for intergeneric conjugation. All *E. coli* strains in this study were grown following standard protocols [48].

PCR Amplification of a Partial GDP-D-Mannose 4,6-Dehydratase Gene

Amplification of a partial *hyg5* gene encoding a putative MDH from *S. hygroscopicus* NRRL 2388 was achieved with a set of degenerate primers: Myco-F1, AARCGHGCRCTGATCACYGGA; and Myco-R1, CGSGGBGATTCGTGRTTGAA. These primers were designed based on highly conserved motifs identified by creating a multiple alignment of deposited putative MDH genes of *pimJ* (CAC20923), *nysDIII* (AAF71765), *amphDIII* (AAK73500), and *ata12* (CAD27644). Using a GC-rich PCR kit (Roche, Indianapolis, IN) with these primers and genomic DNA of *S. hygroscopicus* NRRL 2388, we amplified a PCR product of the expected size (~500–600 bp). This fragment was cloned and sequenced, and a BLAST search revealed the predicted product had greater than 78% amino acid sequence similarity to other putative MDHs. This partial *hyg5* gene was used as probe to screen a cosmid clone genomic library of *S. hygroscopicus* NRRL 2388 and led to the identification of the hygromycin A biosynthetic gene cluster.

Cloning, Sequencing, and Annotation of the Hygromycin A Biosynthetic Gene Cluster

The total genomic DNA of *S. hygroscopicus* NRRL 2388 was prepared following standard protocols [47]. A genomic library was constructed by using Supercos-1 cosmid vector as recommended in the manufacturer's protocol (Stratagene, La Jolla, CA). Approximately 3000 cosmid clones were probed with the DNA of a digoxigenin-labeled partial *hyg5*. Preparation of the digoxigenin probes and the subsequent hybridization and detection were performed as recommended in the manufacturer's protocol (Roche). The overlapping cosmid clones identified by the *hyg5* probe were sequenced to completion with the TOPO shotgun subcloning kit (Invitrogen, Carlsbad, CA). Automated DNA sequencing was performed on an ABI Prism 3700 DNA sequencer at DNA core facility of Medical College of Virginia, Virginia Commonwealth University. The DNA sequences were assembled with SeqMan II (DNASTAR, Inc., Madison, WI). The assembled DNA and deduced protein sequences were analyzed with DS Gene software (Accelrys, San Diego, CA) and Frame program [25] and compared with sequences in the public databases with the BLAST suite of programs [26].

Allelic Replacement of *hyg26* within the Hygromycin A Biosynthetic Gene Cluster

The *hyg26* gene of the hygromycin A biosynthetic gene cluster was replaced with *aac(3)IV* by the PCR-targeted *Streptomyces* gene

replacement method [49]. The *aac(3)IV* resistance marker (conferring apramycin resistance) and *oriT* were amplified from the pIJ773 disruption cassette with the primers [49]. The primers were HYG26-F 5′- GTCACATCCTTGATCGTTGCGGAAAGGGCATGGGC GATG***ATTCCGGGGA-TCCGTCGACC***-3′ and HYG26-R 5′-CGTCG GCGAGCGGCTGACCGCGGACGGTT-GCGCGGCTCA***TGTAGGCT GGAGCTGCTTC***-3′ (sequence homologous to pIJ773 disruption cassette is shown in bold, italicized text). The resulting PCR product from this set of primers was used to replace *hyg26* first in the cosmid clone 17E3 and then in *S. hygroscopicus* NRRL 2388 to generate the SCH30 mutant (Δ*hyg26: aac(3)IV*) by the intergeneric conjugation method [49]. Allelic replacement of the *hyg26* gene in SCH30 mutant was confirmed by PCR amplification and sequencing.

Production and Analysis of Hygromycin A

S. hygroscopicus NRRL 2388 and the SCH30 mutant were cultivated as reported previously [19]. Mycelia were removed by centrifugation, and the resulting supernatant was analyzed directly by HPLC.

HPLC and LC-MS Analyses

The culture filtrates of wild-type and mutant strain were subjected to HPLC and LC-MS analyses with a 5 μm Discovery HS C18 reverse phase column (4.6 × 250 mm, Supelco, Belefonte, PA) and a methanol:water (containing 0.05% formic acid) gradient from 10:90 to 90:10 over 40 min at 1.0 ml/min flow rate. Hygromycin A and related compounds were detected at 272 nm and by MS analyses with a Perkin-Elmer SCIEX API 2000 pneumatically assisted electrospray triple quadrapole mass spectrometer.

Purification and Characterization of Hygromycin A Analogs Produced by the *S. Hygroscopicus* SCH30 Mutant

Three hygromycin A related products observed in the culture filtrate of the SCH30 mutant were purified by semipreparative HPLC with a C18 reverse-phase column (250 × 10.0 mm, 5 μm, Phenomenex, Torrance, CA) with the same solvent gradient as for analytical column and a flow rate of 3 ml/min. The three purified products were characterized by MS (see above) and NMR analyses. ^{1}H-NMR spectra were obtained on a Varian Inova 400 MHz spectrometer with a Sun Microsystems Ultra 1 processor and VNMR version 5.1c software. ^{13}C-NMR spectra were obtained on an AXZ 400 MHz Brucker spectrometer.

5″-Dihydrohygromycin A (3)

M/z 514 $(M + H)^+$; ^{1}H-NMR (CD_3OD, 400MHz) δ 7.26 (1H, d, *J* = 0.8, H-3′), 7.18 (1H, dd, *J* = 8.4, 1.6, H-9′), 6.91 (1H, d, *J* = 1.6, H-5′), 6.86 (1H, d, *J* = 8.4, H-8′), 5.44 (1H, d, *J* = 4.4, H-1″), 5.24, 4.80 (2H, d, *J* = 1.2, -OCH_2O-), 4.51 (1H, m, H-2), 4.1–4.3 (5H, m, H-2″, H-3″, H-4, H-5, H-6), 3.97 (1H, t, *J* = 6.8, H-3), 3.79 (2H, m, H-1, H-5″), 3.63 (1H, m, H-4″), 2.13 (3H, d, *J* = 0.8, α-CH_3), 1.21 (3H, d, *J* = 6.4, H-6″); ^{13}C-NMR (CD_3OD, 100 MHz) δ 172.63, 149.08, 146.31, 135.05, 132.95, 132.17, 122.64, 119.07, 118.18, 103.76, 96.23, 87.65, 79.07, 78.22, 75.79, 72.63, 71.54, 71.33, 69.29, 50.26, 19.32, 14.63.

5″-Dihydromethoxyhygromycin A (4)

M/z 516 $(M + H)^+$; ^{1}H-NMR (CD_3OD, 400MHz) δ 7.24 (1H, d, *J* = 0.8, H-3′), 7.18 (1H, dd, *J* = 8.4, 1.6, H-9′), 6.92 (1H, d, *J* = 1.6, H-5′), 6.87 (1H, d, *J* = 8.4, H-8′), 5.44 (1H, d, *J* = 4.4, H-1″), 4.63 (1H, t, *J* = 4.0, H-2), 4.25 (1H, t, *J* = 8.0, H-3″), 4.16 (1H, dd, *J* = 8.0, 4.4, H-2″) 4.0–4.1 (4H, m, H-1, H-3, H-4, H-6), 3.79 (1H, dq, *J* = 6.4, H-5″), 3.74 (1H, m, H-5), 3.62 (1H, dd, *J* = 8.0, 6.4, H-4″), 3.52 (3H, s, -OCH_3), 2.13 (3H, d, *J* = 0.8, α-CH_3), 1.21 (3H, d, *J* = 6.4, H-6″); ^{13}C-NMR (CD_3OD, 100 MHz) δ 149.10, 146.35, 135.09, 132.96, 132.23, 122.68, 119.10, 118.22, 103.79, 87.68, 79.10, 75.82, 73.27, 69.31, 49.91, 19.34, 14.73.

(E)-3-(3-Hydroxy-4-O-α-Fucofuranosylphenyl)-2-Methylacrylic Acid (5)

M/z 363 $(M + Na)^+$; ^{1}H-NMR (CD_3OD, 400 MHz) δ 7.30 (1H, d, *J* = 0.8, H-3′), 7.18 (1H, dd, *J* = 8.4, 1.6, H-9′), 6.94 (1H, d, *J* = 1.6, H-5′), 6.89 (1H, d, *J* = 8.4, H-8′), 5.45 (1H, d, *J* = 4.4, H-1″), 4.24 (1H, dd, *J* = 8.4, 7.2, H-3″), 4.15 (1H, dd, *J* = 8.4, 4.4, H-2″), 3.79 (1H, dq, *J* = 6.4, 5.6, H-5″), 3.63 (1H, dd, *J* = 7.2, 5.6, H-4″), 2.08 (3H, d, *J* = 0.8, α-CH_3), 1.20 (3H, d, *J* = 6.4, H-6″); ^{13}C-NMR (CD_3OD, 100 MHz) δ 149.06, 146.73, 139.98, 132.71, 131.32, 129.70, 123.17, 118.97, 118.44, 103.64, 87.71, 79.11, 75.82, 69.37, 19.31, 14.32.

Reduction and Hydrolysis of Hygromycin A

1.15 g (2.25 mmol) of hygromycin A was dissolved in water (8.5 ml) and cooled to 0°C. The solution was treated with $NaBH_4$ (85 mg, 2.25 mmol, one equiv.) dissolved in water (1.5 ml). The reaction was allowed to warm to room temperature, and the disappearance of hygromycin A (complete within 3 hr) was monitored by HPLC. A portion (2.5 ml) of the diastereomeric mixture of products (1:1 mixture of 5″-epimers) was purified by the semipreparative HPLC conditions described above. MS and ^{1}H NMR analysis were consistent with this being a diastereomeric mixture of 5″-dihydrohygromycin A (**3**). Another portion (2.5 ml) of this diastereomeric mixture of **3** was treated with 1.5 ml aqueous NaOH solution, to give a final NaOH concentration of 6 M, under reflux conditions for 6 hr. HPLC analysis revealed that the two peaks for the 5″ diastereomers of **3** were lost. Two new peaks were observed, and these were purified by semipreparative HPLC, and MS and ^{1}H NMR analyses were consistent with a diastereomeric mixture of (*E*)-3-(3-hydroxy-4-*O*-α-fucofuranosylphenyl)-2-methylacrylic acid (**5**). The final portion (5 ml) of the remaining diastereomeric mixture of **3** was cooled to 0°C, treated with 0.3 ml concentrated HCl, and stirred overnight at 50°C. The hygromycin A aglycone (**6**, (*E*)-3-(3,4-dihydroxyphenyl)-2-methyl-*N*-(2L-2-amino-2-deoxy-4,5-*O*-methylene-*neo* inositol acrylamide) (Figure 1) was precipitated by cooling the reaction mixture to 0°C and subsequently filtering and rinsing with cold acetone. LC-MS analysis of the product was a single peak with the predicted molecular mass (m/z 368 $[M + H]^+$).

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Accession Numbers

The sequence reported here has been deposited in the GenBank database under the accession number DQ314862.