

Structure-activity Relationships of Hygromycin A and Its Analogs: Protein Synthesis Inhibition Activity in a Cell Free System

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Several analogs of hygromycin A were tested in an *Escherichia coli* cell free protein synthesis inhibition assay and in a *Serpulina hyodysenteriae* whole cell assay. The aminocyclitol moiety is essential for antibacterial activity in both cell free and whole cell assays. However a 4'-*O*-allyl ether of hygromycin A aglycone showed an equivalent MIC to hygromycin A, while having a less potent IC₅₀ in the cell free assay. Hence 6-deoxy-5-keto-D-arabino-hexofuranose can be replaced by a hydrophobic allyl group and still retain antibacterial activity. However, this replacement reduces the intrinsic protein synthesis inhibition activity. The loss of intrinsic activity with replacement by the allyl group may be compensated for by better transport into the bacterial cell. In addition to the SAR analysis, we demonstrated that the ineffectiveness of hygromycin A against Gram-negative enteric bacteria such as *Escherichia coli* is mainly due to the efflux mechanism (Acr A/B pump) existing widely among the enteric bacteria rather than the impermeable barrier of the outer membrane.

Hygromycin A is a fermentation derived natural product first isolated from *Streptomyces hygroscopicus* in 1953^{1,2)}. The mode of action of hygromycin A is peptidyltransferase inhibition and the compound shares the same binding site on the ribosome as chloramphenicol³⁾. The antibiotic possesses moderate antibacterial activity against human pathogens¹⁾. Excellent *in vitro* potency has been reported⁴⁾ against *Serpulina (Treponema) hyodysenteriae*, the causative agent of swine dysentery, an economically significant muco-hemorrhagic disease of swine. Furthermore the antibiotic also demonstrates efficacy in the treatment of an induced dysentery infection model of swine at a level of 5~20 g/ton feed⁵⁾. These results renewed our interest in hygromycin A and led to a semi-synthetic program based on this compound^{6~12)}.

In the present paper, protein synthesis inhibition activities of several hygromycin A analogs have been studied in a cell free system (Zubay's transcription dependent translation system) to analyze their structure activity relationships. These analogs include hygromycin A aglycone (CP-97,523), 5''-dihydrocinnamide (CP-108,072), 5''-dihydro- α -propylhygromycin A (CP-111,907), 4'-*O*-allyl aglycone (CP-111,905), 3'-amino-4'-*O*-allyl aglycone (CP-113,056), 5'-*O*-methyl-4'-*O*-allyl aglycone (CP-119,755), hygromycin A aglycone-4''-*O*-arabinopyranoside (CP-117,542), and dichloroacetamide of aminocyclitol (CP-117,986). The analogs were also tested in a *S. hyodysenteriae* whole cell assay to determine

MICs and the active analogs among them were further studied in an antibacterial spectrum assay.

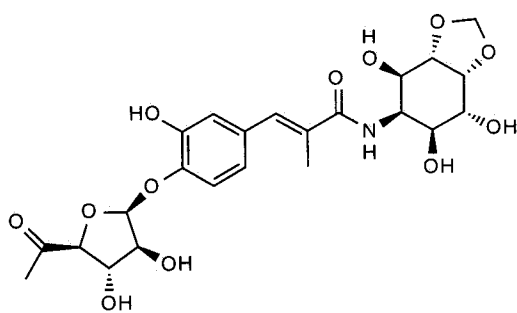
We also demonstrated that the lack of anti-Gram-negative enteric activity of hygromycin A is caused by Acr A/B efflux pump mechanism existing widely among these bacteria.

Materials and Methods

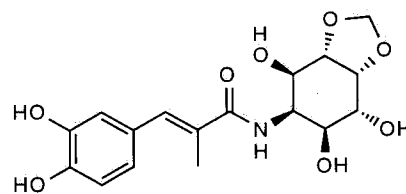
Bacterial Strains

Escherichia coli K12 D10 (*met*, *rna*) was used for the S-30 and chromosomal DNA preparation. *E. coli* W4680 (F⁻ Δ lacZ39, *rpsL45*, *rpsL110*, *melB4*) and WZM120 (same as W4680; *lacrAB::Tn903kan^r*) were obtained from J. E. HEARST, University of California, Berkeley¹³⁾. *S. hyodysenteriae* 94A002 (Pfizer collection) was used for the MIC microdilution assay. The following bacterial strains were used in the antibacterial spectrum agar dilution assay: *Bordetella bronchiseptica* 73A009 (swine isolate), *E. coli* 51A538 (bovine isolate), *Pasteurella haemolytica* 59B018 (bovine isolate), *P. multocida* 59A006, *Salmonella choleraesuis* 58B015 (swine isolate), *S. typhimurium* LT2 SGSC230 (RD2 LPS deep rough mutant), *Staphylococcus aureus* 01A539 (bovine isolate), *Actinomyces pyogenes* 14D002 (swine isolate), *Bacteroides vulgatus* 78E029, *Clostridium perfringens* 10A009 (ATCC strain #3626), and *S. hyodysenteriae* 94A007 (swine isolate, erythromycin resistant).

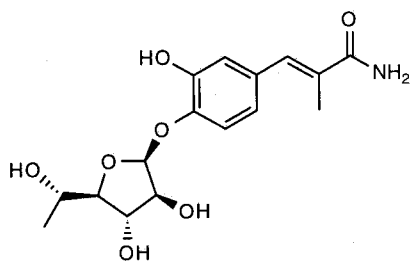
Fig. 1. Structures of hygromycin A analogs.



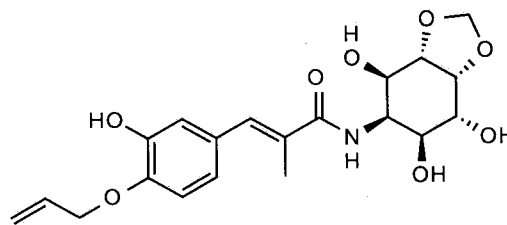
Hygromycin A



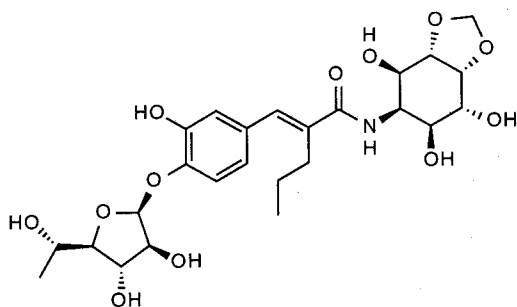
CP-97,523



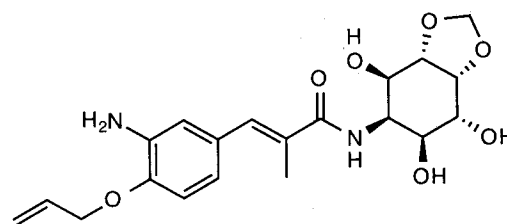
CP-108,072



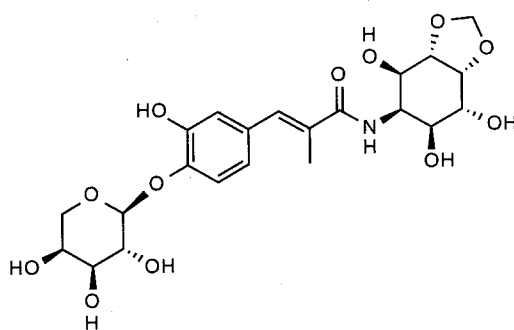
CP-111,905



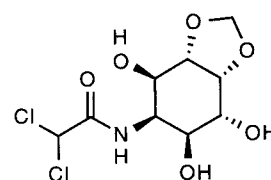
CP-111,907



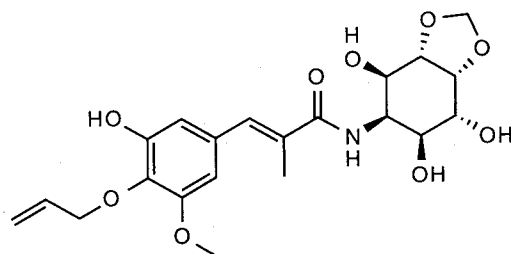
CP-113,056



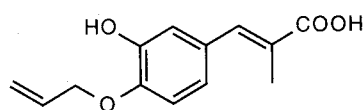
CP-117,542



CP-117,986



CP-119,755



CP-123,800

Antibiotics

Hygromycin A analogs tested in this study were supplied from in-house sample bank. The synthetic methods for these analogs were described in references 6 to 12. Streptomycin, chloramphenicol, novobiocin and erythromycin were commercially obtained (Sigma).

Cell Free Protein Synthesis Inhibition Assay (PSI Assay)

A DNA dependent transcription/translation system (Zubay's system) was used to test the intrinsic protein synthesis inhibition activity of hygromycin A analogs.

1. S-30 preparation: The preparation of S-30 has been previously reported¹⁴⁾. *E. coli* K-12 D10 was grown in 3 liters of L broth supplemented with 0.2% glucose. About 10 g of frozen cells were ground at 4°C with 15 g of alumina and extracted with 20 ml of TKM buffer [10 mM Tris-acetate buffer pH 8.2 containing 14 mM magnesium acetate, 60 mM potassium acetate and 0.1 mM dithiothreitol (DTT)]. The suspension was centrifuged at 10,000 × *g* for 10 minutes and the supernatant was further centrifuged at 30,000 × *g* for 1 hour. The supernatant fraction was treated with 0.5% octylglucoside for 15 minutes on ice, and dialyzed against TKM buffer overnight. The supernatant was aliquoted and frozen at -70°C as S-30 solution.

2. DNA preparation: 500 ml of a fully grown *E. coli* K-12 D10 culture was used for chromosomal DNA preparation. DNA was prepared by standard methods¹⁵⁾. After CsCl centrifugation, DNA was precipitated by ethanol, rinsed several times with 70% ethanol and dissolved in TE buffer.

3. Protein synthesis inhibition assay: *In vitro* protein synthesis with S-30 was carried out by the method described by BRUSLOW *et al.*¹⁶⁾. A typical 400 μl reaction mixture contained; 58 mM of phosphoenolpyruvate, 25 μg of creatine kinase, 19 mM of creatine phosphate, 11.6 μg each of NAD and FAD, 2.4 mM ATP, 0.6 mM each of CTP, UTP, GTP, 42 μg of *E. coli* tRNA, 14 μg of folinic acid, 1 mM DTT, 1.2 mM each of 19 amino acids, 108 mM of Tris-acetate (pH 8.2), 137 mM potassium acetate, 72 mM of ammonium acetate, 7 mM of magnesium acetate, 3.7 mM calcium acetate, 10 μg of *E. coli* chromosomal DNA, 30 ~ 40 μCi of [³⁵S]methionine (sp. act. 1000 Ci/mmole from ICN), 2.9 μg of p-APMSF and S-30 (2 mg of protein). After mixing well, a 20 μl of aliquot was placed in an eppendorf tube and 5 μl of the various concentrations of drug solution was added. The eppendorf tubes were incubated at 40°C for 1 hour. 20 μl of non-radioactive methionine solution (200 mg/ml) was

added to each tube and further incubated for 5 minutes. The sample was heated at 90°C for 5 minutes after the addition of 10 μl of 10% SDS solution in order to stop the reaction. 20 μl of the heated sample was applied to a small piece of filter paper (Whatman 3 MM) and dried at room temperature. The filter papers were individually dropped into a 10% cold TCA (trichloroacetic acid) solution containing 0.1% methionine. After 1 hour with occasional swirling, filter papers were washed 3 ~ 4 times with 5% TCA-0.1% methionine solution. After drying, the radioactivity of each filter paper was counted on a liquid scintillation counter. After plotting radioactivity against the various concentrations of test compound, the concentration of test compound inhibiting the incorporation of radioisotope into protein (TCA precipitates), was determined. The IC₅₀ was determined as the drug concentration which inhibited 50% of protein synthesis (50% of [³⁵S] methionine incorporation into TCA precipitate).

Anti-*S. hyodysenteriae* Microdilution Assay

The assay method used here was reported previously and was based on the measurement of *S. hyodysenteriae* hemolysin production which was well correlated to bacterial growth¹⁷⁾. A hygromycin A analog was considered active if it inhibited the *S. hyodysenteriae*-mediated hemolysis of red blood cells by 50% or more. In this assay, BHI (Brain heart infusion; Difco) medium was supplemented with cysteine-HCl (0.1%) as a reducing agent, resazurin as a redox indicator (0.0001%), fetal bovine serum (12%), and washed bovine red blood cells (7%). The compound solution was serially diluted two fold by automatic pipette in a 96 well microtiter format. After inoculation with *S. hyodysenteriae* (final cell density was approximately 10⁶ CFU/ml), the microtiter plates were incubated anaerobically at 39°C for 24 hours in an anaerobic chamber (Coy Laboratory). The minimum inhibition concentration (MIC) was determined as the concentration of the first well showing activity.

Anti-bacterial Spectrum Agar Dilution Assay

Seven aerobic animal pathogens and four anaerobic animal pathogens were tested. BHI agar and 5% bovine blood tryptose agar were used for the aerobic bacteria and anaerobic bacteria, respectively. The test compound was first dissolved into 1 ml absolute ethanol, then BHI broth was added to adjust to the appropriate starting concentration of each test compound. This original solution was serially diluted two-fold and 1 ml of each

dilution solution was mixed well with molten agar medium and solidified to make a series of drug containing plates. The precultures of all the test bacteria were re-inoculated into fresh media on the morning of the assay and were inoculated onto the drug-containing plates using a Steer's replicator after adjusting their OD_{600nm} to 0.2. The BHI plates inoculated with aerobic bacteria were incubated at 37°C overnight and the blood plates with anaerobic bacteria were incubated in an anaerobic chamber (N_2 80%, H_2 10%, and CO_2 10%) at 39°C for 48 hours. The MICs were determined as the lowest concentration of test compound which inhibited growth completely.

Anti-*E. coli* Microdilution Assay

The isogenic *E. coli* strains W4680 and WZM120 (Δ acrAB) were used in this assay to test the effect of AcrA/B deletion mutation on hygromycin A susceptibility using erythromycin, novobiocin and chloramphenicol as controls. Both strains were grown on BHI plate overnight. Several colonies were suspended into saline and adjusted to $OD_{625nm}=0.09$ (0.5 McFarland unit). The inoculum solution was made by preparing a 1:100 dilution of 0.5 McFarland saline suspension using cation adjusted Mueller-Hinton (CAMH) broth and 100 μ l of this suspension was added to 100 μ l of CAMH broth containing various concentrations of test antibiotics. The test antibiotic solution was serially diluted two-fold by automatic pipette in a 96 well microtiter format (starting at 4000 μ g/ml for hygromycin A and 400 μ g/ml for controls). After inoculation with both strains (final density was approximately 5×10^5 CFU/ml), the microtiter plates were incubated at 37°C for 18 hours. The minimum inhibition concentration (MIC) was determined as the lowest concentration of the test compound in which the absorbance at 600nm is less than or equal to 0.025.

Results

The structure of the hygromycin A analogs tested in the PSI assay are listed in Fig. 1. These include hygromycin A aglycone (CP-97,523), 5''-dihydrocinnamide (CP-108,072), 5''-dihydro- α -propylhygromycin A (CP-111,907), 4'-O-allyl aglycone (CP-111,905), 3'-amino-4'-O-allyl aglycone (CP-113,056), 5'-O-methyl-4'-O-allyl aglycone (CP-119,755), hygromycin A aglycone-4''-O-arabinopyranoside (CP-117,542), and dichloroacetamide of aminocyclitol (CP-117,986). In addition to the above analogs, chloramphenicol and streptomycin

Table 1. Protein synthesis inhibition activity (IC_{50}) with *E. coli* S-30 and anti-*S. hyodysenteriae* activity (MIC) of hygromycin A analogs.

Compound	IC_{50} of cell free protein synthesis inhibition (μ M)	Anti- <i>S. hyodysenteriae</i> activity MIC (μ g/ml)
Streptomycin (control)	1.25	25
Chloramphenicol (control)	7.5	0.78
Hygromycin A	0.6 +/- 0.4	0.78
CP-97,523	>1400	>200
CP-108,072	>1400	200
CP-111,905	28	0.78
CP-113,056	64	0.78
CP-119,755	>1400	>200
CP-111,907	250	>200
CP-117,542	>1400	>200
CP-117,986	>1400	>200

were tested as controls. These analogs and controls were also tested against *S. hyodysenteriae* whole cell assays to obtain anti-*S. hyodysenteriae* activity (MICs). The results of both PSI assays (IC_{50}) and whole cell assays (MIC) are presented in Table 1.

The IC_{50} s of hygromycin A and chloramphenicol were 0.6 μ M and 7.5 μ M, respectively and hygromycin A was about ten times more potent than chloramphenicol. MICs of both antibiotics against *S. hyodysenteriae* were the same in the microdilution assay (0.78 μ g/ml) or very similar in the agar dilution assay (1.56 μ g/ml and 0.78 μ g/ml, Table 2). Hygromycin A did not show any activity against *E. coli* and *Salmonella*, but chloramphenicol does (Table 2).

Of 8 hygromycin A analogs tested, only three showed activity in the PSI assay: CP-111,905, CP-113,056 and CP-111,907. Their IC_{50} s were 28 μ M, 64 μ M and 250 μ M, respectively, and were much higher than that of parent compound, hygromycin A (0.6 μ M). Of the three analogs, CP-111,907 did not show anti-*S. hyodysenteriae* activity and CP-111,905 and CP-113,056 showed equivalent or better anti-*S. hyodysenteriae* activity to hygromycin A (MICs were 0.78 μ g/ml and 0.2 μ g/ml, respectively) in spite of their higher IC_{50} values.

Both active analogs (CP-111,905 and CP-113,056) were tested against a panel of animal pathogens in order to study their antibacterial spectrum with hygromycin A and chloramphenicol as controls. The results are presented in Table 2. Hygromycin A and its analogs possess moderate antibacterial spectrum against several animal pathogens but lack anti-G⁻fam-negative enteric

Table 2. Anti-bacterial spectrum of hygromycin A analogs.
(Agar dilution method)

	MIC ($\mu\text{g/ml}$)			
	Chloramphenicol	Hygromycin A	CP-111,905	CP-113,056
Aerobes				
<i>Bordetella bronchiseptica</i>	12.5	> 400	50	50
<i>Escherichia coli</i>	6.25	> 400	> 100	> 100
<i>Pasteurella haemolytica</i>	0.78	12.5	6.25	25
<i>Pasteurella multocida</i>	< 0.39	1.56	3.13	3.13
<i>Salmonella choleraesuis</i>	1.56	> 400	> 100	> 100
<i>Salmonella typhimurium</i>	6.25	> 400	> 100	> 100
<i>Staphylococcus aureus</i>	1.56	25	25	25
Anaerobes				
<i>Actinomyces pyogenes</i>	1.56	100	1.56/3.13	1.56
<i>Bacteroides vulgatus</i>	1.56	25	12.5	12.5
<i>Clostridium perfringens</i>	3.13	3.13/6.25	1.56/3.13	3.13
<i>Serpulina hyodysenteriae</i>	0.78	1.56/3.13	0.78	< 0.2

Table 3. The effect of $\Delta\text{acrA/B}$ mutation on MICs of hygromycin A.

Antibiotics	<i>E. coli</i> W4680 (parent strain)	<i>E. coli</i> WZM120 ($\Delta\text{acrA/B}$)	Ratios of MIC of parent/ $\Delta\text{acrA/B}$
Hygromycin A	1000 $\mu\text{g/ml}$	15.6 $\mu\text{g/ml}$	64
Erythromycin	100 $\mu\text{g/ml}$	3.13 $\mu\text{g/ml}$	32
Novobiocin	200/400 $\mu\text{g/ml}$	3.13 $\mu\text{g/ml}$	64/128
Chloramphenicol	6.25 $\mu\text{g/ml}$	0.78/1.56 $\mu\text{g/ml}$	4/8

activity. In contrast, chloramphenicol, which is a less potent protein synthesis inhibitor than hygromycin A, showed excellent broad spectrum as well as potency.

The antibacterial activity of hygromycin A (MIC) was measured against AcrA/B deletion mutant (WZM120 ΔacrAB) and its isogenic parent strain (W4680). Erythromycin, novobiocin and chloramphenicol were used as controls. The results are presented in Table 3. The significant reduction in MICs of both erythromycin and novobiocin (32 to 128 fold) were observed in AcrA/B deletion mutant (WZM120) compared to its parent strain (W4680) as reported previously¹³. The MIC of hygromycin A was equally reduced in the ΔacrAB mutant strain (MIC = 15.6 $\mu\text{g/ml}$ vs. 1000 $\mu\text{g/ml}$, 64 fold reduction). These results indicate that hygromycin A is a substrate of the AcrA/B efflux pump.

Discussion

Structure Activity Relationships

The following structure-activity relationships of hygromycin A were derived from the *in vitro* cell free PSI and whole cell assays:

1. The aminocyclitol portion of hygromycin A is

essential for antibacterial activity as well as cell free PSI activity (CP-108,072) but the aminocyclitol itself does not have any biological activity (CP-117,986). These results confirm the SAR obtained in the semisynthetic program^{8,10}. It was also demonstrated that *cis-cis*-2,6-dihydroxycyclohexamide instead of aminocyclitol is enough to gain moderate antibacterial activity albeit the intact aminocyclitol gave the best antibacterial activity¹⁰. The latter study to explore the aminocyclitol region was carried out using 4'-O-allyl aglycone of hygromycin A instead of hygromycin A itself. Therefore there is a possibility that the aminocyclitol is essential only in combination with 6-deoxy-5-keto-D-arabino-hexofuranose moiety; 4'-allyloxy-3'-hydroxy-2-methylcinnamic acid (CP-123,800, Fig. 1) may have antibacterial activity by itself without aminocyclitol. This possibility was excluded by the following experiment: the whole cell assays of CP-123,800 against both *S. hyodysenteriae* and *P. multocida* showed that this analog did not possess any antibacterial activity (MICs were over 200 $\mu\text{g/ml}$ against both bacteria). These results are very consistent with that of CP-108,072 and the SAR of aminocyclitol established in 4'-O-allyl analogs may equally be applicable to hygromycin A.

2. The analog lacking 6-deoxy-5-keto-D-arabino-hexofuranose moiety at the 4' position (CP-97,523) did not show any antibacterial activity or PSI activity. Replacing 6-deoxy-5-keto-D-arabino-hexofuranose with arabino-hexopyranose (CP-117,542) at the 4' position did not regain antibacterial activity *in vitro* or in the cell free system (MIC > 200 $\mu\text{g/ml}$ and IC₅₀ > 1400 μM). The lack of antibacterial activity of CP-117,542 may be originated from the difference in the stereochemistry of sugar moiety. In addition to the different ring size, the stereochemistry of both hydroxyl groups at 2'' and 3'' is opposite from that of the naturally occurring 6-deoxy-5-keto-D-arabino-hexofuranose (Fig. 1).

However, 6-deoxy-5-keto-D-arabino-hexofuranose moiety can be replaced by an allyl group without losing antibacterial activity (CP-111,905). The extensive study on 4' position of hygromycin A by semi-synthetic approach and anti-*S. hyodysenteriae* whole cell assay revealed that small lipophilic groups, which are less than five methylene groups, lead to the most active 4' substituted analogs and longer aliphatic chain substitutions at this position reduced antibacterial activity^{7,11,12}.

3. The OH group at the 3' position of the allyl analog (CP-111,905) can be replaced by an NH₂ without losing *in vitro* antibacterial activity (CP-113,056, MIC 0.78 $\mu\text{g/ml}$). The replacement with other group such as hydrogen, methyl, and *O*-methyl reduced *in vitro* antibacterial activity⁷. However, the intrinsic protein synthesis inhibition activity of CP-113,056 seems to be less than that of CP-111,905 (IC₅₀, 64 μM vs. 28 μM). The 5' position replacement by an *O*-methyl group (CP-119,755) totally abolished the antibacterial activity in both *in vitro* and the cell free system. These results seem to indicate that the 3' and 5' position of the allyl analog of hygromycin A have certain steric restrictions.

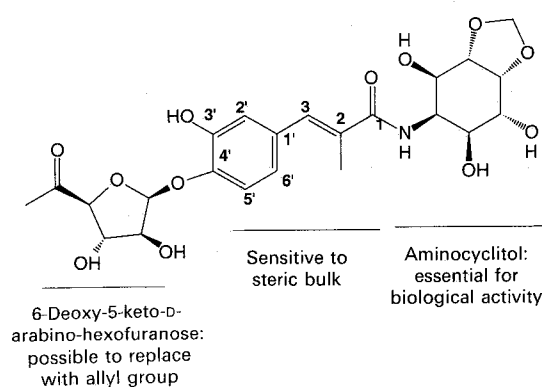
Similar steric restriction has also been observed at the 2 position of hygromycin A. The replacement of the methyl group at the 2 position with a propyl group (CP-111,907) led to a loss of *in vitro* antibacterial activity (MIC > 200 $\mu\text{g/ml}$) and significantly reduced intrinsic PSI activity (IC₅₀ = 250 μM). Only replacement with an ethyl group ensured the same *in vitro* antibacterial activity and replacement with other groups such as hydrogen and allyl reduced the antibacterial activity⁶.

4. The IC₅₀ values of both CP-111,905 and CP-113,056 were much higher than the parent compound, hygromycin A (28 μM , 64 μM vs. 0.6 μM , respectively) yet both analogs showed equivalent or better anti-*S. hyodysenteriae* activity to that of hygromycin A (0.78 $\mu\text{g/ml}$ and < 0.2 $\mu\text{g/ml}$). The discrepancy between the

equivalent susceptibility against *S. hyodysenteriae* and the difference in intrinsic PSI activity may be explained by superior transport of both analogs into the *S. hyodysenteriae* cells due to the replacement of 6-deoxy-5-keto-D-arabino-hexofuranose moiety by allyl group. There is another possibility that the *S. hyodysenteriae* ribosome may be different from *E. coli*, especially the peptidyltransferase center region. This difference may affect the binding of the antibiotics. In *S. hyodysenteriae*, hygromycin A, CP-111,905 and CP-113,056 may bind equally to peptidyltransferase center and may inhibit the protein synthesis equally, leading to nearly equal MIC values. This alternative can be answered only when both analogs as well as hygromycin A can be tested in a *S. hyodysenteriae* cell free PSI system. However, cell free PSI systems are less well established with *S. hyodysenteriae* than *E. coli* and *Bacillus subtilis*.

5. Replacement of 6-deoxy-5-keto-D-arabino-hexofuranose moiety by an allyl group did not affect the antibacterial spectrum significantly compared with its parent compound, hygromycin A except against *A. pyogenes* (Table 2). CP-111,905 showed better potency against this bacterium (> 25 $\mu\text{g/ml}$ vs. 3.13 $\mu\text{g/ml}$). CP-113,056 is the analog of CP-111,905 with the OH group at 3' replaced by an NH₂ group. This compound showed less potency against *P. haemolytica* and *A. pleuropneumoniae* and slightly increased potency against *S. hyodysenteriae* (Table 2). The overall structure-activity relations based on PSI assay and whole cell assay have been summarized in Fig. 2. These conclusions were consistent with the SAR reported previously based on the whole cell assays with over one hundred analogs synthesized^{6~12}.

Fig. 2. The summary of the structure activity relation of hygromycin A.



Hygromycin A Transport through Cell Membranes of *E. coli*

It has been shown that hygromycin A is a peptidyl-transferase inhibitor and strongly inhibits the binding of [¹⁴C]chloramphenicol and [¹⁴C]lincomycin to ribosomes suggesting that the binding sites of these antibiotics are very closely related³⁾. In the same paper, the authors reported that hygromycin A was more effective than chloramphenicol in inhibiting poly(U)-directed synthesis of polyphenylalanine³⁾. A similar result was obtained in our PSI assay based on natural DNA derived from *E. coli* and Zubay's S-30 lysate of *E. coli* (Table 1) where hygromycin A was about ten times more potent than chloramphenicol. However, this potency did not translate to the whole cell assay (MICs) and hygromycin A was inactive against *E. coli* and *Salmonella* but chloramphenicol showed moderate activity (MICs 1.56 µg/ml and 6.25 µg/ml, Table 2).

These discrepancies may be explained by the difference in transport efficiency of both antibiotics into bacterial cells. There are three factors influencing transport of antibiotics into Gram-negative bacterial cells. These are the outer membrane barrier, the efflux pump mechanism and the inner (cytoplasmic) membrane barrier. It is well known that enteric Gram-negative bacteria such as *E. coli* and *Salmonella* have a less permeable outer membrane compared with other Gram-negative bacteria such as *Pasteurella*. This rigid outer membrane is a barrier against certain types of antibiotics such as bulky macrolides and hydrophobic fusidic acid. The *S. typhimurium* LT2 strain used in our spectrum assay is a deep rough mutant of lipopolysaccharide (LPS) lacking O-side chain, outer core and one heptose in the diheptose region. This strain is more susceptible to the antibiotics mentioned above. Historically MICs of macrolides against this strain are equivalent or 2~4 times higher than against *P. multocida*. However, the lack of this enhancement in susceptibility of hygromycin A against *Salmonella* deep rough mutant strain indicates that the outer membrane permeability barrier may not be the major reason for ineffectiveness of hygromycin A against *E. coli* and *Salmonella*. This hypothesis is further supported by the facts that hygromycin A is a hydrophilic compound and its molecular weight is 511 dalton, which is small enough to pass through porins (OmpC and OmpF) by passive diffusion¹⁸⁾.

Recently the universal efflux pump was identified in *Pseudomonas aeruginosa* (Mex A/B¹⁹⁾) as well as in *E. coli* (Acr A/B²⁰⁾). Many antibiotic classes can be substrates for these pumps, which provides *P. aeruginosa*

and *E. coli* an intrinsic antibiotic resistance^{19,20)}. It was reported that the newly developed oxazolidinone antibiotics are a substrate of Acr A/B efflux pump in *E. coli* and their lack of anti-Gram-negative aerobic activity is caused by this efflux mechanism²¹⁾. Oxazolidinones showed significantly improved susceptibility against Δ acrA/B *E. coli* mutant (MIC = 4 µg/ml) compared to its parent strain (MIC > 128 µg/ml²¹⁾). There is a possibility that hygromycin A is a substrate of this efflux pump likewise. In order to test this hypothesis, the antibacterial activity of hygromycin A was measured against Acr A/B deletion mutant (WZM120 Δ acrA/B) and its isogenic parent strain (W4680). As presented in Table 3, MIC of hygromycin A against WZM120 is significantly improved compared to its isogenic parent strain (15.6 µg/ml vs. 1000 µg/ml, 64 folds). These results indicate that hygromycin A is also a substrate of this efflux pump and the ineffectiveness of hygromycin A against enteric Gram-negative bacteria *E. coli* and *Salmonella* is mainly caused by this universal efflux pump.

The MIC of chloramphenicol was also reduced in the Δ acrA/B mutant strain but the degree of reduction in MIC was much less than for erythromycin, novobiocin and hygromycin A (4~8 fold). Chloramphenicol may be a less favorable substrate for the Acr AB efflux pump. Hygromycin A is still less potent against Δ acrA/B mutant than chloramphenicol (15.6 µg/ml vs. 0.78/1.56 µg/ml, 10~20 fold) in spite of its 10-fold higher potency in the cell free system. One explanation could be that hygromycin A passes through the cytoplasmic (inner) membrane less efficiently than chloramphenicol in enteric Gram-negative bacteria, in which case lower permeability through the cytoplasmic membrane and significant efflux activity could combine to render hygromycin A totally inactive against enteric Gram-negative bacteria.

In contrast with the enteric Gram-negative bacteria, hygromycin A showed moderate antibacterial activity against both respiratory Gram-negative bacteria *P. haemolytica* and *P. multocida* (12.5 µg/ml and 1.56 µg/ml, Table 2). These respiratory Gram-negative bacteria may not possess Acr A/B type universal efflux pump, or hygromycin A may not be a substrate for the efflux pump of these bacteria due to different substrate specificity.

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