

**Enzymatic and applied studies on microbial hydrolases
acting on carbon-nitrogen bonds
for pharmaceutical intermediates production**

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ABBREVIATIONS

¹ H-NMR	proton nuclear magnetic resonance
¹³ C-NMR	carbon-13 nuclear magnetic resonance
BLASTP	protein-protein Basic Local Alignment Search Tool
CAPS	<i>N</i> -cyclohexyl-3-aminopropanesulfonic acid
CFE	cell-free extracts
C-N	carbon-nitrogen
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization mass spectrometry
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-thiogalactopyranoside
KPB	potassium phosphate buffer
LB	Luria Bertani
MRS	de Man, Rogosa and Sharpe
MS	mass spectrometry
NH	nucleoside hydrolase
<i>N</i> -MH	<i>N</i> -methylhydantoinase
OD	optical density
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TLC	thin layer chromatography
UV	ultraviolet

2'-OMe-6ClGR	2'-O-methyl-6-chloroguanosine
2'-OMe-AR	2'-O-methyladnosine
2'-OMe-CR	2'-O-methylcytidine
2'-OMe-GR	2'-O-methylguanosine
2'-OMe-IR	2'-O-methylinosine
2'-OMe-NRs	2'-O-methylribonucleosides
2'-OMe-ribose	2'-O-methylribose
2'-OMe-TR	2'-O-methylthymidine
2'-OMe-UR	2'-O-methyluridine
2'-OMe-XR	2'-O-methylxanthosine
NDI	<i>cis</i> -5-norbornene- <i>exo</i> -2,3-dicarboximide
NDM	<i>cis</i> -5-norbornene- <i>exo</i> -2,3-dicarboxylic acid monoamide

GENERAL INTRODUCTION

Carbon-nitrogen (C-N) bonds are usually found in the molecular structure of many medicines, such as amine functional group, amide bond, imide bond and *N*-glycosidic bond. Various enzymatic reactions acting on C-N bonds are used for the formation of C-N bonds in low-molecular drugs and pharmaceutical intermediates. For example, vidarabine is a nucleoside analogue used as an anticancer drug and produced via the enzymatic phosphorolysis of *N*-glycosidic bond in nucleosides (1). L-DOPA is an optically active amino acid used for the treatment of Parkinson's disease, and the efficient enzymatic synthesis of L-DOPA was accomplished with the reverse reaction catalyzed by tyrosine phenol-lyase (2,3). These enzymatic reactions offer several advantages such as regio- and stereo-selectivity and substrate specificity under the mild conditions over chemical reactions. Therefore, the exploitation and the utilization of enzymes acting on C-N bonds are expected to contribute the development of novel production methods for chiral pharmaceutical intermediates.

2'-*O*-methylribonucleosides (2'-OMe-NRs) are promising raw materials of nucleic acid drugs (4-7), but enzymes acting to 2'-OMe-NRs and metabolic fates of 2'-OMe-NRs are still unknown. To exploit biocatalysts for the enzymatic synthesis of 2'-OMe-NRs, the author screened 2'-OMe-NRs-metabolizing microorganisms and investigated the microbial enzymes to be applied for the production of 2'-OMe-NRs. In Chapter I, a screening of laboratory culture collection for 2'-OMe-NRs-metabolizing microorganisms was carried out, and *Lactobacillus buchneri* LBK78 was found to hydrolyze 2'-*O*-methyluridine (2'-OMe-UR) to produce uracil and 2'-*O*-methylribose. The responsible enzyme was partially purified from *L. buchneri* LBK78 cells and identified to a novel nucleoside hydrolase termed as *LbNH*. In Chapter II, *LbNH* was found to be the first nucleoside hydrolase catalyzing transribosylation between 2'-OMe-UR and various nucleobases. The author investigated the application of *LbNH* for the enzymatic production of 2'-*O*-methyladenosine (2'-OMe-AR) via the transribosylation between 2'-OMe-UR and adenine. In Chapter III, *Agromyces* sp. MM-1 was isolated microorganisms from environmental samples and found to utilize 2'-OMe-UR as a sole carbon source. The responsible enzyme of *Agromyces* sp. MM-1 was identified to a nucleoside hydrolase family enzyme termed as *AgNH*. *AgNH* also catalyzed the transribosylation between 2'-OMe-UR and nucleobases and showed different catalytic properties from *LbNH*. Thus, the author investigated its application for the enzymatic production of 2'-*O*-methyl-2-amino-6-chloropurine riboside via

the transribosylation between 2'-OMe-UR and 2-amino-6-chloropurine.

Optically active half amide, *cis*-5-norbornene-*exo*-2,3-dicarboxylic acid monoamide (NDM), is a valuable intermediates for the synthesis of chiral drugs (8-10). Enzymatic regio-selective hydrolysis of a cyclic imide, *cis*-5-norbornene-*exo*-2,3-dicarboximide (NDI), using imidases is a promising strategy to produce optically active half amides (11). In Chapter IV, the author screened microorganisms assimilating NDI for the hydrolyzing activity towards NDI. Then, *Mesorhizobium* sp. A9-2-3 was isolated from environmental samples and found to catalyze the regio-selective hydrolysis towards NDI to produce NDM. According to the comparative proteome analysis, an imide-inducible protein was identified to a novel type of microbial imidase, termed as *MeIH*, catalyzing the regio-selective hydrolysis of NDI.

Chapter I

A novel nucleoside hydrolase from *Lactobacillus buchneri* LBK78 catalyzing hydrolysis of 2'-O-methylribonucleosides

Introduction

Nucleic acid drugs, particularly those comprising modified DNA/RNA oligomers, have recently attracted considerable attention as a next-generation medicine of low molecular mass drugs and antibody drugs. Nucleic acid drugs have been suggested for various applications including as antisense drugs, ribozymes, small interfering RNA drugs, and as aptamers (12,13). However, foreign oligonucleotides are rapidly decomposed by host nucleases and their uptake into target organs or cells is poor (14). Therefore, there is a need for modified oligonucleotides that are resistant to nucleases, in order to improve the delivery and stability of nucleic acid drugs.

2'-O-Methylribonucleosides (2'-OMe-NRs) are nucleoside analogues that have a sugar 2'-modification introduced by substituting a methoxy group for the 2'-hydroxyl group of the ribose moiety. It is known that the oligonucleotides containing 2'-OMe-NRs have high thermal stability and nuclease tolerance, and so 2'-OMe-NRs are prospective raw materials for nucleic acid drugs (4-7). To date 2'-OMe-NRs are synthesized by several chemical methods (15-17), but chemical synthesis generally requires time-consuming, expensive, and environmentally harmful multi-step processes. Synthesis of 2'-OMe-NRs also requires the use of organic solvents to protect/deprotect the nucleobase and/or pentose moiety, and to purify the desired product and remove side products, in almost every step (18,19). In contrast, biocatalytic synthesis of nucleoside analogues offers several advantages over chemical methods, such as mild reaction conditions and high stereo- and regio-selectivity (20). Although the enzymatic synthesis of 2'-OMe-NRs is a promising alternative to chemical methods, there are no reports regarding the metabolic fate and enzymes involved in the metabolism of 2'-O-alkyl nucleosides.

In the present study we carried out a comprehensive screening to identify microorganisms metabolizing 2'-OMe-NRs and determine the metabolic enzyme with catalytic activities towards 2'-OMe-NRs. *Lactobacillus buchneri* LBK78 (NITE P-01581) was found to decompose 2'-O-methyluridine (2'-OMe-UR), and the responsible enzyme was partially purified and characterized, and determined to be a novel type of nucleoside hydrolase, *LbNH*.

Materials and Methods

Analysis of nucleosides, nucleobases, and sugars

For thin-layer chromatography (TLC) analysis, 2 μ L of each sample was spotted onto a silica gel TLC plate (Kieselgel 60 F₂₅₄; Merck Millipore, MA, USA), and developed with a solvent system containing ethyl acetate/methanol/0.1N hydrochloric acid (15/2/2, v/v/v). Nucleobases and nucleosides were detected qualitatively by UV absorbance with a wavelength at 254 nm. Sugars and nucleosides were visualized by spraying the plates with *p*-anisaldehyde/acetic acid/sulfuric acid/ethanol (2/1/4/96, v/v/v/v) and heating at 105°C. For high-performance liquid chromatography (HPLC) analysis, an LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a COSMOSIL 5C₁₈-ARII column (4.6 $\times$ 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) was used for separation at 40°C. The mobile phase was 10 mM ammonium acetate (pH 4.5, eluent A) and acetonitrile (eluent B), and the flow rate was 1.5 mL/min. The eluent gradients were 0% (v/v) B for 0-5 min, 0-5% (v/v) B over 5-10 min, 5-60% (v/v) B over 10-17.5 min, and 60% (v/v) B for 17.5-22.5 min. Nucleobases and nucleosides were quantitatively detected by UV absorbance at 254 nm.

Reaction conditions for 2'-OMe-UR decomposition

For the microbial screening by the whole-cell reaction, dried cells of microorganisms from our existing laboratory stock (2-10 mg [dried weight]) were added into 200 μ L of a reaction mixture comprising 30 mM 2'-OMe-UR and 100 mM potassium phosphate (KPB) (21), pH 7.0. After cultivating at 28°C for 24 h, with shaking, the mixture was centrifuged at 20,400 $\times g$ for 15 min, and 2 μ L and 100 μ L of the supernatant was subjected to TLC and HPLC analysis, respectively. During the purification of the enzyme, 10 μ L of each protein fraction was added into 1 mL of a reaction mixture comprising 60 mM 2'-OMe-UR and 50 mM KPB, pH 7.0. After shaking at 50°C for 30 min, the mixture was centrifuged at 20,400 $\times g$ for 15 min and the supernatant was subjected to TLC and HPLC analysis.

Purification and N-terminal amino acid sequence analysis of the enzyme

L. buchneri LBK78 was cultivated in 2 L of MRS medium (Becton, Dickinson and Company, NJ, USA) for 72 h at 28°C without shaking. Cells from 2 L of culture (6.7 g [wet weight]) were harvested by centrifugation at 15,200 $\times g$ for 20 min, washed and re-suspended with 25 mM KPB, pH 7.0 (purification buffer). The cell suspension was disrupted by ultrasonication for 40 min with an Insonator 201M (KUBOTA, Osaka, Japan). The disrupted cell suspension was centrifuged at 20,700 $\times g$ for 20 min and the resulting supernatant (14 mL) was used as the cell-free extracts (CFE). The CFE was separated by ammonium sulfate fractionation. Solid ammonium sulfate (3.4 g) was added to the CFE (14 mL), and the precipitate formed was removed by centrifugation. Additional solid ammonium sulfate (1.6 g) was added to the supernatant (12 mL),

and the precipitate with 60 % saturation of ammonium sulfate was collected by centrifugation and dissolved in purification buffer (10 mL). The resultant enzyme solution was dialyzed twice against 2 L of purification buffer and then applied to a MonoQ HR10/100 column (GE Healthcare, Buckinghamshire, UK) equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions (eluted with 0.35 to 0.4 M NaCl) were collected and concentrated by ultrafiltration using an Ultracel-10K membrane (Millipore, MA, USA) to a final volume of 0.7 mL. The concentrated sample was applied to a Superdex200 HR10/30 column (GE Healthcare) equilibrated with purification buffer containing 0.2 M NaCl, and then eluted with the same buffer. The active fractions were collected and concentrated to a final volume of 0.6 mL. The concentrated fractions were applied to a MonoQ HR5/5 column (GE Healthcare) equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions (eluted with 0.4 to 0.5 M NaCl) were combined, and concentrated to a final volume of 0.45 mL. The purity of the proteins in the active fractions was checked by SDS-PAGE using a 12.5% polyacrylamide gel stained with Coomassie Brilliant Blue R-250. The protein bands were cut from the gel and transferred to a PVDF membrane. The *N*-terminal amino acid sequences of the proteins were determined by automated Edman degradation with a PSQQ-30 Protein Sequencer (Shimadzu).

Expression of recombinant *LbNH* in *Escherichia coli*

The gene encoding *LbNH* of *L. buchneri* LBK78 was amplified by PCR using the following primers: 5' AAAAAACATATGTTGAAAAATGTTTACTTTGACCATGAC 3' (*NdeI* site underlined) and 5' AAAAAACTCGAGTTAATGACCCTGAGTCAGTA 3' (*XhoI* site underlined). The PCR product was cloned into the expression vector pET21a (Novagen, WI, USA) under transcriptional control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *lac* promoter, and the resultant plasmid was introduced into *E. coli* Rosetta2 (DE3) cells (Novagen). The transformant was cultured at 20°C for 24 h in Luria Bertani (LB) medium containing 1% (w/v) tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract, supplemented with 5 μ g/mL ampicillin and 1 mM IPTG. The cells were harvested by centrifugation, washed twice with 0.85 % NaCl, suspended with two volumes (w/w) of purification buffer, and disrupted by ultrasonication for 30 min. The lysate was centrifuged at 13,000 $\times g$ for 20 min at 4°C and the supernatant was used for the following purification steps.

Purification and molecular mass determination of recombinant *LbNH*

The supernatant prepared as described above was dialyzed against 10 L of purification buffer and applied to a MonoQ HR10/10 column equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active

fractions were collected and concentrated by ultrafiltration. The concentrated protein solution was applied to a Superdex200 HR10/30 column equilibrated with purification buffer, containing 0.2 M NaCl and then eluted with same buffer. The relative molecular mass of the purified recombinant *LbNH* was determined using a LC-10A HPLC system equipped with a gel filtration chromatography column (TSKgel G3000SW, 7.5 × 600 mm; Tosoh, Tokyo, Japan). The recombinant protein and the molecular mass standards (Gel Filtration Calibration Kit LMW and HMW; GE Healthcare) were eluted with 100 mM Na₂SO₄, 200 mM NaCl, and 100 mM KPB, pH 7.0, at a flow rate of 0.3 mL/min, and detected by UV absorbance at 280 nm. The purified enzyme was subjected to SDS-PAGE analysis (12.5% gel stained with coomassie brilliant blue).

Enzymatic activity assay of *LbNH*

For characterization of 2'-OMe-UR-catalyzing, hydrolysis with the recombinant *LbNH*, a standard reaction mixture containing 60 mM 2'-OMe-UR and 0.015 mg/mL purified recombinant *LbNH* in 50 mM KPB, pH 7.0, was used and the reaction was allowed to proceed at 50°C for 15 min. After incubation, the reaction mixture was diluted five-fold with methanol to terminate the reaction, and centrifuged at 20,400×*g* for 15 min at 4°C. The supernatant was collected and nucleosides and nucleobases in the supernatant were evaluated by HPLC analysis. Enzyme activity was determined as the production of uracil. In order to determine kinetic constants, 2'-OMe-UR was used at concentrations of 0.1 to 10 mM. The kinetic constants were determined using R software (<http://www.R-project.org/>) and the drc package (22) to fit the Michaelis-Menten equation for K_m and k_{cat} . The effects of pH on the enzyme activity were measured using the standard reaction mixture incubated at 50°C for 15 min, but with 50 mM sodium citrate buffer (pH 3-5), 50 mM KPB (pH 6-8), 50 mM Tris/HCl buffer (pH 6-9), and CAPS/NaOH buffer (pH 10-11) as reaction buffers. The pH stability was examined by measuring the residual activity after incubation in each reaction buffer at 4°C for 1 h. The effects of temperature on the enzyme activity were measured using the standard reaction mixture incubated at 4-90°C for 15 min. The temperature stability was examined by measuring the residual activity after incubation at each temperature for 1 h. For substrate specificity analysis of *LbNH*, the 2'-OMe-UR in the standard reaction mixture was replaced with 2.5-60 mM of various 2'-OMe-NRs, ribonucleosides, and 2'-deoxyribonucleosides, and the reaction was allowed to proceed at 50°C for 1 h. The enzyme activity of *LbNH* towards these substrates was determined as the production of corresponding nucleobases measured by HPLC analysis.

Identification of products from the hydrolysis of 2'-OMe-UR by *LbNH*

Eight milliliters of a reaction mixture comprising 100 mM 2'-OMe-UR and 0.015 mg/mL purified recombinant *LbNH* in 50 mM KPB, pH 7.0, was incubated at 50°C for 90 h. The

mixture was then centrifuged and the supernatant was applied to anion exchange resin (DOWEX 1W×8; Dow Chemical, MI, USA) and eluted with 24 mL of KPB. The fractions containing a sugar compound were collected, lyophilized (FreeZone 6; Labconco, MO, USA), and dissolved in D₂O. The spectra of ¹H NMR and ¹³C NMR were recorded on an Avance 500 (BrukerBioSpin, MA, USA). Alternatively, the lyophilized samples were dissolved in distilled water, and analyzed by electrospray ionization mass spectrometry (ESI-MS) using a LCMS-2010A (Shimadzu, Kyoto, Japan). MS conditions were as follows: block temperature, 200°C; curved desolvation line (CDL) temperature, 250°C; detector voltage, 1.5 kV; nebulizing gas flow, 1.5 L/min.

Results

Screening of 2'-OMe-UR-metabolizing microorganisms

To screen for 2'-OMe-UR-metabolizing microorganisms, a total of 1,026 strains from our existing stocks of cultures were used, consisting of 267 strains of lactic acid bacteria, 46 strains of actinomycetes, 183 strains of other bacteria, 144 strains of yeasts, 135 strains of basidiomycetes, 181 strains of other fungi, and 70 strains of insect parasite fungi. Among the microbial strains tested, 5 strains of lactic acid bacteria, *Lactobacillus buchneri* LBK78 (NITE P-01581), JCM1069, and JCM1115, *Lactobacillus mali* JCM1116, and *Pediococcus pentosaceus* NRIC0122, were found to have 2'-OMe-UR-degrading activity. Of these, *L. buchneri* LBK78 showed the strongest activity and was selected for further study.

Identification of a 2'-OMe-UR-degrading enzyme of *L. buchneri* LBK78

In order to identify the enzyme responsible for 2'-OMe-UR degradation, the CFE of *L. buchneri* LBK78 was subjected to a four-step purification procedure. Following the final purification step, the enzyme purity was increased approximately 133-fold (Table 1-1). Several protein bands were observed by SDS-PAGE analysis of the partially purified enzyme (data not shown). According to the N-terminal amino acid sequence analysis of these proteins, the sequence of one of the protein bands, MKNVYFDHHDGNVDDLVS, matched that of a putative nucleoside hydrolase Lbuc_0117 of *L. buchneri* NRRL B-30929. When compared to the full-length nucleotide sequence, the protein obtained from the *L. buchneri* LBK78 genome was found to be completely identical to Lbuc_0117. Thus, the protein, termed *LbNH*, was heterologously expressed in *E.coli*, and the recombinant protein was purified and assayed for its enzymatic activity. *LbNH* was found to have 2'-OMe-UR-degrading activity, and was confirmed to be the enzyme responsible for 2'-OMe-UR degradation in *L. buchneri* LBK78.

Table 1-1. Purification of *LbNH* from *L. buchneri* LBK78

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold
Cell-free extracts (CFE)	455	2.30	0.00506	1.00
Ammonium sulfate fractionation	76.9	2.46	0.0320	6.34
MonoQ HR 10/100 chromatography	5.95	1.01	0.170	33.6
Superdex200 HR10/30 chromatography	1.07	0.624	0.583	115
MonoQ HR 5/50 chromatography	0.487	0.326	0.671	133

Enzymatic characterization of *LbNH*

The purified recombinant *LbNH* enzyme appeared as a single band on SDS-PAGE analysis, corresponding to a subunit molecular mass of 34,000 (Fig. 1-1 (A)). When analyzed by gel filtration chromatography the enzyme showed a single symmetrical peak corresponding to a native molecular mass of 130,000 (Fig. 1-1 (B)). These results suggest that *LbNH* is a homotetrameric enzyme. When the reaction mixture containing *LbNH* and 2'-OMe-UR was analyzed by TLC, two spots were detected as degradation products derived from 2'-OMe-UR. Only one spot had UV absorbance at 254 nm, while the other spot was stained with the *p*-anisaldehyde reagent. In comparison to the retention times of the standard reagents by HPLC analysis, the product having UV absorbance was determined to be uracil (Fig. 1-2). The other product of 2'-OMe-UR degradation was purified by anion exchange chromatography, and subjected to NMR and ECI-MS analysis. The chemical shifts detected by NMR analysis were as follows: ^1H NMR (500 MHz, D_2O) δ (ppm): 4.87 (d, $J = 6.60$ Hz, 1H), H'-1; 4.22 (m, 1H), H'-3; 3.74 (m, 2H), H-4' and -5'; 3.58 (m, 1H), H-5''; 3.36 (s, 3H), 2'-O-CH₃; 3.12 (dd, $J=6.58$, 2.70 Hz, 1H), H-2'. ^{13}C NMR (125 MHz, D_2O) δ (ppm): 92.25, C-1'; 80.00, C-2'; 66.97, C-4'; 66.33, C-3'; 62.96, C-5'; 56.93, 2'-O-CH₃. Based on the information from these NMR spectra, the other reaction product of 2'-OMe-UR was determined to be 2'-O-methylribose (2'-OMe-ribose). This sugar compound was detected at m/z 199 [$\text{M}+^{35}\text{Cl}$] $^-$ and 201 m/z [$\text{M}+^{37}\text{Cl}$] $^-$ in the ESI-MS analysis. Therefore, *LbNH* was determined to have 1'-hydrolysis activity towards 2'-OMe-UR to form 2'-OMe-ribose and uracil as the reaction products.

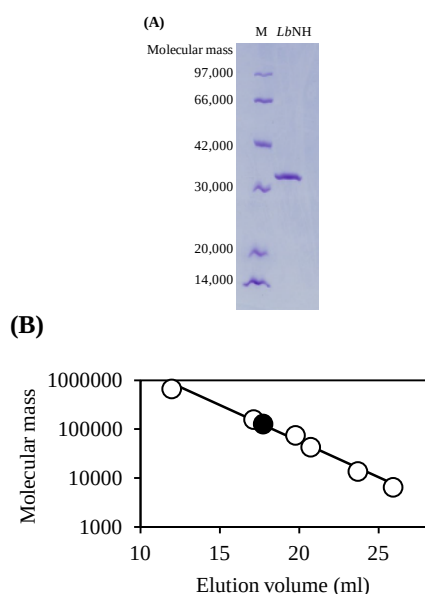


Fig. 1-1. Molecular mass determination of recombinant *LbNH*

A, SDS-PAGE analysis of recombinant *LbNH*. Lane M indicates molecular mass standards: phosphorylase *b* (97,000), BSA (66,000), aldolase (42,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), and lysozyme (14,000) from top of gel. B, Gel filtration chromatography analysis of recombinant *LbNH*. *LbNH* (closed circles) and molecular mass standards (open circles): thyroglobulin (670,000), aldolase (160,000), conalbumin (75,000), ovalbumin (43,000), ribonuclease A (14,000), and aprotinin (6,500).

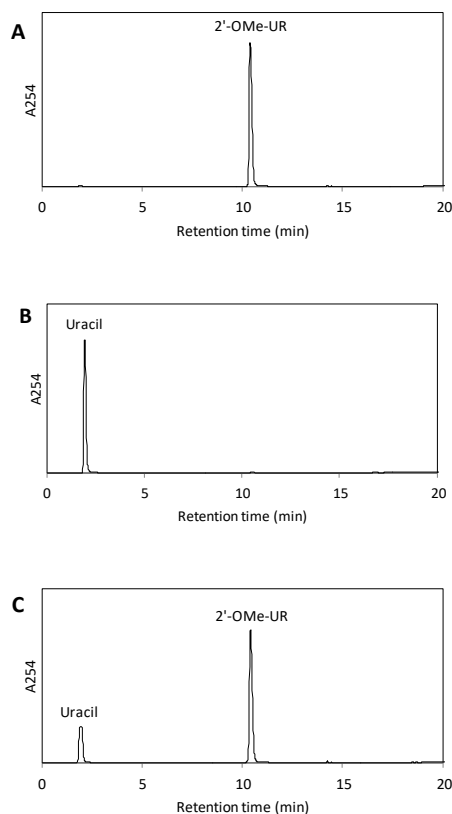


Fig. 1-2. HPLC analysis of degradation products of 2'-OMe-UR produced by *LbNH*

A, Chromatogram of standard 2'-OMe-UR. B, Chromatogram of standard uracil. C, Chromatogram of *LbNH* reaction mixture with 2'-OMe-UR after incubation at 50°C for 15 min.

Catalytic properties of *LbNH*

Normal hyperbolic kinetics was observed in the 2'-OMe-UR hydrolysis by *LbNH*. Non-linear regression analysis using Michaelis-Menten model of the data yielded apparent K_m values for 2'-OMe-UR of 0.040 mM, k_{cat} of 0.49 s⁻¹, and k_{cat}/K_m of 12 mM⁻¹·s⁻¹. The effect of pH on 2'-OMe-UR hydrolysis was examined using pH values ranging from 3.0 to 11.0. *LbNH* showed activity at all pH values tested, and the maximum activity was observed at pH 7.0 (Fig. 1-3 (A)). *LbNH* retained over 70% of its activity after 1 h of incubation at pH 3.0-10.0, and *LbNH* activity decreased sharply after incubation at pH 11.0 (Fig. 1-3 (B)). The effect of temperature on 2'-OMe-UR hydrolysis was also examined using incubation temperatures ranging from 4°C to 90°C. *LbNH* showed over 80% activity between 30°C and 70°C, and the maximum activity was

observed at 70°C (Fig. 1-4). However, *LbNH* activity markedly decreased after 1 h of incubation at temperatures above 60 °C. From these observations, the optimal conditions for 2'-OMe-UR hydrolysis by *LbNH* were determined to be pH 7.0 and 50°C. Substrate specificity of *LbNH* towards various nucleosides in the hydrolysis reaction was determined by analyzing the corresponding nucleobases released from these substrates by HPLC. Relative enzyme activities against 18 different nucleosides are shown in Table 1-2. *LbNH* tended to prefer 2'-OMe-NRs and ribonucleosides as hydrolytic substrates, but little activity was observed when tested with 2'-deoxyribonucleosides. All 2'-OMe-NRs tested were accepted as substrates, and in particular, *LbNH* showed strong activity (383%) towards 2'-O-methylguanosine (2'-OMe-GR) when compared to 2'-OMe-UR. *LbNH* hydrolyzed 2'-O-methylinosine (2'-OMe-IR) and 2'-O-methylcytidine (2'-OMe-CR) with the relative activities of 43% and 30%, respectively, but showed less activity towards 2'-O-methyladenosine (2'-OMe-AR) (9%). All ribonucleosides tested were hydrolyzed by *LbNH*, and *LbNH* activity was markedly increased in the presence of guanosine, with the relative activity of 1,600%. Relative activities towards inosine and uridine was 42% and 41%, respectively, while weaker relative activity was detected with the other ribonucleosides tested (adenosine, cytidine, 5-methyluridine, and xanthosine). Of the 2'-deoxyribonucleoside compounds tested, little to no hydrolysis by *LbNH* was detected, and only very weak activity was observed towards 2'-deoxyuridine, 2'-deoxyinosine, and thymidine. These results suggest that *LbNH* recognized the 2'-hydroxy or 2'-methoxy group in the sugar moiety of the substrate, and while the preferred nucleobase was guanine, uracil and hypoxanthine were also acceptable.

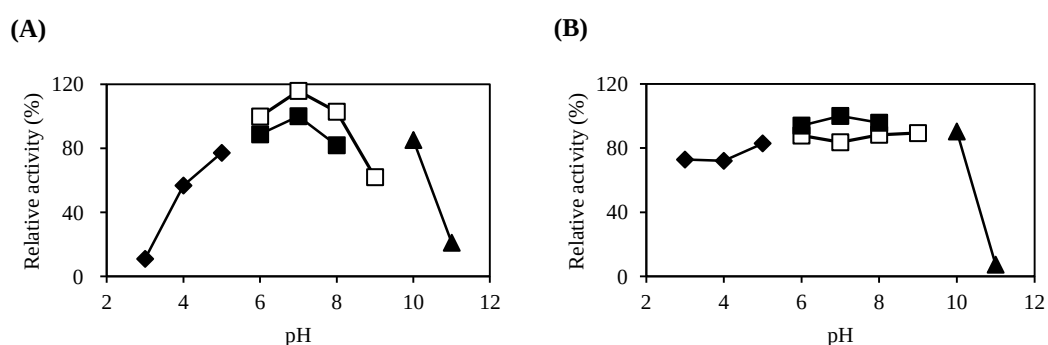


Fig. 1-3. Effect of pH on the activity and stability of *LbNH* in 2'-OMe-UR hydrolysis

A, The effect of pH on the activity of *LbNH*. B, The effect of pH on the stability of *LbNH*. Sodium citrate buffer (pH 3.0-5.0, closed diamonds), Tris/HCl buffer (pH 6.0-9.0, open squares), KPB (pH 6.0-8.0, closed squares), and CASP/NaOH buffer (pH 10.0-11.0, closed triangles) are indicated. Relative activity was defined as the percent increase in mass amounts of uracil in the case of pH 7.0 as 100%.

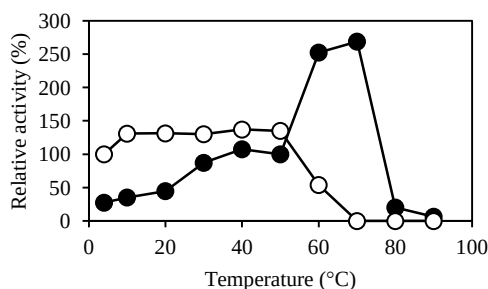


Fig. 1-4. Effect of temperature on the activity and stability of *LbNH* in 2'-OMe-UR hydrolysis

Effect of temperature on the activity (closed circles) and on the stability (open circles) of *LbNH* are shown. Relative activity was defined as the percent increase in mass amounts of uracil in the case of 50°C as 100%.

Table 1-2. Substrate specificity of hydrolysis by *LbNH*

Substrate		Starting concentration (mM)	Relative activity (%)
2'-OMe-NRs	2'-OMe-UR	60	100
	2'-OMe-AR	10	9
	2'-OMe-GR	2.5	383
	2'-OMe-IR	10	43
	2'-OMe-CR	60	30
Ribonucleosides	Uridine	60	41
	Adenosine	10	7
	Guanosine	2.5	1,600
	Inosine	10	42
	Cytidine	60	8
	5-Methyluridine	60	2
	Xanthosine	2.5	15
	Thymidine	60	5
2'-Deoxyribonucleosides	2'-Deoxyuridine	60	1
	2'-Deoxyadenosine	10	<i>n. d.</i>
	2'-Deoxyguanosine	2.5	<i>n. d.</i>
	2'-Deoxyinosine	10	1
	2'-Deoxycytidine	60	<i>n. d.</i>

Relative activity was defined as the percent increase in mass amounts of corresponding heterocyclic base using 2'-OMe-UR as 100%.

n. d., not detected

Amino acid sequence analysis of *LbNH*

Using BLASTP (protein-protein Basic Local Alignment Search Tool) to search a database of reference proteins, 16 putative nucleoside hydrolases which had high homology with *LbNH* were found. *LbNH* and these homologous enzymes were subjected to phylogenetic analysis together with 16 well-known nucleoside hydrolases characterized by their substrate

specificity, such as pyrimidine-specific, purine-specific, non-specific, and 6-oxopurine-specific nucleoside hydrolases (Fig. 1-5) (23-35). *LbNH* and putative nucleoside hydrolases formed a cluster with two purine-specific nucleoside hydrolases, IAG-NHs, found in trypanosomes which had about 32 % homology (23,24). The alignment of the amino acid sequences of *LbNH* and representative well-characterized nucleoside hydrolases is shown in Fig. 1-6. Three amino acid motifs highly conserved among members of the nucleoside hydrolase superfamily were also found in the sequence of *LbNH* (36). The first is an aspartic acid-rich *N*-terminal motif (DXDXXXDD) corresponding to amino acids 7 to 14 of *LbNH*. This motif is involved in the binding of the catalytic Ca^{2+} ion and is conserved in all nucleoside hydrolases. The second is the AEXNXXDPXAA motif corresponding to amino acids 171 to 182 of *LbNH*. The third motif is a catalytic aspartic residue corresponding to Asp245 in *LbNH* that is also conserved in all nucleoside hydrolases, and completes the positioning of the catalytic Ca^{2+} ion. The histidine residue adjacent to this aspartic residue, for example His244 in *Crithidia fasciculata* IU-NH, is found in almost all purine-specific, non-specific, and pyrimidine-specific nucleoside hydrolases (37). However, in *LbNH* and IAG-NHs of trypanosomes, the histidine residue is replaced by a tryptophan. IAG-NHs possess hydrolysis activity towards guanosine and inosine (23,24), and *LbNH* was also found here to prefer guanosine and inosine as its hydrolytic substrates (Table 1-2). These results indicate that *LbNH* is similar to trypanosome IAG-NHs, even though *LbNH* also had hydrolytic activity towards pyrimidine ribonucleosides such as uridine.

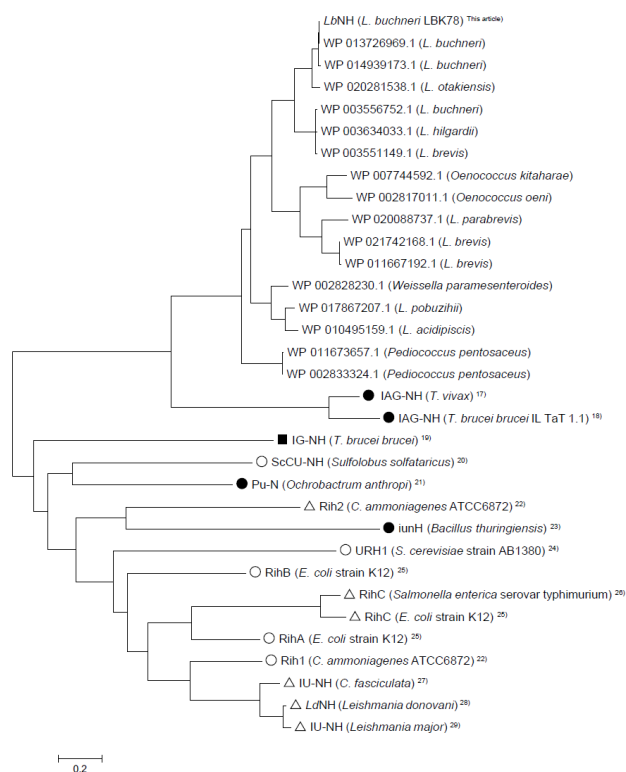


Fig. 1-5. Phylogenetic analysis of *LbNH*

LbNH and its homologous enzymes were subjected to phylogenetic analysis with well-known hydrolases using the neighbor-joining method. The scale bar indicates 0.2 substitutions per nucleotide. These well-known hydrolases were classified into pyrimidine-specific (open circles), purine-specific (closed circles), non-specific (open triangles), and 6-oxopurine-specific (closed squares) nucleoside hydrolases.

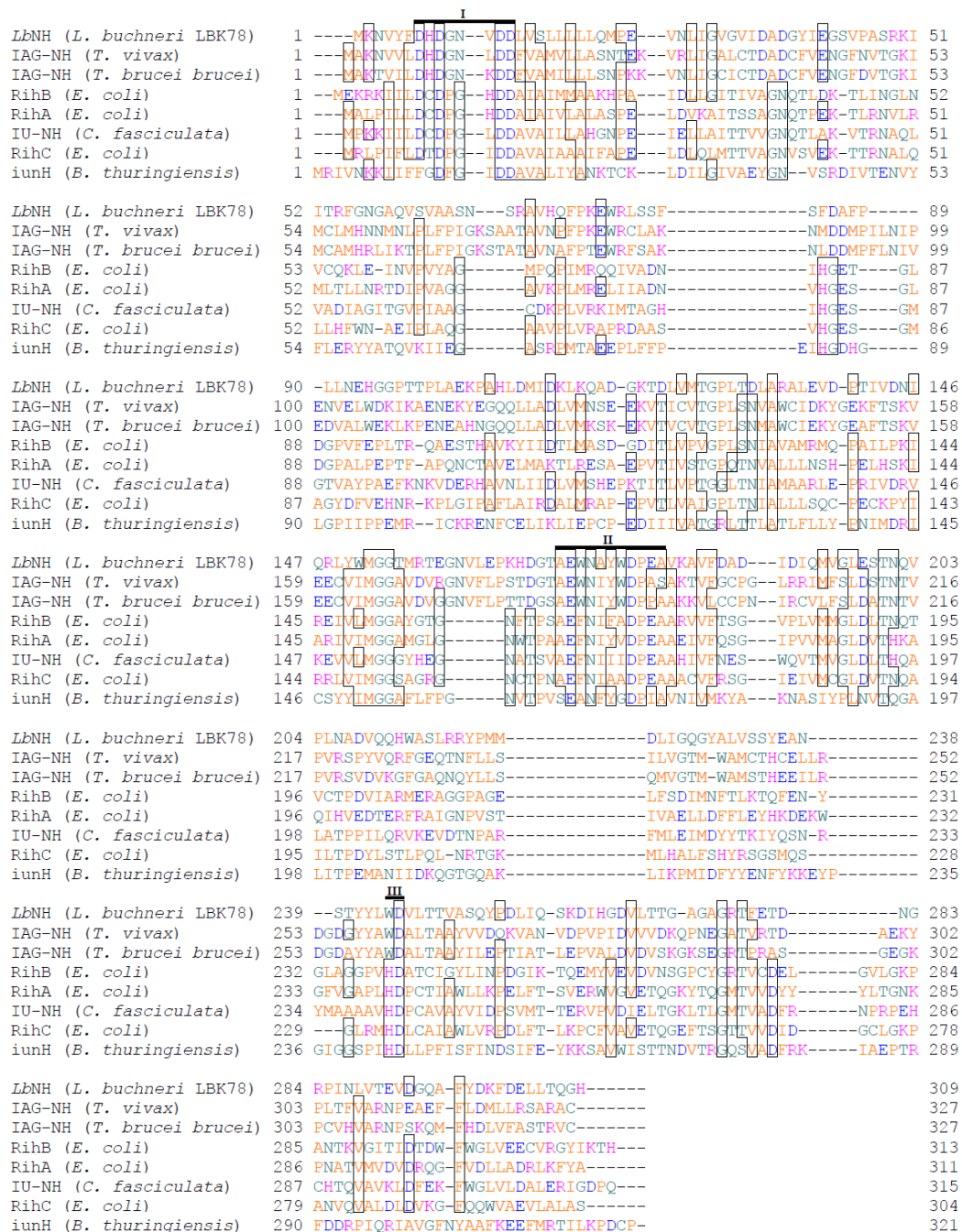


Fig. 1-6. Amino acid sequence alignment of *LbNH* and well-known nucleoside hydrolases

The highly conserved regions and residues within the nucleoside hydrolase superfamily are the Aspartic acid-rich *N*-terminal motif (I), the AEXNXXDPXAA motif (II), and the region containing Trp244 and Asp245 in *LbNH* (III).

Discussion

A considerable number of studies have been performed on microbial nucleoside hydrolases. Nucleoside hydrolases are classified based on their substrate specificities into purine-specific, pyrimidine-specific, non-specific, and 6-oxopurine-specific, and several characteristic amino acid residues reflect these substrate specificities. Based on the crystal structure of *C. fasciculate* IU-NH, the His241 residue acts as a general proton donor (36-38). The corresponding histidine residue is conserved in most nucleoside hydrolases which possess hydrolysis activity towards uridine. However, the catalytic histidine is replaced with a tryptophan residue in *LbNH* and trypanosomal IAG-NHs. From the crystal structure of IAG-NH from *Trypanosoma vivax*, only four amino acids, Asn12, Asp40, Trp83, and Trp260, are involved in distinction between purine and pyrimidine nucleosides as substrates (39). Of these, Asp40, Trp83, and Trp260 are characteristically conserved residues in trypanosomal IAG-NHs and *LbNH*. In particular, Trp83 and Trp260 are involved in catalytic efficiency and substrate recognition (39,40), and IAG-NHs have strict specificity towards purine ribonucleosides such as guanosine and inosine. However, because *LbNH* demonstrated hydrolysis activity towards uridine as well as guanosine and inosine, this would suggest that other amino acid residues were responsible for catalysis and the efficient hydrolysis of pyrimidine nucleosides.

So far, only two types of enzymes, deoxyribosyltransferase and nucleoside phosphorylase have been reported to react with 2'-modified ribonucleosides, such as 2'-fluoro-deoxyribonucleosides and 2'-amino-deoxyribonucleosides (41,42), however there is no information regarding enzymes reacting with 2'-OMe-NRs. Therefore, this is the first report demonstrating the enzyme activity towards 2'-OMe-NRs. Interestingly, 2'-OMe-NRs exist naturally as components in modified rRNA and tRNA in various organisms (43-46). Thus *LbNH* and homologous enzymes may play a physiological role in the metabolism of 2'-OMe-NRs caused by degradation of this rRNA and tRNA. Besides the physiological interests, application of *LbNH* to 2'-OMe-NRs modifications, e.g., base exchange reaction etc., are also promising and targets for future investigations.

Summary

2'-O-Methylribonucleosides (2'-OMe-NRs) are promising raw materials for nucleic acid drugs because of their high thermal stability and nuclease tolerance. In the course of microbial screening for metabolic activity towards 2'-OMe-NRs, *Lactobacillus buchneri* LBK78 was found to decompose 2'-O-methyluridine (2'-OMe-UR). The enzyme responsible was

partially purified from *L. buchneri* LBK78 cells by a four-step purification procedure, and identified as a novel nucleoside hydrolase. This enzyme, *LbNH*, belongs to the nucleoside hydrolase superfamily, and formed a homotetrameric structure composed of subunits with a molecular mass of around 34,000. *LbNH* hydrolyzed 2'-OMe-UR to 2'-O-methylribose and uracil, and the kinetic constants were K_m of 0.040 mM, k_{cat} of 0.49 s⁻¹, and k_{cat}/K_m of 12 mM⁻¹·s⁻¹. In a substrate specificity analysis, *LbNH* preferred ribonucleosides and 2'-OMe-NRs as its hydrolytic substrates, but reacted weakly with 2'-deoxyribonucleosides. In a phylogenetic analysis, *LbNH* showed a close relationship with purine-specific nucleoside hydrolases from trypanosomes.

CHAPTER II

Enzymatic synthesis of 2'-O-methylribonucleosides with a nucleoside hydrolase family enzyme from *Lactobacillus buchneri* LBK78

Introduction

Nucleic acid drugs have attracted considerable attention as next-generation medicines, suggested for various applications such as antisense drugs, ribozymes, small interfering RNA drugs, and aptamers (12,13). In order to increase their stability, target affinity, biological potency, and intracellular uptake, modified nucleoside analogues have been introduced into nucleic acid drugs, for example 2'-O-(2-methoxyethyl)ribonucleosides in Mipomersen (Kynamro; Genzyme, Cambridge, MA, USA) (14).

2'-O-Methylribonucleosides (2'-OMe-NRs) are 2'-modified nucleoside analogues that are promising as raw materials for nucleic acid drug productions, since oligonucleotides containing 2'-OMe-NRs show high thermal stability and nuclease tolerance (4-7). Although 2'-OMe-NRs are synthesized by chemical methods (15-17), such chemical synthesis usually requires multi step processes for the selective modification and purification of products. Moreover, it is more difficult to synthesize purine nucleoside analogues such as 2'-O-methyladenosine (2'-OMe-AR) than pyrimidine nucleoside analogues such as 2'-O-methyluridine (2'-OMe-UR) due to their low solubility and nucleobase structures (47). In contrast, biocatalytic synthesis of nucleoside analogues offers high stereo- and regio-selectivity under mild reaction conditions (20). 2'-Deoxyribosyltransferases, nucleoside phosphorylases, and phosphoribosyltransferases have been used for the interconversion between pyrimidine nucleosides and purine heterocyclic nucleobases (48-53). There are no reports on the enzyme acting on 2'-OMe-NRs, however, three enzymes have been reported for the interconversion of other kinds of 2'-modified ribonucleosides. The 2'-deoxyribosyltransferase of *Lactobacillus reuteri* was shown to have the transribosylation activity between 2'-fluoro-deoxyribouridine and cytosine (41,54), and phosphorylases of *Geobacillus thermoglucosidasius* and *Thermus thermophilus* were found to have the phosphorolytic activities towards 2'-fluoro- and 2'-amino-deoxyribonucleosides, respectively (42).

We previously reported that *LbNH* isolated from *Lactobacillus buchneri* LBK78 (NITE P-01581) is the first nucleoside hydrolase found to react on 2'-OMe-NRs, and it degraded various 2'-OMe-NRs into nucleobases and 2'-O-methylribose (2'-OMe-ribose) (55). In the present study, we found that *LbNH* is a bi-functional enzyme possessing the transribosylation activity towards 2'-OMe-NRs as well as the hydrolytic activity. Furthermore,

the enzymatic production process of 2'-OMe-AR was accomplished using *LbNH* as the biocatalyst via the transribosylation reaction between 2'-OMe-UR and adenine.

Materials and Methods

Chemicals

Standard reagents of 2'-OMe-NRs other than 2'-OMe-ribose were obtained from Carbosynth Limited (Compton, Berkshire, UK). All other reagents and solvents were obtained from commercial sources.

Analysis of nucleosides and nucleobases

For high-performance liquid chromatography (HPLC) analysis, an LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a COSMOSIL 5C₁₈-ARII column (4.6 × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) was used for separation at 40°C. The mobile phase was 10 mM ammonium acetate (pH 4.5, eluent A) and acetonitrile (eluent B), and the flow rate was 1.5 mL/min. The eluent gradients were 0% (v/v) B for 0-5 min, 0-5% (v/v) B over 5-10 min, 5-60% (v/v) B over 10-17.5 min, and 60% (v/v) B for 17.5-22.5 min. Nucleobases and nucleosides were quantitatively detected by UV absorbance at 254 nm. To identify products from the transribosylation reaction between 2'-OMe-UR and nucleobases, the samples were analyzed by electrospray ionization mass spectrometry (ESI-MS) using a LCMS-2010A mass spectrometer (Shimadzu, Kyoto, Japan). MS conditions were as follows: block temperature, 200°C; curved desolvation line (CDL) temperature, 250°C; detector voltage, 1.5 kV; nebulizing gas flow, 1.5 L/min, and eluent gradients were modified to 1% (v/v) B for 0-38 min, 1-5% (v/v) B over 38-75 min, 5-60% (v/v) B over 75-130 min, and 60% (v/v) B for 130-140 min.

Recombinant bacterial strain and preparation of *LbNH*

Escherichia coli Rosetta2 (DE3) carrying pET21a-*LbNH* was used for enzyme expression (22). Recombinant *E. coli* cells were grown aerobically at 28°C with shaking at 120 rpm in 500 mL Luria Bertani (LB) medium containing 1% (w/v) tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract, supplemented with 50 ng/mL ampicillin. When the culture reached an OD₆₀₀ of approximately 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for 24 h at 20°C with shaking at 120 rpm. The cells were harvested by centrifugation (13,000×g, 20 min, 4°C) and washed in physiological saline twice. The cells were then suspended in purification buffer containing 20 mM potassium phosphate buffer (KPB), pH 7.0, and disrupted by ultrasonication for 30 min.

The lysate was centrifuged at $13,000\times g$ for 20 min at 4°C , and the supernatant was used as the cell-free extract. The cell-free extract was applied to a MonoQ HR10/10 column (GE Healthcare, IL, USA) equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions were collected and concentrated by ultrafiltration. The concentrated protein solution was applied to a Superdex200 HR10/30 column (GE Healthcare) equilibrated with purification buffer, containing 0.2 M NaCl and eluted with the same buffer. The fractions containing recombinant *LbNH* were pooled, concentrated by ultrafiltration and used as the purified enzyme. One unit of *LbNH* was defined as the amount of enzyme that produced one micro mole of uracil from 60 mM 2'-OMe-UR per min at 50°C in 50 mM KPB, pH 7.0, and the enzyme activity was determined from the HPLC analysis.

Measurement of the transribosylation reaction with *LbNH*

A standard reaction mixture containing 60 mM 2'-OMe-UR, 100 mM adenine and 0.03-0.25 U/mL purified recombinant *LbNH* in 50 mM KPB, pH 7.0, was used and the reaction was allowed to proceed at 50°C for 24 h. After incubation, the reaction mixture was diluted five-fold with 1% (v/v) trifluoroacetic acid to terminate the reaction and centrifuged at $20,400\times g$ for 15 min at 4°C . The nucleosides and nucleobases in the supernatant were evaluated by HPLC analysis. The transribosylation activity was determined as the amount of corresponding 2'-OMe-NRs produced as measured by HPLC analysis. For the substrate specificity analysis, adenine was replaced with various nucleobases in the standard reaction mixture. The effect of hydrophilic solutes on the transribosylation was measured using the standard mixture containing various concentrations of NaCl, sugars, alcohols, and polysaccharides. To examine the effect of temperature, the standard mixture was used with or without 50% (v/v) glycerol and the reaction was carried out at $4-70^{\circ}\text{C}$ for 60 min. The effect of 2'-OMe-UR concentration on the transribosylation was measured in the standard reaction mixture containing 30-2000 mM 2'-OMe-UR with 50% (v/v) glycerol. Similarly, the effect of adenine concentrations was measured in the standard reaction mixture using adenine at concentrations of 20-1000 mM and 120 mM 2'-OMe-UR, in the presence of 50% (v/v) glycerol. The yield of 2'-OMe-AR produced was calculated as the amount of 2'-OMe-AR divided by the starting concentration of 2'-OMe-UR.

Kinetic analysis of *LbNH* activity

To determine the kinetic constants, 2'-OMe-UR and adenine were reacted with 0.041 U/mL of purified recombinant *LbNH* at 50°C in 50 mM KPB, pH 7.0. In order to determine the kinetic constants for the transribosylation, 2'-OMe-UR was used at concentrations of 0.1-20 mM with 20 mM adenine, and adenine was used at concentrations of 0.25-15 mM with 60 mM

2'-OMe-UR. Each reaction was allowed to proceed for 5 min. The transribosylation activity was determined as the production of 2'-OMe-AR. In order to determine kinetic constants for the hydrolysis, 2'-OMe-UR was used at concentrations of 0.01-5 mM, and the reaction was allowed to proceed for 3 min. The hydrolytic activity was determined as the production of uracil, or was calculated by subtracting the amount of 2'-OMe-AR from the amount of uracil in the case of adenine addition. Kinetic constants were determined using R software (<https://www.R-project.org/>) and the software package drc (22) to fit the Michaelis-Menten equation for K_m and k_{cat} . In order to determine the inhibition constants of the hydrolysis, adenine was used at concentrations of 0-6 mM with 5, 10, or 60 mM 2'-OMe-UR, and the reaction was allowed to proceed for 10 min. Four inhibition mechanism models were constructed based on Eq. 1, 2, 3 and 4, and the fitting of these inhibition models and inhibition constants were calculated using R software based on the least squares method.

Eq. 1

$$v = \frac{V_{max}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i} \right)}$$

Eq. 2

$$v = \frac{V_{max}[S]}{([S] + K_m) \left(1 + \frac{[I]}{K_i} \right)}$$

Eq. 3

$$v = \frac{V_{max}[S]}{K_m + [S] \left(1 + \frac{[I]}{K'_i} \right)}$$

Eq. 4

$$v = \frac{V_{max}[S]}{[S] \left(1 + \frac{[I]}{K'_i} \right) + K_m \left(1 + \frac{[I]}{K_i} \right)}$$

Enzymatic synthesis of 2'-OMe-AR under optimal conditions

Production of 2'-OMe-AR was carried out in the reaction mixture containing 60 mM 2'-OMe-UR, 200 mM adenine, 50% glycerol (v/v), 20% (w/v) dextrin and 0.04 U/mL purified recombinant *LbNH* in 50 mM KPB, pH 7.0. The reaction was allowed to proceed at 50°C for 96 h.

Results

Bi-functional transribosylation activity of *LbNH*

The purified recombinant *LbNH* enzyme showed the hydrolytic activity in the reaction mixture containing 2'-OMe-UR, and produced uracil and 2'-OMe-ribose as shown in our previous report (55). However, when adenine was added into the reaction mixture along with 2'-OMe-UR, a novel peak other than uracil was detected by HPLC analysis after 24 h (Fig. 2-1 (A)). The retention time of this new product corresponded to the 2'-OMe-AR standard reagent. Moreover, its molecular weight was determined to be m/z 282 $[M+H]^+$ by ESI-MS analysis, which was the same as that of 2'-OMe-AR (Fig. 2-1 (B)). Therefore, *LbNH* was revealed to be a bi-functional enzyme with the transribosylation activity between 2'-OMe-UR and adenine to form 2'-OMe-AR and uracil, in addition to 1'-hydrolytic activity towards 2'-OMe-UR to form uracil and 2'-OMe-ribose.

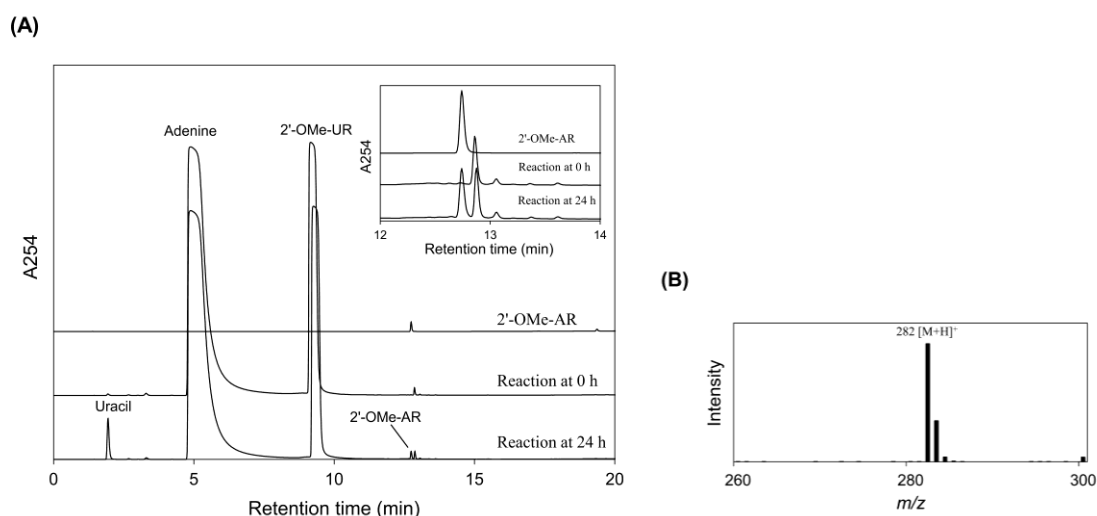


Fig. 2-1. HPLC analysis of transribosylation products of 2'-OMe-UR and adenine catalyzed by *LbNH*

A, Chromatograms of the standard reagent 2'-OMe-AR (top) and *LbNH* reaction mixture containing 2'-OMe-UR and adenine at 0 h (middle) and 24 h (bottom). The peaks detected at 1.9 min, 5.0 min, 9.3 min and 12.7 min corresponded to standard reagents of uracil, adenine, 2'-OMe-UR and 2'-OMe-AR, respectively. The peak of 2'-OMe-AR was detected at 12.7 min. Peaks detected at 12.9 min and 13.1 min were seemed to be contaminants derived from substances, and any change of these peaks were observed during the reaction. The inset indicated enlarged chromatograms of the region of 2'-OMe-AR. B, Positive ESI-MS spectrum of the *LbNH* reaction product detected at 12.7 min by HPLC analysis.

Kinetic analysis of *LbNH* activity

Normal hyperbolic kinetics were observed in the *LbNH* reaction, and the resulting kinetic constants are summarized in Table 2-1. In the transribosylation reaction between 2'-OMe-UR and adenine, non-linear regression analysis using the Michaelis-Menten model of the data yielded an apparent K_m of 1.8 ± 0.14 mM for 2'-OMe-UR and of 1.3 ± 0.081 mM for adenine, and a k_{cat} of 0.099 ± 0.0022 s⁻¹ for 2'-OMe-UR and 0.10 ± 0.0016 s⁻¹ for adenine. The catalytic efficiency (k_{cat}/K_m) was 0.055 mM⁻¹·s⁻¹ for 2'-OMe-UR and 0.076 mM⁻¹·s⁻¹ for adenine. The initial velocity of the hydrolysis reaction towards 2'-OMe-UR was measured with or without adenine in the reaction mixture. In the absence of adenine, non-linear regression analysis using the Michaelis-Menten model of the data yielded an apparent K_m of 0.32 ± 0.0075 mM, k_{cat} of 3.6 ± 0.030 s⁻¹, and k_{cat}/K_m of 11 mM⁻¹·s⁻¹. On the other hand, the data yielded an apparent K_m of 0.66 ± 0.054 mM, k_{cat} of 1.4 ± 0.027 s⁻¹, and k_{cat}/K_m of 2.2 mM⁻¹·s⁻¹ in the presence of a saturated concentration of adenine, which indicated that adenine inhibited the 2'-OMe-UR hydrolytic activity of *LbNH*. Therefore, four typical schemes were designed to account for the inhibition of the hydrolytic activity by adenine (Eq. 1, 2, 3, 4), where [S] and [I] corresponded to the concentration of 2'-OMe-UR and adenine, respectively. In the comparison of these inhibition models, the mixed non-competitive inhibition model based Eq. 4 was the best fit (Fig. 2-2), and the data yielded an apparent K_i value of 0.45 mM and K_i' value 8.3 mM.

Table 2-1. Kinetic constants of hydrolysis of 2'-OMe-UR and transribosylation between 2'-OMe-UR and adenine by *LbNH*

Reaction	Substrate	Additive	Kinetic constants			Inhibitory constants	
			K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	K_i (mM)	K_i' (mM)
Hydrolysis	2'-OMe-UR		0.32 ± 0.0075	3.6 ± 0.030	11		
	2'-OMe-UR	Adenine	0.66 ± 0.054	1.4 ± 0.027	2.2	0.45	8.3
Transribosylation	2'-OMe-UR		1.8 ± 0.14	0.099 ± 0.0022	0.055		
	Adenine		1.3 ± 0.081	0.10 ± 0.0016	0.076		

Data are presented as mean $\pm$ standard error. To determine the kinetic constants in the hydrolysis reaction, the reaction was carried out with 0.01-5 mM 2'-OMe-UR at 50°C for 3 min. To determine the inhibitory constants, the reaction was carried out with 5, 10 or 60 mM 2'-OMe-UR and 0-6 mM adenine at 50°C for 10 min. In the transribosylation reaction, the reaction mixture containing 0.1-20 mM 2'-OMe-UR and 20 mM adenine was used to determine the kinetic constants of 2'-OMe-UR, and incubated at 50°C for 5 min. The reaction mixture containing 60 mM 2'-OMe-UR and 0.25-15 mM adenine was used to determine the kinetic constants of adenine in the transribosylation reaction, and the reaction was allowed to proceed at 50°C for 5 min.

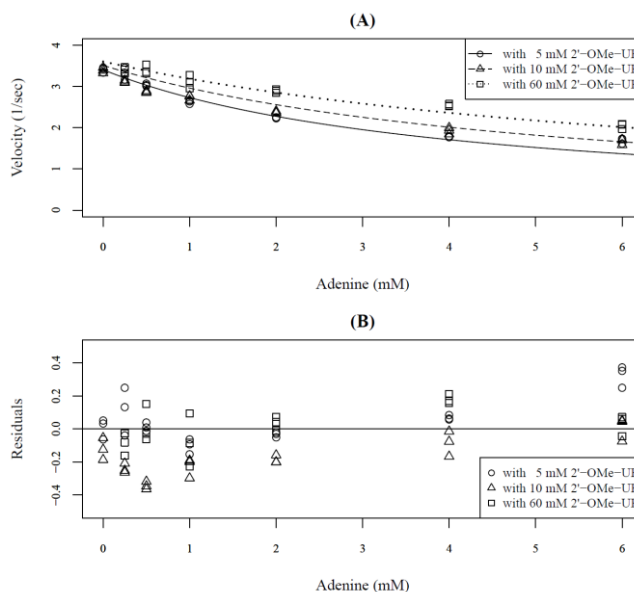


Fig. 2-2. Fittings and residuals of a mixed non-competitive inhibition model of the 2'-OMe-UR hydrolysis by adenine based on Eq. 4

To determine the inhibitory constants in the hydrolysis reaction towards 2'-OMe-UR, the reaction was carried out with 5, 10 or 60 mM 2'-OMe-UR and 0-6 mM adenine at 50°C for 10 min. A, Michaelis-Menten plots of experimentally determined (plots) and theoretically fit (curves) rate values of 2'-OMe-UR hydrolysis velocity in the presence of adenine. B, The residuals from a fitted model defined as the differences between the measured and the predicted values at each adenine concentration. The data obtained using 5 mM (open circles), 10 mM (open triangles), or 60 mM 2'-OMe-UR (open squares) and fitting curves based on the data obtained with 5 mM (solid line), 10 mM (dashed line), or 60 mM 2'-OMe-UR (dotted line) are indicated.

Substrate specificity analysis of *LbNH* transribosylation

In order to analyze the specificity of the acceptor substrate in the transribosylation by *LbNH*, the reaction was carried out using eleven different nucleobases with 2'-OMe-UR as the donor substrate (Table 2-2). From these results, purine nucleobases were preferable acceptor substrates for the transribosylation with 2'-OMe-UR as a donor substrate. *LbNH* reacted with hypoxanthine and 2,6-diaminopurine with 27% and 21% relative activities to that with adenine set as 100%, respectively. *LbNH* also reacted with 2-amino-6-chloropurine, guanine, and xanthine with relative activities of 4.1%, 1.0%, and 0.16%, respectively. Exceptionally, *LbNH* showed the transribosylation activity towards cytosine (4.1%) among pyrimidine nucleobases tested. The reaction products from hypoxanthine, guanine, and cytosine had the same retention times and molecular weights as the standard reagents 2'-O-methylinosine, 2'-O-methylguanosine, and 2'-O-methylcytidine, respectively. Although standard reagents for the other predicted products were not available, the molecular weights of the new products

derived from 2,6-diaminopurine, 2-amino-6-chloropurine, xanthine, thymine, and 4-chlorouracil corresponded to those of 2'-*O*-methyl-2,6-diaminopurine riboside, 2'-*O*-methyl-2-amino-6-chloropurine riboside, 2'-*O*-methylxantosine, 2'-*O*-methylthymine, and 2'-*O*-methyl-4-chlorouridine, respectively. Dihydrothymine and 6-thioxanthine were not suitable as acceptor substrates for the transribosylation reaction, and no products were detected with these nucleobases.

Table 2-2. Substrate specificity of the transribosylation reaction by *LbNH* using nucleobases as acceptor substrates and 2'-OMe-UR as a donor substrate

Acceptor	Relative activity (%)
Adenine	100
Hypoxanthine	27
2,6-Diaminopurine	21
2-Amino-6-chloropurine	4.1
Cytosine	4.1
Guanine	1.0
Xanthine	0.16
Thymine	0.04
4-Chlorouracil	0.012
6-Thioxanthine	<i>n. d.</i>
Dihydrothymine	<i>n. d.</i>

The reaction was carried out with 60 mM 2'-OMe-UR, 100 mM nucleobase and 0.041 U/mL purified *LbNH* at 50°C for 0.5-24 h. Relative activity was defined as the percent increase in mass amount of corresponding 2'-OMe-NR relative to adenine coupling with 60 mM 2'-OMe-UR (defined as 100%). *n. d.*, not detected.

Effect of the reaction mixture compositions on the production of 2'-OMe-AR

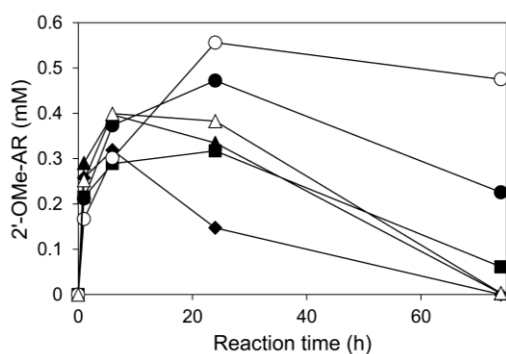
According to Kramers' theory, solvent viscosity is reported to affect enzyme catalysis by restricting the mobility of the enzyme and small molecules (56-58). Therefore, the effect of various hydrophilic solutes to increase solvent viscosity was investigated. The addition of 2 M NaCl, 10 or 30% (v/v) glycerol, or a 20, 40, 60, 80 or 100% saturated concentration of glucose were found to increase the amount of 2'-OMe-AR produced by 15%, 6%, 3%, 9%, 12%, 13%, 41% and 14%, respectively, after 3 h (Table 2-3). The effects of these solutes on the reaction were then examined over a longer time period (Fig. 2-3). Among the solutes tested, 50% (v/v) glycerol produced the highest amount of 2'-OMe-AR after 24 h, and thus was used as the

hydrophilic solute in subsequent experiments. Addition of 80% (w/v) saturated glucose increased the initial velocity of 2'-OMe-AR production, however, the amount of 2'-OMe-AR was rapidly decreased after 6 h. The effect of temperature on the transribosylation and the hydrolysis reaction was compared with and without 50% (v/v) glycerol (Fig. 2-4). The viscosity of aqueous glycerol solutions strongly depends on solvent temperature, with viscosity decreasing as temperature increases (59). The addition of 50% (v/v) glycerol to the reaction lowered the initial velocities of both the activity of the transribosylation and the hydrolysis below 60°C, but the difference in enzyme activity between the reaction with and without 50% (v/v) glycerol was negligible at 70°C. Regardless of the addition of glycerol, the maximum transribosylation activity was obtained at 60°C or more. However, *LbNH* is known to be inactivated by heating at 60°C or more for over 1 h (55). Therefore, the reaction temperature was maintained at 50°C for 2'-OMe-AR production during longer reaction times. Additionally, the effect of saccharides on solution viscosity was also examined in the presence of glycerol, and the addition of 20% (w/v) dextrin most increased the amount of 2'-OMe-AR produced by 24% compared to the control without saccharide added (Table 2-4). The effect of 2'-OMe-UR concentration is shown in Fig. 2-5 (A). *LbNH* showed a maximum molar yield in the presence of 60 mM 2'-OMe-UR. The amount of 2'-OMe-AR produced increased as the concentration of 2'-OMe-UR increased, up to a concentration of 480 mM 2'-OMe-UR. However, substrate inhibition of the transribosylation reaction was observed above 240 mM 2'-OMe-UR, and the time required to reach the maximum yield of 2'-OMe-AR was delayed with increasing 2'-OMe-UR concentration (Table 2-5). The effect of adenine concentration is shown in Fig. 2-5 (B). *LbNH* showed a maximum molar yield in the presence of 200 mM adenine. Although the detailed mechanism is unclear, excess amounts of adenine, most of which precipitated in the reaction mixture, only inhibited the hydrolysis of 2'-OMe-AR, and the initial velocity of 2'-OMe-AR production was not affected (Fig. 2-6).

Table 2-3. The effect of hydrophilic solutes on 2'-OMe-AR production by *LbNH*

Hydrophilic solute	Concentration	Relative activity (%)
Control		100
Glycerol	10% (v/v)	106
	30% (v/v)	103
	50% (v/v)	79
	70% (v/v)	28
	90% (v/v)	0
1,3-Propanediol	50% (v/v)	68
(R)-1,2-Propanediol	50% (v/v)	4
(S)-1,2-Propanediol	50% (v/v)	6
NaCl	0.5 M	84
	1 M	90
	2 M	115
	3 M	84
	4 M	65
	5 M	45
Glucose	6 M	41
	20% (w/v) saturated	109
	40% (w/v) saturated	112
	60% (w/v) saturated	113
	80% (w/v) saturated	141
	100% (w/v) saturated	114

The reaction was carried out with 60 mM 2'-OMe-UR, 100 mM adenine and 0.25 U/mL purified *LbNH* at 50°C for 3 h. Relative activity was defined as the percent increase in mass amount of 2'-OMe-AR produced relative to the control without hydrophilic solute (defined as 100%).

**Fig. 2-3. Effect of hydrophilic solutes on 2'-OMe-AR production by *LbNH*-catalyzing transribosylation**

The effect of the addition of the hydrophilic solutes glycerol and glucose on 2'-OMe-AR production by *LbNH* is shown. The reaction was carried out with 60 mM 2'-OMe-UR and 100 mM adenine at 50°C for 74 h. The reaction without solute (closed diamonds), in the presence of 2 M NaCl (closed squares), 30% or 50% (v/v) glycerol (closed and open circles, respectively), or with an 80% or 100% saturated concentration of glucose (closed and open triangles, respectively) are indicated.

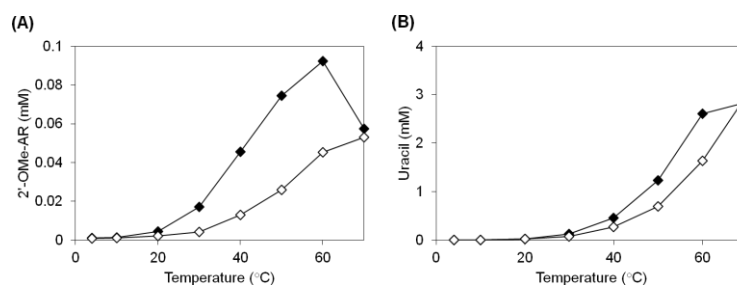


Fig. 2-4. Effect of temperature on the activity of *LbNH* in 2'-OMe-AR production and 2'-OMe-UR degradation

To examine the effect of temperature on the transribosylation and the hydrolytic activity, the reaction was carried out with 60 mM 2'-OMe-UR, 100 mM adenine and 0.044 U/mL purified *LbNH* at 4-70°C for 60 min, with or without 50% (v/v) glycerol. A, The effect of temperature on 2'-OMe-AR production by *LbNH*. Control reaction without glycerol (closed diamonds), and the reaction with 50% (v/v) glycerol (open diamonds) are shown. B, The effect of temperature on 2'-OMe-UR degradation by *LbNH*. Control reaction without glycerol (closed triangles), and the reaction with 50% (v/v) glycerol (open triangles) are shown.

Table 2-4. The effect of saccharides on 2'-OMe-AR production by *LbNH*

Saccharide	Concentration	Relative activity (%)
Control		100
Trehalose	100% saturated	97
Agarose	2% (w/v)	80
Methyl cellulose 15	2% (w/v)	93
Carboxymethyl cellulose	2% (w/v)	83
Dextrin	2% (w/v)	110
	5% (w/v)	115
	10% (w/v)	117
	20% (w/v)	124
Dextran	20% (w/v)	102

The reaction was carried out with 60 mM 2'-OMe-UR, 100 mM adenine, 50% (v/v) glycerol, 0.044 U/mL purified *LbNH* and various concentrations of saccharides at 50°C for 73 h. Relative activity was defined as the percent increase in mass amount of corresponding 2'-OMe-AR produced relative to the control without any saccharide added (defined as 100%).

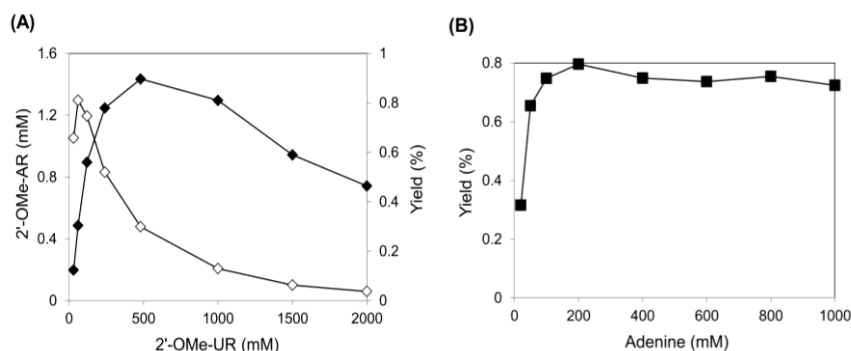


Fig. 2-5. Effect of substrate concentrations on 2'-OMe-AR production by *LbNH*-catalyzing transribosylation

A, The effect of 2'-OMe-UR concentration on 2'-OMe-AR production by *LbNH*. The reaction was carried out with 100 mM adenine, 50% (v/v) glycerol and 30-2000 mM 2'-OMe-UR, and the total amount (closed diamonds), and yield of 2'-OMe-AR produced (open diamonds) at 50°C after 72 h are shown. B, The effect of adenine concentration on the molar yield of 2'-OMe-AR production by *LbNH* with 120 mM 2'-OMe-UR, 50% (v/v) glycerol and 20-1000 mM adenine at 50°C after 24 h is shown. The molar yield (%) was defined as the amount of 2'-OMe-AR produced divided by the starting concentration of 2'-OMe-UR.

Table 2-5. The effect of 2'-OMe-UR concentration on 2'-OMe-AR production by *LbNH*

2'-OMe-UR (mM)	Reaction time (h)	Maximum amount of 2'-OMe-AR (mM)	Yield (%)
30	24	0.23	0.78
60	72	0.49	0.81
120	100	0.97	0.81
240	189	1.7	0.72
480	189	2.4	0.49
1000	189	2.3	0.23
1500	189	2.0	0.13
2000	189	1.6	0.081

The reaction was carried out with 30-2000 mM 2'-OMe-UR, 100 mM adenine, 50% (v/v) glycerol and 0.044 U/mL *LbNH* at 50°C for a maximum of 189 h. Reaction time (h) indicates the time when the maximum yield (%) was obtained. The yield (%) was defined as the amount of 2'-OMe-AR produced divided by the stating amount of 2'-OMe-UR.

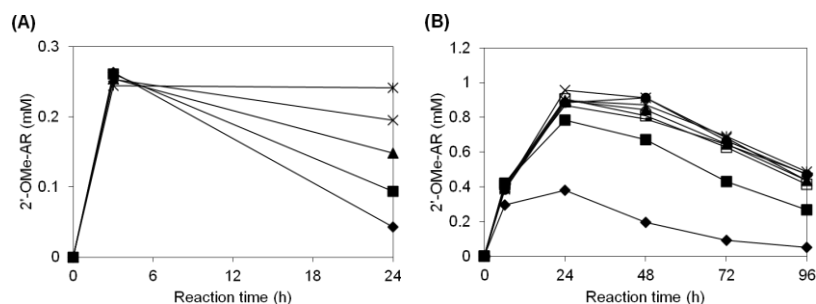


Fig. 2-6. Effect of adenine concentration on 2'-OMe-AR production by *LbNH* with increased reaction time

A, The effect of adenine concentration on 2'-OMe-AR production without glycerol is shown. The reaction was carried out with 60 mM 2'-OMe-UR, 30-2000 mM adenine and 0.044 U/mL purified *LbNH* at 50°C for 24 h. 30 mM (closed diamonds), 100 mM (closed squares), 500 mM (closed triangles), 1000 mM (x-marks) and 2000 mM (asterisks) are indicated. B, The effect of adenine concentration on 2'-OMe-AR production in the presence of 50% (v/v) glycerol is shown. The reaction was carried out with 120 mM 2'-OMe-UR, 20-1000 mM adenine and 0.044 U/mL purified *LbNH* at 50°C for 96 h. 20 mM (closed diamonds), 50 mM (closed squares), 100 mM (closed triangles), 200 mM (x-marks), 600 mM (asterisks) and 1000 mM (closed circles) are indicated.

Optimized bioprocess production of 2'-OMe-AR by *LbNH*-catalyzing transribosylation

Based on the result obtained above, the optimal conditions for 2'-OMe-AR production with *LbNH* were determined to be 60 mM 2'-OMe-UR and 200 mM adenine with 50% (v/v) glycerol and 20% (w/v) dextrin at 50°C. Under these conditions, *LbNH* produced a maximum of 0.16 g/L (0.58 mM) of 2'-OMe-AR after 72 h, and its molar yield reached 0.97% towards 2'-OMe-UR (Fig. 2-7).

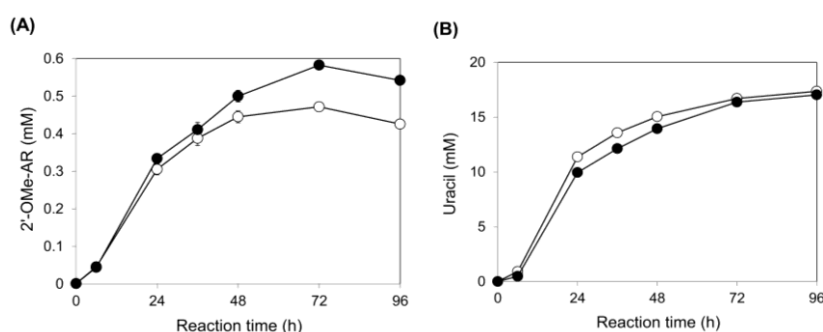


Fig. 2-7. Enzymatic synthesis of 2'-OMe-AR with *LbNH* using optimal conditions

2'-OMe-UR production was carried out using a reaction mixture containing 60 mM 2'-OMe-UR, 200 mM adenine, 50% glycerol (v/v), and 0.04 U/mL *LbNH* in 50 mM KPB, pH 7.0, at 50°C, with or without 20% (w/v) dextrin. A, 2'-OMe-AR production and B, uracil production are shown. Reactions with 20% (w/v) dextrin (closed circles) and without dextrin (open circles) are indicated. The y-axis error bars represent the standard deviation calculated from three measurements.

Discussion

We have shown here that *LbNH* is a bi-functional enzyme catalyzing both the hydrolysis and the transribosylation reactions towards 2'-OMe-UR. The hydrolytic activity of *LbNH* was affected by the mixed non-competitive inhibition by adenine, suggesting that adenine could bind to both the free enzyme and the enzyme-substrate complex. K_i and K_i' are dissociation constants for the enzyme-inhibitor complex and the enzyme-substrate-inhibitor complex, and the observation here that $K_i \ll K_i'$ indicates that adenine bound to the enzyme-substrate complex more readily than the free enzyme. Moreover, this indicated that the conformational change of the enzyme structure occurred after the binding between *LbNH* and 2'-OMe-UR. Several enzymes belonging to the glycoside hydrolase superfamily are known to be bi-functional enzymes, catalyzing both the hydrolysis and the transglycosylation towards disaccharides, and substrate inhibition is observed at high disaccharide concentrations (60,61). According to the catalytic mechanism of glycoside hydrolases, we hypothesized a reaction scheme for *LbNH* (Fig. 2-8). In this scheme, *LbNH* has two substrate binding sites, for a ribose moiety as donor and a nucleobase as acceptor. The *LbNH*-2'-OMe-uridine complex was predicted to be an active intermediate for both the hydrolysis and the transribosylation, and adenine appeared to compete for this complex with a water molecule in the course of the transribosylation.

2'-OMe-AR and 2'-OMe-GR are important raw materials of nucleic acid drugs such as Pegaptanib (Macugen; Pfizer, New York, NY, USA), and 2'-OMe-GR is easily converted from 2'-OMe-AR by chemical methods (62). To improve the yield of 2'-OMe-AR production by bioprocess, the first approach was to modify solvent viscosity according to Kramers' theory. In this study, the addition of glycerol increased the yield of 2'-OMe-AR production by *LbNH*, which appeared to result from the increase in solvent viscosity. However, higher concentration of glycerol than 50% (v/v) inactivated *LbNH* irreversibly (Table 2-3). The second approach was to shift the equilibrium to 2'-OMe-AR accumulation. For example, replacing water as the reaction solvent with an organic solvent increasing adenine solubility can be a useful method. However, *LbNH* immediately lost activity in high concentrations of organic solvents such as pyrrolidine, which dissolved fifty times more adenine than distilled water (data not shown). Another approach is the utilization of homologous bi-functional enzymes which have weaker hydrolytic activity than that of *LbNH*. Studies of fungal β -glucosidases revealed that the transglycosylation activity varied significantly among enzymes belonging to the glycoside hydrolase family GH3 (63,64). *LbNH* was previously shown to form a new sub-cluster among

the nucleoside hydrolase superfamily with function-unknown enzymes (55). Thus, the exploitation of homologous enzymes of *LbNH* from the environment may be useful for the production of various 2'-OMe-NRs.

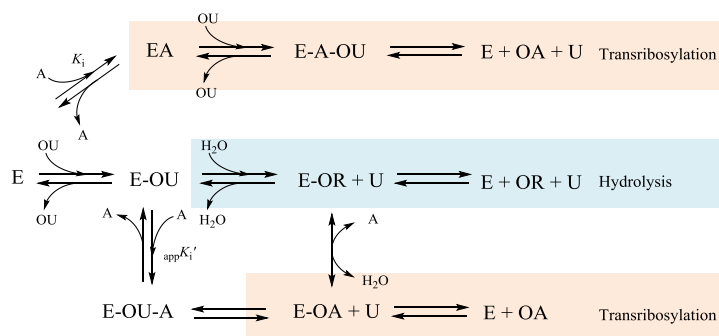


Fig. 2-8. Hypothetical reaction mechanism of *LbNH*-catalyzing reaction

A hypothetical reaction mechanism for the 2'-OMe-UR coupling with adenine catalyzed by *LbNH* is shown. *LbNH* (E), 2'-OMe-UR (OU), 2'-OMe-ribose (OR), uracil (U), and adenine (A) are indicated. K_i and K_i' denotes dissociation constants yielding an enzyme-inhibitor (E-A) complex and an enzyme-substrate-inhibitor complex (E-OU-A), respectively.

Summary

2'-O-Methylribonucleosides (2'-OMe-NRs) are promising raw materials for the production of nucleic acid drugs. We previously reported that *LbNH*, a nucleoside hydrolase from *Lactobacillus buchneri* LBK78 (NITE P-01581), was the first enzyme found to act on 2'-OMe-NRs. In the present study, we determined that *LbNH* also has the transribosylation activity between 2'-OMe-NRs and nucleobases, in addition to the hydrolyzing activity towards 2'-OMe-NRs. When 2'-O-methyluridine (2'-OMe-UR) and adenine were reacted with *LbNH*, 2'-O-methyladenosine (2'-OMe-AR) was produced. *LbNH* preferred purine nucleobases as its acceptor substrates for the transribosylation with 2'-OMe-UR as a donor substrate. Kinetic analysis of *LbNH* revealed that adenine behaved as a mixed inhibitor of the hydrolysis of 2'-OMe-UR. Under the optimal reaction conditions, the maximum molar yield of enzymatic 2'-OMe-AR produced reached 0.97% towards 2'-OMe-UR, corresponding to 0.16 g/L.

CHAPTER III

A new nucleoside hydrolase with transribosylation activity from *Agromyces* sp. MM-1 and its application for enzymatic synthesis of 2'-O-methylribonucleosides

Introduction

2'-O-Methylribonucleosides (2'-OMe-NRs) are raw materials of nucleic acid drugs. Nucleic acid drugs are composed of oligonucleotides and promising next-generation medicines applied for the various applications (12,13). Introduction of 2'-OMe-NRs provide oligonucleotides high thermal stability and metabolic resistance *in vivo* (4-7). Currently, all 2'-OMe-NRs are synthesized by chemical methods. However, such chemical methods usually acquire wasteful multi-step processes especially in the production of purine nucleosides such as 2'-O-methyladenosine (2'-OMe-AR) (15-17,47). Thus, enzymatic reactions such as transribosylation and phosphorolysis are promising alternatives to chemical synthesis in 2'-OMe-NRs production because of their high stereo- and regio-selectivity under mild conditions (20,48-53).

In the previous study, we identified a novel nucleoside hydrolase, *LbNH*, of *Lactobacillus buchneri* LBK78 as the first enzyme acting to 2'-O-methyluridine (2'-OMe-UR) (55). Surprisingly, *LbNH* was a bi-functional enzyme catalyzing the hydrolysis and the transribosylation towards 2'-OMe-NRs, and the enzymatic synthesis of 2'-OMe-AR was achieved via the transribosylation between 2'-OMe-UR and adenine with *LbNH* (65). Although *LbNH* also hydrolyzed 2'-OMe-AR, the addition of hydrophilic solutes such as glycerol and dextrin suppressed its hydrolytic activity and increased the production yield 1.8 times more than without any solutes. However, the maximum yield of 2'-OMe-AR reached no more than 1%, and *LbNH* was difficult to accept nucleobase other than adenine as its acceptor substrate in the transribosylation. Therefore, we conducted a microbial screening to develop novel biocatalyst for the transribosylation to produce 2'-OMe-NRs and identified an actinomycete nucleoside hydrolase family enzyme, *AgNH*, of *Agromyces* sp. MM-1. *AgNH* had differences in amino acid sequence and catalytic properties from *LbNH* and was especially suitable as the transribosylation biocatalyst for the production of 2'-O-methyl-6-chloroguanosine (2'-OMe-6ClGR).

Materials and Methods

Chemicals

Standard reagents of 2'-OMe-NRs other than 2'-O-methylribose (2'-OMe-ribose) were obtained from Carbosynth Limited (Compton, Berkshire, United Kingdom). The standard reagent of 2'-OMe-ribose was purified from hydrolysates of 2'-OMe-UR according to the previous study (55). All other reagents and solvents were obtained from commercial sources.

Analysis of nucleosides, nucleobases, and sugars

For thin-layer chromatography (TLC) analysis, 2 μ L of each sample was spotted onto a silica gel TLC plate (Kieselgel 60 F₂₅₄; Merck Millipore, MA, USA), and developed with a solvent system containing ethyl acetate/methanol/0.1N hydrochloric acid (15/2/2, v/v/v). Nucleobases and nucleosides were detected qualitatively by UV absorbance with a wavelength at 254 nm. Sugars and nucleosides were visualized by spraying the plates with *p*-anisaldehyde/acetic acid/sulfuric acid/ethanol (2/1/4/96, v/v/v/v) and heating at 105°C. For high-performance liquid chromatography (HPLC) analysis, an LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a COSMOSIL 5C₁₈-ARII column (4.6 $\times$ 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) was used for separation at 40°C. The mobile phase was 10 mM ammonium acetate (pH 4.5, eluent A) and acetonitrile (eluent B), and the flow rate was 1.5 mL/min. The eluent gradients were 0% (v/v) B for 0-5 min, 0-5% (v/v) B over 5-10 min, 5-60% (v/v) B over 10-17.5 min, and 60% (v/v) B for 17.5-22.5 min. Nucleobases and nucleosides were quantitatively detected by UV absorbance at 254 nm. For LC-MS analysis of products from the transribosylation reaction between 2'-OMe-UR and nucleobases, the samples were analyzed by electrospray ionization mass spectrometry using a LCMS-2010A (Shimadzu). MS conditions were as follows: block temperature, 200°C; curved desolvation line (CDL) temperature, 250°C; detector voltage, 1.5 kV; nebulizing gas flow, 1.5 L/min.

Screening for 2'-OMe-AR-producing microorganisms

All soil samples used for the screening were collected in the north campus of Kyoto University (Kyoto, Japan). For the isolation of microorganisms utilizing 2'-OMe-UR, an isolation culture medium containing 0.1% (w/v) 2'-OMe-UR, 0.1% (w/v) K₂HPO₄, 0.1% (w/v) KH₂PO₄, 0.01% (w/v) yeast extract, 0.03% (w/v) MgSO₄·7H₂O, and 0.1% (w/v) NH₄Cl in distilled water, pH 7.2, was used. Soil-isolated microorganisms were inoculated into an isolation culture medium, and shaken with 300 rpm for 24 h at 28°C. After the incubation, the mixture was centrifuged at 20,400 $\times g$ for 15 min, and 2 μ L and 100 μ L of the supernatant was subjected to TLC and HPLC analysis, respectively. To preserve 2'-OMe-UR-utilizing microorganisms, a 2% (w/v) plate of isolation culture medium was used. During the microbial screening, the reaction mixture containing 30 mM 2'-OMe-UR, 10 mM adenine and each resting cells (2-10 mg [wet weight]) in 50 mM KPB, pH 7.0, was used. After incubation for 24 h at 50°C with shaking at 300 rpm, the

mixture was centrifuged at $20,400\times g$ for 15 min and the supernatant was subjected to TLC and HPLC analyses.

Identification of Strain MM-1

Strain MM-1 was cultivated in 30 mL TGY broth containing 0.015% (w/v) tryptone, 0.015% (w/v) yeast extract, 0.003% (w/v) glucose and 0.003% (w/v) K_2HPO_4 , pH 7.0. Cells were harvested by centrifugation at $15,200\times g$ for 20 min, and total DNA from cells was isolated from the cells using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Nearly full length of the 16S ribosomal DNA (16S rDNA) gene was amplified by PCR using the following primers: 27f primer; 5' AGAGTTTGTATCMTGGCT CAG 3' (M is A or C) and 1492r primer; 5' GGTTACCTTGTTACGACTT 3' with a C1000 Thermal Cycler (Bio-Rad Laboratories KK, Tokyo, Japan). The resulting PCR product was purified using FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), and DNA sequencing was performed using the Genome Lab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Brea, CA, USA) with a DNA sequencer CEQ 2000XL DNA Analysis System (Beckman Coulter). In order to obtain the 16S rDNA sequence, following primers were used: 27f primer, 357f primer; 5' CTCCTACGGGAGGCAGCAG 3', 519r primer; 5' GWATTACCGCGGCKGCTG 3' (W is A or T, and K is G or T), 530f primer; 5' GTGCCAGCMGCCGCGG 3', 907r primer; 5' CCGTCAATTCMTTTRAGTTT 3', 1100r primer; 5' GGGTTGCGCTCGTTG 3', 1392r primer 5' ACGGGCGGTGTGTRC 3' (R is G or A), and 1492r primer. Sequence data were assembled by Genetyx (Genetyx Corporation, Tokyo, Japan).

Purification and gene cloning of the enzyme

Agromyces sp. MM-1 was cultivated in 10 L of a culture medium containing 0.1% (w/v) 2'-OMe-UR, 0.1% (w/v) K_2HPO_4 , 0.1% (w/v) KH_2PO_4 , 0.01% (w/v) yeast extract, 0.03% (w/v) $MgSO_4\cdot 7H_2O$, 0.2% (w/v) NH_4Cl , 0.05% (w/v) glucose and 0.15% (w/v) peptone in distilled water, pH 7.0, for 120 h at 28°C with shaking at 120 rpm. Cells from 10 L of culture (11 g [wet weight]) were harvested by centrifugation at $15,200\times g$ for 20 min, washed and re-suspended with 30 mL of 20 mM KPB, pH 7.0 (purification buffer). The cell suspension was disrupted by ultrasonication for 30 min with an Insonator 201M (KUBOTA, Osaka, Japan). The disrupted cell suspension was centrifuged at $20,700\times g$ for 20 min and the resulting supernatant (33 mL) was used as the cell-free extracts (CFE). The CFE was separated by ammonium sulfate fractionation. Solid ammonium sulfate (7.7 g) was added to the CFE (33 mL), and the precipitate formed was removed by centrifugation. Additional solid ammonium sulfate (1.6 g) was added to the supernatant (12 mL), and the precipitate with 40 % saturation of ammonium sulfate was collected by centrifugation and dissolved in purification buffer (10 mL). The resultant enzyme solution was

dialyzed twice against 2 L of purification buffer and then applied to a MonoQ HR10/100 column (GE Healthcare, Buckinghamshire, UK) equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions (eluted with 0.40 to 0.45 M NaCl) were collected and concentrated by ultrafiltration using an Ultracel-10K membrane (Millipore, MA, USA). The concentrated sample was applied to a Superdex200 HR10/30 column (GE Healthcare) equilibrated with purification buffer containing 0.2 M NaCl, and then eluted with the same buffer. The active fractions were collected and diluted to a final volume of 10 mL by purification buffer. The diluted fractions were applied to a MonoQ HR5/5 column (GE Healthcare) equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions (eluted with 0.40 to 0.45 M NaCl) were collected, desalted, and diluted by 5 mL of containing 5 mM sodium phosphate buffer, pH 6.8. The diluted fractions were applied to a CHT Type I column (Nippon Bio-Rad Laboratories, Tokyo, Japan) equilibrated with 5 mM sodium phosphate buffer, pH 6.8. The enzyme was eluted with a linear gradient of 5-500 mM sodium phosphate buffer, pH 6.8. The active fractions (eluted with 5 mM of sodium acetate buffer, pH 6.8) were collected, and replaced the buffer with 5 mL of purification buffer. The active fractions were applied to a MonoQ HR5/5 column (GE Healthcare) equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions (eluted with 0.36 to 0.38 M NaCl) were collected and concentrated to 100 μ L by ultrafiltration using an Ultracel-10K membrane (Millipore). The purity of the proteins in the active fractions was checked by SDS-PAGE using a 12.5% polyacrylamide gel stained with coomassie brilliant blue R-250. The N-terminal amino acid sequences of the proteins were determined as described previously (55). To determine the 5'-terminal sequence of the gene encoding AgNH, the genomic DNA of *Agromyces* sp. MM-1 was extracted and amplified using the following primers: 5' ATGACCCGAATGATCATCGA 3' and 5' TCGGCGGCTGCGGTGATGTT 3'. The remaining sequence of the gene was determined by inverse PCR as described below. The extracted genomic DNA was subjected to completely digestion by restriction endonuclease XhoI (Takara Bio) followed by circularization. Inverse PCR was performed using the following primers: 5' TCGATGATCATTCGGGTCAT 3' and 5' GGCCGAGTACCACGTCGCAA 3'. DNA sequences of all PCR products were determined, assembled, and analyzed by Genetyx (Genetyx Corp.).

Expression of recombinant AgNH in *Escherichia coli*

The gene encoding AgNH of *Agromyces* sp. MM-1 was amplified by PCR using the following primers:

5' GAATTCATTAAAGAGGAGAAATTAACCATGACCCGAATGATCATCGA 3' and 5' CAACAGGAGTCCAAGCTCAGCTAATTATCATGCGGTGCCGCCGAACG 3'. The PCR

product was cloned into the expression vector pQE60 (Qiagen) under transcriptional control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *lac* promoter, and the resultant plasmid was introduced into *E. coli* JM109 (DE3) cells (Novagen, Madison, WI, USA). The transformant was cultured at 20°C for 24 h in Luria Bertani (LB) medium containing 1% (w/v) tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract, supplemented with 5 μ g/mL ampicillin and 1 mM IPTG. The cells were harvested by centrifugation, washed twice with 0.85 % NaCl, suspended with two volumes (w/w) of purification buffer, and disrupted by ultrasonication for 30 min. The lysate was centrifuged at 13,000 $\times g$ for 20 min at 4°C and the supernatant was used for the following purification steps.

Purification and molecular mass determination of recombinant AgNH

The supernatant prepared as described above was applied to a MonoQ HR10/10 column equilibrated with purification buffer. The enzyme was eluted with a linear gradient of 0-1.0 M NaCl in purification buffer. The active fractions were collected and concentrated by ultrafiltration. The concentrated protein solution was applied to a Superdex200 HR10/30 column equilibrated with purification buffer, containing 0.2 M NaCl and then eluted with same buffer. The relative molecular mass of the purified recombinant AgNH was determined from the relative mobility of molecular mass standards (Gel Filtration Calibration Kit LMW and HMW; GE Healthcare) using a LC-10A HPLC system equipped with a gel filtration chromatography column (TSKgel G3000SW, 7.5 $\times$ 600 mm; Tosoh, Tokyo, Japan) as described previously (55). The purity of the recombinant enzyme checked by SDS-PAGE analysis with 12.5% gel stained with coomassie brilliant blue.

Assay for the hydrolysis reaction with AgNH

To characterize the hydrolysis of 2'-OMe-UR with the recombinant AgNH, a standard reaction mixture A containing 60 mM 2'-OMe-UR and 0.15-0.50 U/mL purified recombinant AgNH in 50 mM KPB, pH 7.0, was used and the reaction was allowed to proceed at 50°C for 10 min. After incubation, the reaction mixture was diluted five-fold with 1% (v/v) trifluoroacetic acid to terminate the reaction, and centrifuged at 20,400 $\times g$ for 15 min at 4°C. Nucleosides and nucleobases in the supernatant were evaluated by HPLC analysis, and the hydrolysis activity was determined as the production of corresponding nucleobases. The effect of pH on the enzyme activity was measured using the standard reaction mixture A, but with 50 mM sodium citrate buffer (pH 3-6), 50 mM KPB (pH 7), 50 mM Tris/HCl buffer (pH 7-9), CAPS/NaOH buffer (pH 10-11), 50 mM NaOH/KCl buffer (pH 12-13), and 50 mM NaOH (pH > 14) as reaction buffers at 50°C for 10 min. The pH stability was examined by measuring the residual activity after incubation in each reaction buffer at 4°C for 1 h. The effect of temperature on the enzyme activity

was measured using the standard reaction mixture A incubated at 4-90°C for 10 min. The temperature stability was examined by measuring the residual activity after incubation at each temperature for 1 h. For substrate specificity analysis of AgNH, the 2'-OMe-UR in the standard reaction mixture A was replaced with 2.5-60 mM of various 2'-OMe-NRs, ribonucleosides, 2'-deoxyribonucleosides, and other nucleotide derivatives, and the reaction was allowed to proceed at 50°C for 1 h.

Assay for the transribosylation reaction with AgNH

To characterize the transribosylation of 2'-OMe-UR with the recombinant AgNH, a standard reaction mixture B containing 60 mM 2'-OMe-UR, 100 mM adenine, and 0.4-5.8 U/mL purified recombinant AgNH in 50 mM KPB, pH 7.0, was used and the reaction was allowed to proceed at 50°C for 24 h. The transribosylation activity was determined as the production amount of corresponding 2'-OMe-NRs measured by the HPLC analysis. For the substrate specificity analysis, adenine was replaced with various nucleobases in the standard reaction mixture. The effect of hydrophilic solutes was measured using the standard reaction mixture B containing 240 mM 2'-OMe-UR, 200 mM adenine and various concentrations of NaCl, amino acid, sugars, and alcohols. The effect of pH on the transribosylation in the presence of 50% (v/v) glycerol was measured using the standard reaction mixture B, but with 50 mM KPB (pH 7), 50 mM Tris/HCl buffer (pH 8), 50 mM NaOH/KCl buffer (pH 13), and 50 mM NaOH (pH > 14) as reaction buffers at 50°C. The effect of 2'-OMe-UR concentrations on the transribosylation was measured in the standard reaction mixture containing 15-240 mM 2'-OMe-UR with 50% (v/v) glycerol. Similarly, the effect of adenine concentrations on the transribosylation was measured in the standard reaction mixture containing 25-200 mM adenine with 50% (v/v) glycerol. The molar yield was determined as the value that is the amount of 2'-OMe-AR divided by the starting concentration of 2'-OMe-UR.

Kinetic analysis of AgNH

To determine the kinetic constants, 0.37 U/mL purified recombinant AgNH was reacted at 50°C in 50 mM KPB, pH 7.0. In order to determine kinetic constants for the hydrolysis, 2'-OMe-UR was used at concentrations of 0.05-2 mM, and the reaction was allowed to proceed for 3 min. The hydrolytic activity was determined as the production of uracil. In order to determine kinetic constants for the transribosylation, 2'-OMe-UR was used at concentrations of 0.25-60 mM with 20 mM adenine, and adenine was used at concentration of 0.25-20 mM with 60 mM 2'-OMe-UR. Each reaction was allowed to proceed for 15 min. The transribosylation activity was determined as the production of 2'-OMe-AR. The simultaneous hydrolytic activity was calculated by subtracting the amount of 2'-OMe-AR from the amount of uracil. Kinetic constants

were determined using R software (<http://www.R-project.org/>) and the drc package (22) to fit the Michaelis-Menten equation for K_m and k_{cat} . In order to determine the inhibition constants of the hydrolysis, adenine was used at concentration of 0-5 mM with 5, 10 or 60 mM 2'-OMe-UR, and the reaction was allowed to proceed for 3 min. Four inhibition mechanism models were constructed based on Eq. 1, 2, 3 and 4, and the fitting of these inhibition models, where [S] and [I] corresponded to the concentration of 2'-OMe-UR and adenine, respectively. Inhibition constants were calculated using R software based on least square method.

Eq. 1

$$v = \frac{V_{max}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)}$$

Eq. 2

$$v = \frac{V_{max}[S]}{([S] + K_m) \left(1 + \frac{[I]}{K_i}\right)}$$

Eq. 3

$$v = \frac{V_{max}[S]}{K_m + [S] \left(1 + \frac{[I]}{K'_i}\right)}$$

Eq. 4

$$v = \frac{V_{max}[S]}{[S] \left(1 + \frac{[I]}{K'_i}\right) + K_m \left(1 + \frac{[I]}{K_i}\right)}$$

Enzymatic synthesis of 2'-OMe-NRs under optimal conditions

Production of 2'-OMe-AR was carried out in the reaction mixture containing 60 mM 2'-OMe-UR, 100 mM adenine, 50% glycerol (v/v), 20% (w/v) dextrin and 3.7 U/mL purified recombinant AgNH in 50 mM KPB, pH 7.0. Reaction was allowed to proceed at 50°C for 293 h. Production of 2'-OMe-6ClGR was carried out in the reaction mixture containing 60 mM 2'-OMe-UR, 100 mM 6-chloroguanine, 50% glycerol (v/v) and 3.7 U/mL purified recombinant AgNH in 50 mM KPB, pH 7.0. Reaction was allowed to proceed at 50°C for 217 h.

Identification of products in the transribosylation between 2'-OMe-UR and 6-chloroguanine with AgNH

Two milliliters of a reaction mixture comprising 60 mM 2'-OMe-UR, 100 mM

6-chloroguanine and 3.7 U/mL purified recombinant AgNH in 50 mM KPB, pH 7.0, was incubated at 50°C for 169 h. The mixture was then applied to the HPLC, and the peak fractions (eluted with 12% (v/v) to 15% (v/v) B) were collected. The fractions containing a nucleoside were lyophilized by FreeZone 6 (Labconco, MO, USA), and dissolved in D₂O. The spectra of ¹H NMR and ¹³C NMR were recorded on an Avance 500 (BrukerBioSpin, MA, USA). Alternatively, the lyophilized samples were dissolved in distilled water, and applied to the ESI-MS analysis.

Results

Isolation of 2'-OMe-UR-utilizing strain MM-1

We isolated more than 1,000 microorganisms assimilating 2'-OMe-UR from environmental samples. These microorganisms were screened for the transribosylation activity between 2'-OMe-UR and adenine to produce 2'-OMe-AR by using TLC and HPLC analyses. As the result, whole cells of an isolate labeled as MM-1 were found to significantly degrade 2'-OMe-UR and to produce 2'-OMe-AR in the reaction mixture containing 2'-OMe-UR and adenine. Based on the phylogenetic analysis of 16S rDNA sequence, strain MM-1 was identified as *Agromyces* sp. MM-1, and then was used in the further investigation.

Characterization of a 2'-OMe-AR-producing enzyme of *Agromyces* sp. MM-1

In order to identify the enzyme responsible for 2'-OMe-AR production, the CFE of *Agromyces* sp. MM-1 was subjected to a six-step purification procedure. Then, several protein bands were observed by SDS-PAGE analysis of the partially purified enzyme (data not shown). According to the N-terminal amino acid sequence analysis of these proteins, the sequence of one of the protein bands, TRMIIDTDTAQDDCIALLVGLLDPAATLEAITMVAGNVGF, matched at 85% to that of a putative nucleoside hydrolase of *Nocardiopsis valliformis* (WP 017581380.1). Thus, this protein was targeted to obtain the complete nucleotide sequence and termed as AgNH. When compared to the deduced full-length amino acid sequence, AgNH had homology to a nucleoside hydrolase of *Microbacterium resistens* (WP 067247535.1) with an identity of 97%. Because the recombinant AgNH showed 2'-OMe-AR-producing activity from 2'-OMe-UR and adenine, it was confirmed that AgNH was the enzyme responsible for the transribosylation in *Agromyces* sp. MM-1. When the reaction mixture containing 2'-OMe-UR, AgNH also showed the hydrolysis activity towards 2'-OMe-UR and produced uracil and 2'-OMe-ribose like as LbNH (55). The gene of AgNH was composed of 966 bp and encoded a protein containing 322 amino acids, corresponding to a theoretical subunit molecular mass of 34,000. When analyzed by gel filtration chromatography, the purified recombinant enzyme showed a single symmetrical peak

corresponding to a native molecular mass of 145,000 (Fig. 3-1). From these observations, AgNH was seemed to be a homotetrameric enzyme.

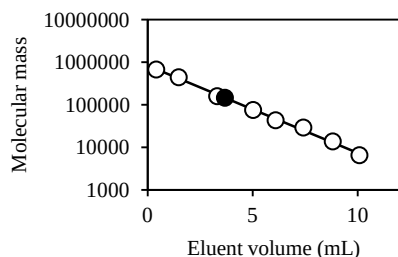


Fig. 3-1. Molecular mass determination of recombinant AgNH

Gel filtration chromatography analysis of recombinant AgNH. AgNH (closed circle) and molecular mass standards (open circles): thyroglobulin (670,000), ferritin (440,000), aldolase (160,000), conalbumin (75,000), ovalbumin (43,000), carbonic anhydrase (29,000), ribonuclease A (14,000), and aprotinin (6,500).

Kinetic analysis of AgNH

Normal hyperbolic kinetics was observed in the AgNH reaction, and the resulting kinetic constants are summarized in Table 3-1. In the transribosylation reaction between 2'-OMe-UR and adenine, non-linear regression analysis using the Michaelis-Menten model of the data yielded an apparent K_m of 60 ± 29 mM for 2'-OMe-UR and of 16 ± 7 mM for adenine, and a k_{cat} of $4.2 \times 10^{-4} \pm 1.2 \times 10^{-4} \text{ s}^{-1}$ for 2'-OMe-UR and of $3.1 \times 10^{-4} \pm 0.8 \times 10^{-4} \text{ s}^{-1}$ for adenine. The catalytic efficiency (k_{cat}/K_m) was 7.0×10^{-6} for 2'-OMe-UR and 1.9×10^{-5} for adenine. In the hydrolysis reaction towards 2'-OMe-UR without adenine, non-linear regression analysis using the Michaelis-Menten model of the data yielded an apparent K_m of 3.6 ± 0.4 mM, k_{cat} of $9.4 \pm 0.5 \text{ s}^{-1}$, and $k_{cat}/appK_m$ of $2.6 \text{ mM}^{-1} \cdot \text{s}^{-1}$. On the other hand, the initial velocity of the hydrolysis reaction towards 2'-OMe-UR lowered with adenine. In the presence of a saturated concentration of adenine, the data yielded an apparent K_m of 5.3 ± 0.3 mM, k_{cat} of $6.3 \pm 0.1 \text{ s}^{-1}$, and $k_{cat}/appK_m$ of $1.2 \text{ mM}^{-1} \cdot \text{s}^{-1}$. In the comparison of inhibition models accounting for the inhibition of the hydrolytic activity by adenine, the mixed non-competitive inhibition model based on Eq. 4 was the best fit as well as LbNH (Fig. 3-2). The data yielded an apparent K_i value of 14 mM and K_i' value of 26 mM.

Table 3-1. Kinetic constants on the hydrolysis towards 2'-OMe-UR and the transribosylation towards 2'-OMe-UR and adenine with AgNH

Reaction	Enzyme	Substrate	Additive	Kinetic constants			Inhibitory constants	
				K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	K_i (mM)	K_i' (mM)
Hydrolysis	AgNH	2'-OMe-UR	Adenine	3.6 ± 0.4 5.3 ± 0.3	9.4 ± 0.5 6.3 ± 0.1	2.6 1.2	14	26
			Adenine	0.32 ± 0.01 0.66 ± 0.05	3.6 ± 0.0 1.4 ± 0.0	11 2.2	0.45	8.3
	LbNH	2'-OMe-UR	Adenine	0.66 ± 0.05	1.4 ± 0.0	2.2		
Transribosylation	AgNH	2'-OMe-UR		60 ± 29	$4.2 \times 10^{-4} \pm 1.2 \times 10^{-4}$	7.0×10^{-6}		
		Adenine		16 ± 7	$3.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$	1.9×10^{-5}		
	LbNH	2'-OMe-UR		1.8 ± 0.1	$9.9 \times 10^{-2} \pm 0.2 \times 10^{-2}$	5.5×10^{-2}		
		Adenine		1.3 ± 0.1	$1.0 \times 10^{-1} \pm 0.0 \times 10^{-1}$	7.6×10^{-2}		

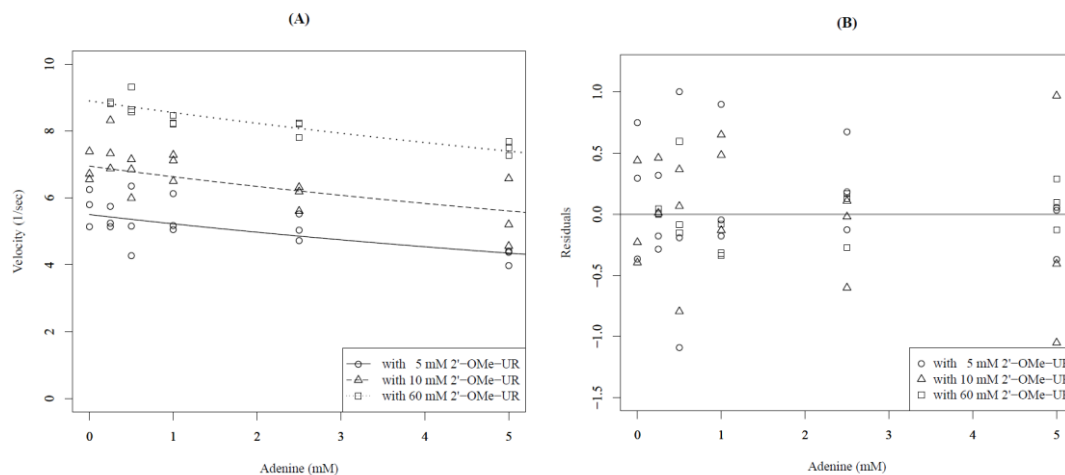


Fig. 3-2. Fittings and residuals of a mixed non-competitive inhibition model of the 2'-OMe-UR hydrolysis by adenine based on Eq. 4

To determine the inhibitory constants in the hydrolysis reaction towards 2'-OMe-UR, the reaction was carried out with 5, 10 or 60 mM 2'-OMe-UR and 0-5 mM adenine at 50°C for 3 min. A, Michaelis-Menten plots of experimentally determined (plots) and theoretically fit (curves) rate values of 2'-OMe-UR hydrolysis velocity in the presence of adenine. B, The residuals from a fitted model defined as the differences between the measured and the predicted values at each adenine concentration. The data obtained using 5 mM (open circles), 10 mM (open triangles), or 60 mM 2'-OMe-UR (open squares) and fitting curves based on the data obtained with 5 mM (solid line), 10 mM (dashed line), or 60 mM 2'-OMe-UR (dotted line) are indicated.

Characterization of AgNH-catalyzing hydrolysis

The effect of pH on 2'-OMe-UR hydrolysis was examined using pH values ranging from 3.0 to over 14.0. AgNH showed activity at all pH values tested, and the maximum activity was observed at pH 10.0 (Fig. 3-3 (A)). AgNH retained over 60% of its activity after 1 h of incubation at pH 5.0-12.0 (Fig. 3-3 (B)). Surprisingly, AgNH was not completely inactivated in 50 mM NaOH (pH > 14.0), and showed 26% of relative activity. The effect of temperature on 2'-OMe-UR hydrolysis was also examined using incubation temperatures ranging from 4°C to 90°C (Fig. 3-3 (C)). AgNH showed over 60% activity between 40°C and 60°C, and the maximum activity was observed at 50°C. AgNH activity markedly decreased at 40°C or below and 70°C or above. Incubation at temperatures above 50 °C also significantly reduced the hydrolysis activity of AgNH. The result of substrate specificity on the hydrolysis towards various nucleoside analogues was shown in Table 3-2. AgNH accepted various nucleoside analogues as its hydrolytic substrates as well as *Lb*NH. Among 2'-OMe-NRs tested, 2'-O-methylguanosine (2'-OMe-GR)

was the most preferable hydrolyzing substrate for AgNH with the relative activity 2,752% when compared to 2'-OMe-UR. AgNH also strongly hydrolyzed 2'-O-methylcytidine (2'-OMe-CR) (103%), but showed less activity towards 2'-O-methylinosine (2'-OMe-IR) and 2'-OMe-AR (6.6% and 0.82%, respectively). Among ribonucleosides tested, AgNH acted towards guanosine with the relative activity of 900%. Weaker relative activity was detected with the other ribonucleosides tested (5-methyluridine, cytidine, inosine, uridine and adenosine), and little or no hydrolysis activities were observed towards xanthosine and purine riboside. All 2'-deoxyribonucleoside compounds tested were accepted as hydrolyzing substrates of AgNH, but only very weak activity was observed except 2'-deoxyguanosine and 2'-deoxycytidine (9.9% and 1.8%, respectively).

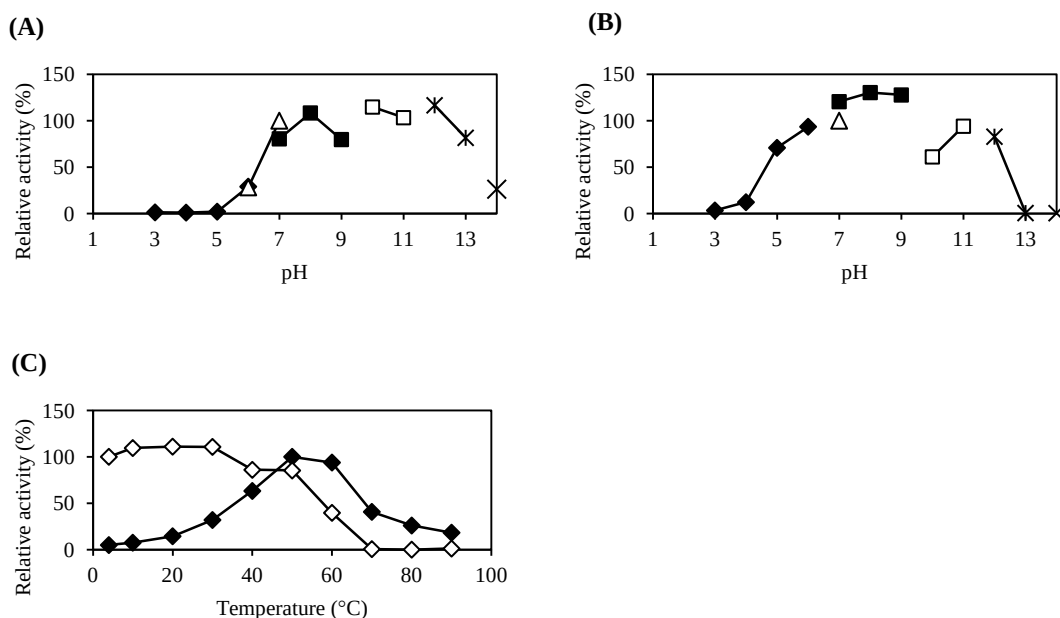


Fig. 3-3. Effects of pH and temperature on the activity and stability of AgNH in the hydrolysis towards 2'-OMe-UR

A, The effect of pH on the hydrolytic activity and B, The effect of pH on the stability of AgNH are shown. The reaction was carried out using a reaction mixture with 60 mM 2'-OMe-UR and 0.15-0.50 U/mL AgNH in 50 mM Sodium citrate buffer (pH 3.0-6.0, closed diamonds), KPB (pH 6.0-7.0, open triangles), Tris/HCl buffer (pH 7.0-9.0, closed squares), CASP/NaOH buffer (pH 10.0-11.0, open squares), NaOH/KCl buffer (pH 12.0-13.0, asterisks) and 50 mM NaOH (pH > 14.0, cross mark) at 50°C for 15min. Relative activity was defined as the percent increase in mass amounts of uracil in the case of pH 7.0 as 100%. C, Thermal effect on the hydrolytic activity (closed diamonds) and on the stability (open diamonds) of AgNH is shown. The reaction was carried out using a reaction mixture with 60 mM 2'-OMe-UR and 0.15 U/mL AgNH in 50 mM KPB, pH 7.0, for 15min. Relative activity was defined as the percent increase in mass amounts of uracil in the case of reaction at 50°C after incubation at 4°C as 100%.

Table 3-2. Substrate specificity on the hydrolysis towards nucleoside analogues

Substrate		Concentration (mM)	Relative activity (%)	
			AgNH	LbNH
2'-OMe-NRs	2'-OMe-UR	60	100	100
	2'-OMe-AR	10	0.82	9.3
	2'-OMe-GR	2.5	2752	383
	2'-OMe-IR	10	6.6	43
	2'-OMe-CR	60	103	30
Ribonucleosides	Uridine	60	0.89	41
	Adenosine	10	0.50	7.2
	Guanosine	2.5	900	1600
	Inosine	10	2.1	42
	Cytidine	60	4.1	8.0
	5-Methyluridine	60	4.4	1.6
	Xanthosine	2.5	trace	15
	Purine riboside	10	<i>n. d.</i>	<i>n. t.</i>
	2'-Deoxyribonucleosides			
	2'-Deoxyuridine	60	0.068	0.90
	2'-Deoxyadenosine	10	0.082	<i>n. d.</i>
	2'-Deoxyguanosine	2.5	9.9	<i>n. d.</i>
	2'-Deoxyinosine	10	0.078	0.89
	2'-Deoxycytidine	60	1.8	<i>n. d.</i>
	Thymidine	60	0.079	4.5

Relative activity was defined as the percent increase in mass amounts of corresponding heterocyclic base using 2'-OMe-UR as 100%.

n. d., not detected, *n. t.*, not tested

Amino acid sequence analysis of AgNH

Using BLASTP (protein-protein Basic Local Alignment Search Tool) to search a database of reference proteins, 5 putative nucleoside hydrolases were found to have high homology with AgNH. AgNH and these homologous enzymes were subjected to phylogenetic analysis together with representative 22 nucleoside hydrolase family enzymes including LbNH (Fig. 3-4) (23-35). AgNH and its homologous enzymes formed a cluster with ScCU-NH of *Sulfolobus solfataricus* which had about 32% homology with AgNH. However, substrate specificity between AgNH and ScCU-NH were greatly different. ScCU-NH was classified as a strictly pyrimidine-specific nucleoside hydrolase having no activity towards adenosine and guanosine (26), despite AgNH showed the highest hydrolysis activity towards 2'-OMe-GR. Although both of AgNH and LbNH preferred 2'-OMe-UR and guanosine analogues as their hydrolyzing substrate, their homology was rather low (24%). The alignment of the amino acid sequences of AgNH, LbNH and seven well-known nucleoside hydrolases is shown in Fig. 3-5. AgNH also had three amino acid motifs highly conserved among nucleoside hydrolase family enzymes as well as LbNH (36,55). A highly conserved motif, an aspartic acid-rich N-terminal motif (DXDXXXDD), corresponded to amino acids 7 to 14 of AgNH, which involved in the

binding of the catalytic Ca^{2+} ion. The AEXNXXDXPXAA sequence was the second conserved motif corresponding to amino acids 163 to 174 of AgNH. A catalytic aspartic residue was also conserved at Asp241 in AgNH, which completes the positioning of the catalytic Ca^{2+} ion in all nucleoside hydrolases. In *Crithidia fasciculata* IU-NH, a histidine residue was adjacent to this aspartic residue. This histidine residue was highly conserved in almost all purine-specific, non-specific, and pyrimidine-specific nucleoside hydrolases (37). However, this histidine residue was replaced by a proline in AgNH and ScCU-NH.

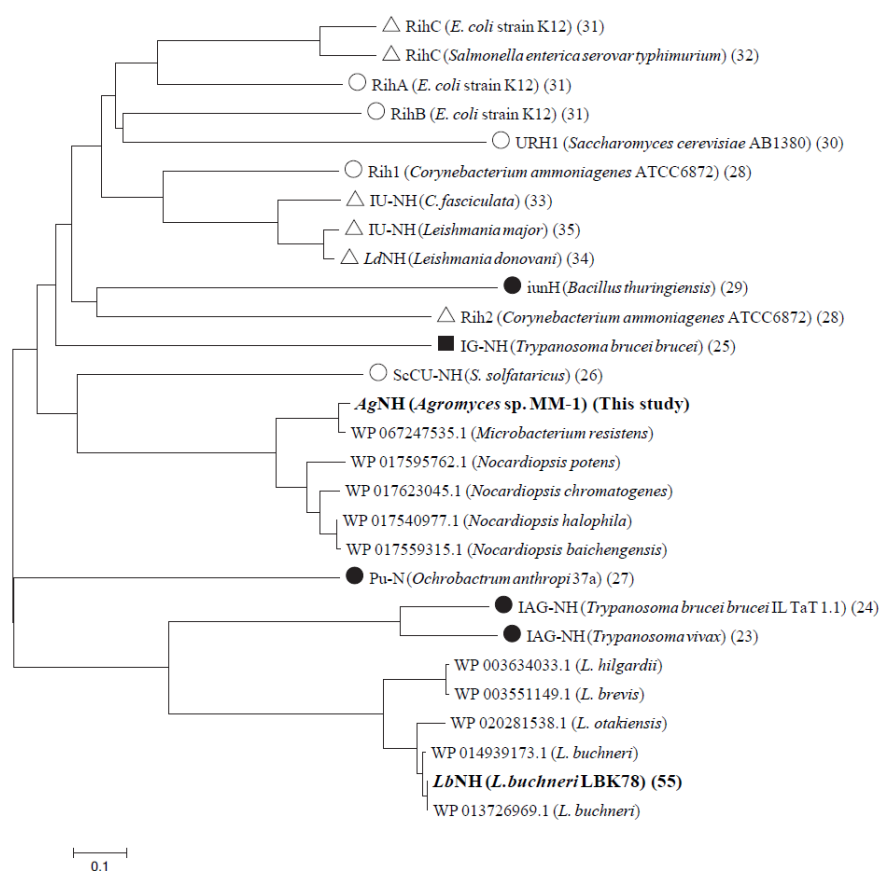


Fig. 3-4. Phylogenetic analysis of AgNH

AgNH, LbNH and their homologous enzymes were subjected to phylogenetic analysis with well-known hydrolases using the neighbor-joining method. The scale bar indicates 0.1 substitutions per nucleotide. These well-known hydrolases were classified into pyrimidine-specific (open circles), purine-specific (closed circles), non-specific (open triangles), and 6-oxopurine-specific (closed square) nucleoside hydrolases.

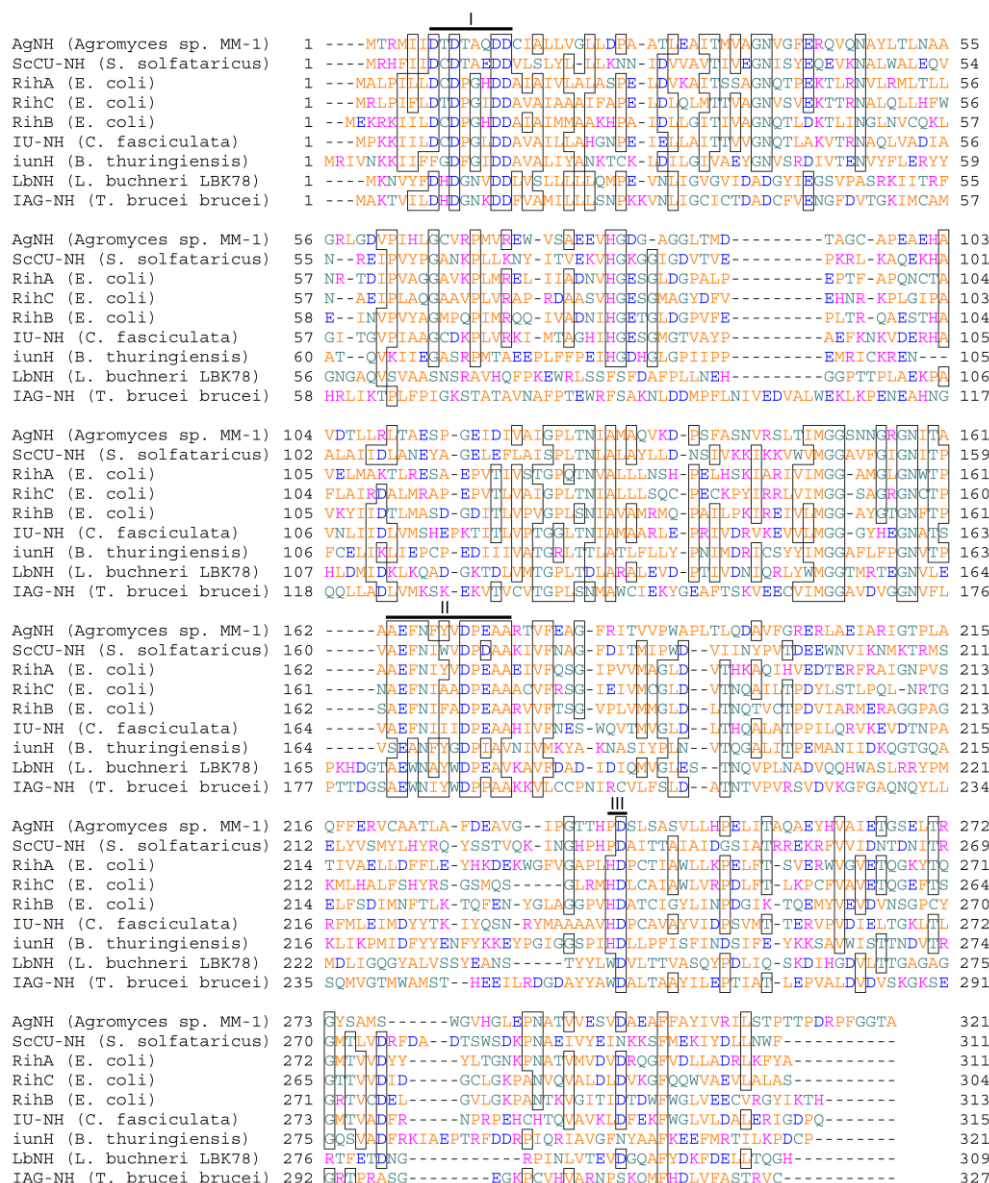


Fig. 3-5. Amino acid sequence alignment of AgNH and well-known nucleoside hydrolases

The highly conserved regions and residues within the nucleoside hydrolase superfamily are the Aspartic acid-rich *N*-terminal motif (I), the AEXNXXDXPXAA motif (II), and the region containing Pro240 and Asp241 in AgNH (III).

Substrate specificity analysis of AgNH-catalyzing transribosylation

In order to analyze the specificity of acceptor substrate in the transribosylation of AgNH, eleven nucleobases was reacted together with 2'-OMe-UR as a donor substrate (Table 3-3). Surprisingly, AgNH remarkably reacted to 6-chloroguanine with the relative activity of 520% compared to adenine. AgNH also preferred cytosine as its acceptor substrate with the relative activities of 70%, but showed less activity towards thymine, hypoxanthine, xanthine and

2,6-diaminopurine (23%, 13%, 5.4% and 5.1%, respectively). In the LC-MS analysis, reaction products from cytosine and hypoxanthine showed same retention times and molecular weights of standard reagents of 2'-*O*-methylcytidine and 2'-*O*-methylinosine, respectively. Although other standard reagents of predicted products were not available, molecular weights of new products derived from 6-chloroguanine, thymine, xanthine and 2,6-diaminopurine corresponded to those of 2'-OMe-6ClGR, 2'-*O*-methylthymine, 2'-*O*-methylxantosine, 2'-*O*-methyl-2,6-diaminopurine riboside, respectively. With other nucleobases such as guanine, 4-chlorouracil, dihydrothymine and 6-thioxanthine, any transribosylation activities were not detected. Thus, *AgNH* showed transribosylation activities towards 6-chloroguanine and pyrimidine nucleobases such as cytosine and thymine far more than *LbNH*.

Table 3-3. Acceptor substrate specificity on the transribosylation with 2'-OMe-UR as the donor substrate

Acceptor substrate	Relative activity (%)	
	<i>AgNH</i>	<i>LbNH</i>
6-Chloroguanine	520	4.1
Adenine	100	100
Cytosine	70	4.1
Thymine	23	0.04
Hypoxanthine	13	27
Xanthine	5.4	0.16
2,6-Diaminopurine	5.1	21
Guanine	<i>n. d.</i>	1.0
4-Chlorouracil	<i>n. d.</i>	0.012
Dihydrothymine	<i>n. d.</i>	<i>n. d.</i>
6-Thioxanthine	<i>n. d.</i>	<i>n. d.</i>

Relative activity was defined as the percent increase in mass amounts of corresponding 2'-OMe-NR using adenine coupling with 60 mM 2'-OMe-UR as 100%. *n. d.*, not detected.

Effect of reaction conditions on the production of 2'-OMe-AR with *AgNH*

Among hydrophilic solutes tested, the addition of 80-100% (w/v) saturated concentration of glucose and 30-50% (v/v) glycerol were found to significantly increase the production amount of 2'-OMe-AR in 24-h reaction (Table 3-4). Then, the effects of these solutes were estimated in the long-time reaction (Fig. 3-6), and the maximum production amount of 2'-OMe-AR was obtained with 50% (v/v) glycerol. Therefore, 50% (v/v) glycerol was used as a suitable hydrophilic solute in the subsequent experiments. The effects of 2'-OMe-UR concentration with 50% (v/v) glycerol were shown in Fig. 3-7 (A), and *AgNH* showed the maximum molar yield with 30 mM 2'-OMe-UR. The production amount of 2'-OMe-AR continuously increased as the concentration of 2'-OMe-UR increased up to 240 mM, but the molar yield decreased above 30 mM 2'-OMe-UR. The effect of adenine concentration with 50%

(v/v) glycerol was shown in Fig. 3-7 (B), and AgNH showed the maximum molar yield with 100 mM or more concentration of adenine. As observed in the case of *Lb*NH, excess amount of adenine above saturated concentration inhibited the hydrolysis of 2'-OMe-UR and 2'-OMe-AR by AgNH (data not shown).

Table 3-4. The effect of hydrophilic solute on 2'-OMe-AR production with AgNH

Hydrophilic solute	Concentration	Relative activity (%)
Control		100
Glucose	100% (w/v) saturated	177
	80% (w/v) saturated	165
	50% (w/v) saturated	145
Glycine	100% (w/v) saturated	61
	80% (w/v) saturated	67
	50% (w/v) saturated	80
NaCl	0.5 M	86
	1.0 M	80
	2.0 M	63
	3.0 M	52
	4.0 M	46
	5.0 M	40
Glycerol	6.0 M	36
	10% (v/v)	129
	30% (v/v)	221
	50% (v/v)	243
	70% (v/v)	119
	90% (v/v)	0.0

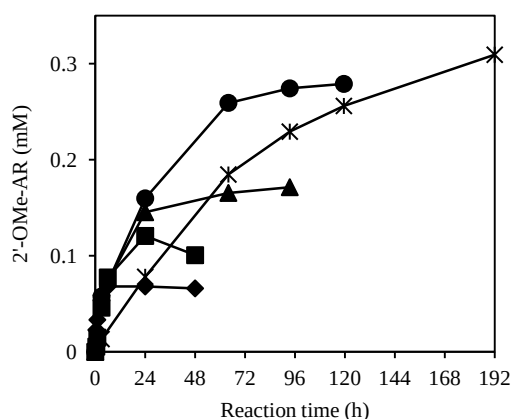


Fig. 3-6. Effect of hydrophilic solutes on 2'-OMe-AR production in the transribosylation of AgNH

The effect of the addition of hydrophilic solutes on 2'-OMe-AR production by AgNH is shown. The reaction was carried out with 240 mM 2'-OMe-UR and 200 mM adenine at 50°C. Without solute (closed diamonds), 100% saturated concentration of glucose (closed squares), 30%, 50% and 70% (v/v) glycerol (closed triangles, circles and asterisks, respectively) are indicated.

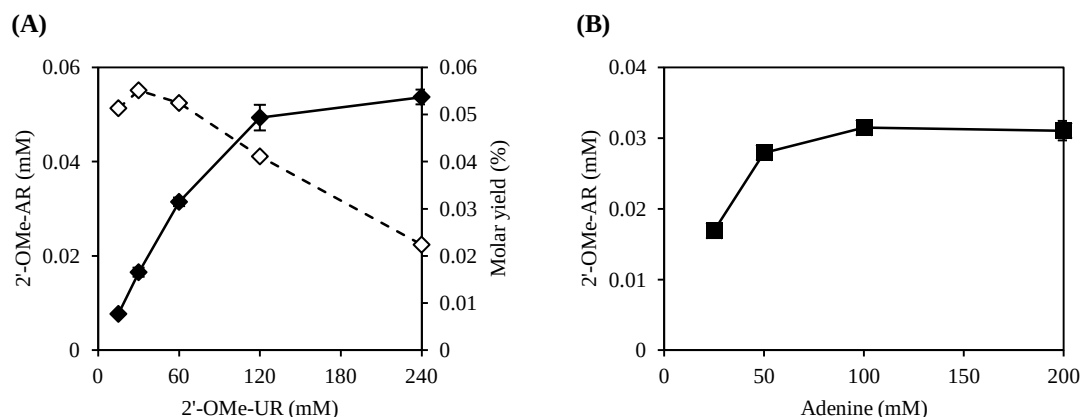


Fig. 3-7. Effect of substrate concentrations on 2'-OMe-AR production by AgNH-catalyzing transribosylation

A, The effect of 2'-OMe-UR concentration on 2'-OMe-AR production by AgNH. The reaction was carried out with 100 mM adenine, 50% (v/v) glycerol and 15-240 mM 2'-OMe-UR, and the total amount (closed diamonds) and the yield of 2'-OMe-AR produced (open diamonds) at 50°C after 24 h are shown. B, The effect of adenine concentration on 2'-OMe-AR production by AgNH with 60 mM 2'-OMe-UR, 50% (v/v) glycerol and 25-200 mM adenine at 50°C after 24 h is shown (closed squares). The molar yield (%) was defined as the value that is the amount of 2'-OMe-AR divided by the starting concentration of 2'-OMe-UR.

Optimized bioprocess production of 2'-OMe-NRs by AgNH-catalyzing transribosylation

Based on the result obtained above, 2'-OMe-AR was produced with AgNH in the reaction mixture containing 60 mM 2'-OMe-UR, 100 mM adenine, 50% (v/v) glycerol and 20% (w/v) dextrin at 50°C. Under the conditions, AgNH produced up to 17 mg/L (0.059 mM) of 2'-OMe-AR for 293 h, and its molar yield reached 0.098% towards 2'-OMe-UR (Fig. 3-8 (A)). Similarly, 2'-OMe-6ClGR was produced with AgNH in the reaction mixture containing 60 mM 2'-OMe-UR, 100 mM 6-chloroguanine and 50% (v/v) glycerol at 50°C. Under the conditions, AgNH produced up to 34 mg/L (0.11 mM) of 2'-OMe-6ClGR for 293 h, and its molar yield reached 0.18% towards 2'-OMe-UR (Fig. 3-8 (C)).

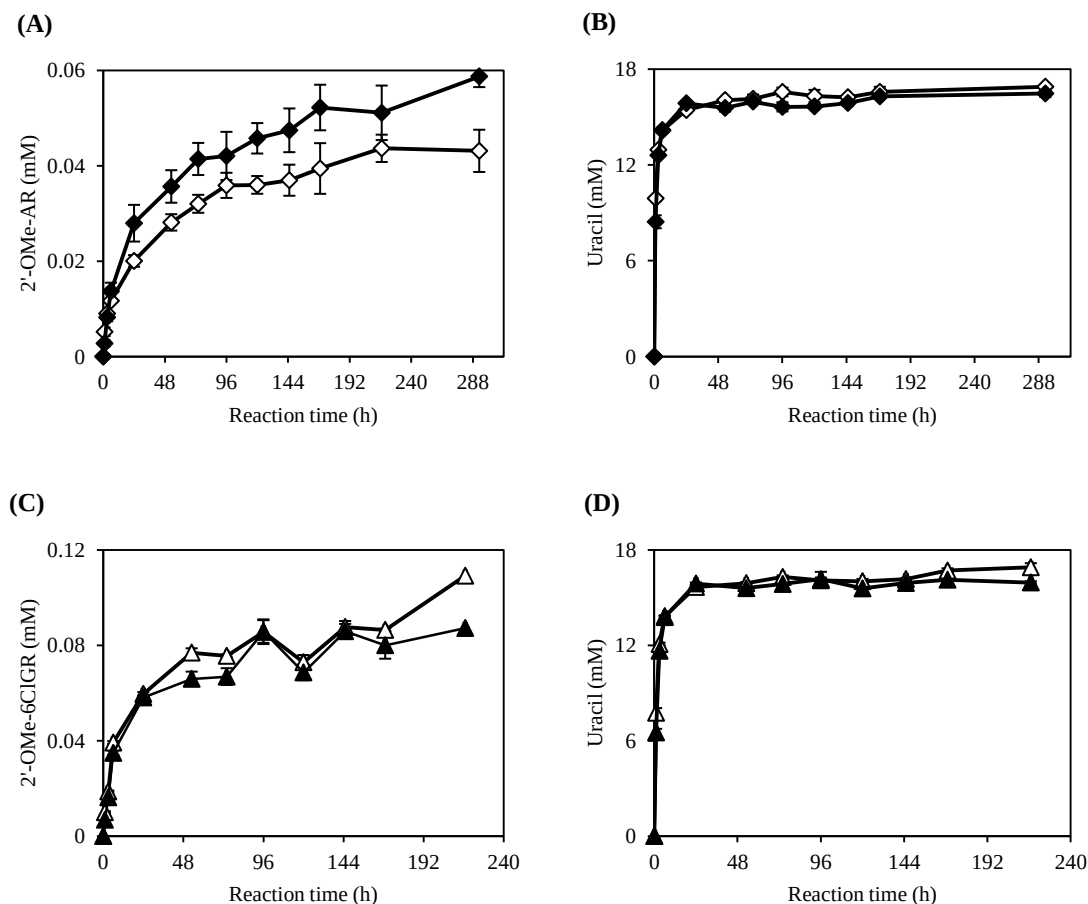


Fig. 3-8. Enzymatic synthesis of 2'-OMe-NRs with AgNH under the optimal conditions

The enzymatic syntheses of 2'-OMe-NRs were carried out using reaction mixtures containing 60 mM 2'-OMe-UR, 100 mM corresponding nucleobases, 50% glycerol (v/v), and 3.7 U/mL AgNH in 50 mM KPB, pH 7.0, at 50°C with or without 20% (w/v) dextrin. A, 2'-OMe-AR production and B, uracil production with adenine are shown. Without and with 20% (w/v) dextrin (open diamonds and closed diamonds, respectively) are indicated. C, 2'-OMe-6ClGR production and D, uracil production with 6-chloroguanine are shown. Without and with 20% (w/v) dextrin (open triangles and closed triangles, respectively) are indicated. The y-axis error bars represent the standard deviation calculated from three measurements.

Discussion

2'-OMe-6ClGR and its analogues are non-natural nucleosides and important intermediates for purine nucleoside synthesis. For example, 2'-OMe-GR is an important building block of nucleic acid drugs such as Pegaptanib (Macugen; Pfizer, New York, NY, USA) and easily converted from 2'-OMe-6ClGR by the dechlorination and the oxidation with adenosine deaminase (66,67). AgNH and LbNH showed little activity towards guanine in the

transribosylation with 2'-OMe-UR as the donor substrate. In contrast, 6-chloroguanine was the most preferable acceptor substrate for AgNH, and no hydrolytic activity was observed towards 2'-OMe-6ClGR in the long-time reaction (Fig. 3-8 (C)). Thus, the coupling reaction of AgNH and deaminases via the transribosylation between 2'-OMe-UR and 6-chloroguanosine is a promising alternative for 2'-OMe-GR production.

According to kinetic studies, the hypothetical reaction scheme catalyzed by AgNH and LbNH was designed and compared based (Fig. 3-9). AgNH showed the lower yield of 2'-OMe-AR production than that of LbNH and has an apparent K_m value of adenine nearly equal to an inhibition constant of K_i (Table 3-1). This indicates that the transribosylation with AgNH progresses via a pathway where the AgNH-adenine complex is a starting intermediate to generate 2'-OMe-AR, and the AgNH-2'-OMe-UR barely contributed to 2'-OMe-AR production. Although the detailed mechanism to produce 2'-OMe-AR was unknown, there are also two possible reaction model of nucleobase exchange between 2'-OMe-UR and adenine. In the hydrolysis-like mechanism, a water molecule acts as a nucleophile and proton donor of releasing uracil (68), and adenine attacks the 1'-carbon of 2'-OMe-ribose. Second model is 'the direct nucleobase transribosylation' without water like the reaction catalyzed by tRNA-guanine transglycosylase (69). In this reaction model, the catalytic aspartyl acid of the enzyme directly contributes the departure of uracil without a water molecule, and nucleophilic adenine attacks an oxocarbenium ion intermediate to generate 2'-OMe-AR.

AgNH had 97% homology with a putative nucleoside hydrolase of *M. resistens* NBRC 103078 which formed a gene cluster together with nucleoside-metabolizing enzymes such as 2-deoxyribose-5-phosphate aldolase, formamidopyrimidine-DNA glycosylase and ribokinase in its genome. However, no enzyme predicted to be involved in 2'-OMe-ribose metabolism was found in this cluster. Therefore, AgNH seemed to be a nucleoside hydrolase to metabolize some kinds of nucleosides, with its incidental activity towards 2'-OMe-NRs. *Agromyces* sp. MM-1 would mainly utilize uracil which was a hydrolysate of 2'-OMe-UR in the course of microbial screening.

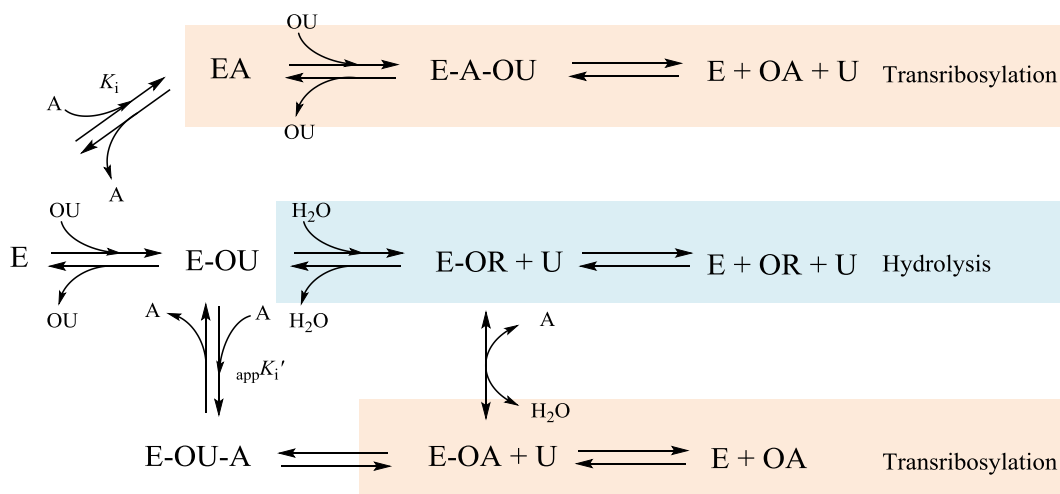


Fig. 3-9. Hypothetical reaction scheme of *AgNH*- and *LbNH*-catalyzing reactions

A hypothetical reaction scheme for reactions catalyzed by *AgNH* and *LbNH* in the presence of 2'-OMe-UR and adenine is shown. *AgNH* or *LbNH* (E), 2'-OMe-UR (OU), 2'-OMe-ribose (OR), uracil (U), and adenine (A) are indicated. K_i and K_i' denotes dissociation constants yielding an enzyme-inhibitor (E-A) complex and an enzyme-substrate-inhibitor complex (E-OU-A), respectively.

Summary

Microorganisms were screened for the transribosylation activity between 2'-O-methyluridine (2'-OMe-UR) and nucleobases, for the purpose to develop biotransformation process into various 2'-O-methylribonucleosides (2'-OMe-NRs), which are raw materials for nucleic acid drugs. Then, an actinomycete, *Agromyces* sp. MM-1 was found to produce 2'-O-methyladenosine (2'-OMe-AR) with its whole cells in the reaction mixture containing 2'-OMe-UR and adenine. The responsible enzyme for the transribosylation was partially purified from *Agromyces* sp. MM-1 cells through a six-step separation procedure, and identified to a nucleoside hydrolase family enzyme termed *AgNH*. *AgNH* was a bi-functional enzyme catalyzing the hydrolysis towards 2'-OMe-NRs and the transribosylation between 2'-OMe-UR and various nucleobases as well as adenine. In the hydrolysis, *AgNH* especially preferred guanosine analogues as its substrates. As for the transribosylation, *AgNH* showed the remarkable activity towards 6-chloroguanine with 520% relative activity to adenine as the acceptor substrate. The transribosylation reaction product from 2'-OMe-UR and 6-chloroguanine was determined to 2'-O-methyl-6-chloroguanosine (2'-OMe-6ClGR). Under the optimal conditions, the maximum molar yield of 2'-OMe-6ClGR reached at 0.18% in 216-h reaction, corresponding to 34 mg/L.

CHAPTER IV

Identification and characterization of new microbial imidase stereo-selectively hydrolyzing *cis*-5-norbornene-*exo*-2,3-dicarboximide**Introduction**

Optically active half amides are valuable pharmaceutical intermediates and easily converted to chiral amino acids via the Hofmann rearrangement (70-72). For example, one kind of the half amides, *cis*-5-norbornene-*exo*-2,3-dicarboxylic acid monoamide (NDM), is changed into 3-amino-5-norbornene-2-carboxylic acid, which is a structural component of several molecular targeting drugs such as ALK inhibitor CEP-28122 (8-10). However, such optically active half amides are difficult to be synthesized chemically because of by-produced undesirable stereoisomers to cause the decrease of the production yield and the requirement of separation processes. On the other hand, enzymatic reactions generally have high stereo- and regio-selectivity. Thus, the enzymatic regio-selective hydrolysis of cyclic imides with imide hydrolyzing enzyme (imidase) is a useful method for the dissymmetric synthesis of corresponding optically active half amides (11). Thus, regio-selective imidase-catalyzing hydrolysis of a cyclic imide, *cis*-5-norbornene-*exo*-2,3-dicarboximide (NDI), is a promising approach for the dissymmetric production of NDM (Fig. 4-1).

In this study, two NDI-metabolizing bacteria isolated from environmental samples were found to degrade NDI. These strains were identified to *Mesorhizobium* sp. A9-2-3 and *Arthrobacter oxydans* E2-4-2, and they showed the hydrolytic activities towards NDI to produce NDM. Especially, *Mesorhizobium* sp. A9-2-3 showed remarkable regio-selectivity in the hydrolysis. The imidase of *Mesorhizobium* sp. A9-2-3 was identified and termed as *MeIH*. *MeIH* consisted of two subunits with a similarity to *N*-methylhydantoinase (EC 3.5.2.16) (73).

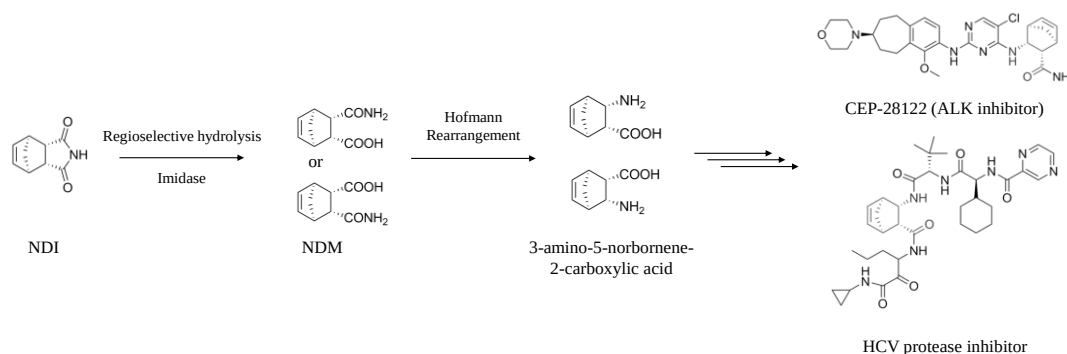


Fig. 4-1. The application of imidase catalyzing the regio-selective hydrolysis towards NDI for the dissymmetric synthesis of NDM, a pharmaceutical intermediate

An application of microbial imidase for the production of NDM from NDI is shown. Imidases catalyze the regio-selective hydrolysis towards cyclic imides such as NDI, which is a potent precursor of an optically active half amide NDM.

Materials and Methods

Chemicals

NDI and NDM were kindly gifted from Biotechnology Development Laboratories, Kaneka Corporation (Hyogo, Japan). All other reagents and solvents were obtained from commercial sources.

Strains and cultures

Mesorhizobium sp. A9-2-3 and *Arthrobacter oxydans* E2-4-2 were cultivated in MG medium consisted of 0.1% (w/v) glutarimide, 0.1% NH_4Cl , 0.1% (w/v) KH_2PO_4 , 0.1% (w/v) K_2HPO_4 , 0.03% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.02% (w/v) Bacto yeast extract (pH 7.0). For the proteome analysis, *Mesorhizobium* sp. A9-2-3 was cultivated in MYP medium consisted of 0.05% (w/v) glucose, 0.1% NH_4Cl , 0.1% (w/v) KH_2PO_4 , 0.1% (w/v) K_2HPO_4 , 0.03% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% (w/v) Bacto yeast extract and 0.01% (w/v) Bacto peptone (pH 7.0), with or without 0.15% (w/v) glutarimide. *Escherichia coli* JM109 was used as the host strain for gene cloning and protein expression, and cultivated in LB medium consisted of 1.0% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract and 1.0% (w/v) NaCl (pH 7.0).

Strain identification of NDI-assimilating microorganisms

Total DNA from each strain was isolated from the cells using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Nearly full length of the 16S rRNA gene was amplified by PCR

using PrimeSTAR GXL DNA Polymerase (Takara Bio, Shiga, Japan) with 27f primer (5' AGAGTTTGATCMTGGCTCAG 3', where M is A or C) and 1492r primer (5' GGTTACCTTGTTACGACTT 3'). The amplification conditions were 30 cycles of 98°C for 10 sec, 55°C for 15 sec, and 68°C for 85 sec. The resulting PCR product was purified and sequenced by a DNA sequencer CEQ 2000XL DNA Analysis System (Beckman Coulter, Brea, CA, USA). Following primer was used in order to obtain the 16S rRNA gene sequence: 27f, 357f (5' CTCCTACGGGAGGCAGCAG 3'), 519r (5' GWATTACCGCGGCKGCTG 3', where W is A or T, and K is G or T), 530f (5' GTGCCAGCMGCCGCGG 3'), 907r (5' CCGTCAATTCMTTTRAGTTT 3'), 1100r (5' GGGTTGCGCTCGTTG 3'), 1392r (5' ACGGGCGGTGTGTRC 3', where R is G or A), and 1492r.

Analytical methods

The reaction mixture was analyzed by reversed-phase HPLC using a Shimadzu LC-VP system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil 5C₁₈-AR-II column (4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan). HPLC was conducted using acetonitrile/water (2/8 by vol., pH 2.5, adjusted with phosphoric acid) as the mobile phase, the flow rate of 0.5 mL/min, the column temperature of 30°C, and UV detection at 210 nm. The enantiomeric excess (% e.e.) of NDM was determined by reversed-phase HPLC using a Shimadzu LC-VP system equipped with two Sumichiral OA-7000 columns (4.6 × 250 mm; Sumika Chemical, Osaka, Japan). The mobile phase was acetonitrile/water (2/8 by vol., pH 2.5, adjusted with phosphoric acid), the flow rate was 0.5 mL/min, and the column temperature was 30°C. The mobile phase was monitored with UV detector at 210 nm.

Hydrolysis reaction of NDI with the resting cells of NDI-assimilating microorganisms

Strains of A9-2-3 and E2-4-2 were cultivated in 250 mL of the MG medium in a Sakaguchi flask at 28°C with shaking at 120 rpm for 50 h. The cells were collected by centrifugation and washed with 20 mM potassium phosphate buffer (pH 7.0). The wet cells (about 9 mg) were resuspended in 600 µL of reaction mixture containing 20 mM NDI and 20 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 28°C with the shaking at 300 rpm. Production amount and optical activity of NDM in the reaction mixture were analyzed by the HPLC analysis.

Draft genome sequencing of *Mesorhizobium* sp. A9-2-3

Genomic DNA of *Mesorhizobium* sp. A9-2-3 was sequenced by Illumina MiSeq (Illumina, San Diego, CA, USA). The draft genome sequence was assembled with CLC Genomics Workbench 7.5 (CLC bio, Aarhus, Denmark). Clustering of the DNA sequences

resulted in 8,863,903 base pairs with 390 contigs and 8,106 open reading frames (ORFs). Functional annotation was carried out on Rapid Annotation using Subsystem Technology (RAST) server (<http://rast.nmpdr.org/>).

Comparative proteomic analysis of *Mesorhizobium* sp. A9-2-3

Isolation of proteins: *Mesorhizobium* sp. A9-2-3 was cultured in 500 mL of MYP medium with or without glutarimide at 28°C with shaking at 300 rpm for 70 h, and then cells were harvested by centrifugation at $7,000 \times g$ for 15 min. The harvested cells were resuspended in 50 mM Tris-HCl (pH 8.0) buffer comprising 7.0 M urea, 2.0 M thiourea, 2.0% (w/v) CHAPS, 10 mM dithiothreitol, and appropriate amount of cOmplete protease inhibitor cocktail (Roche, Basel, Switzerland). The cells were disrupted with 0.1 mm beads by Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), and centrifuged at $20,000 \times g$ for 15 min at 4°C. The supernatants were used as cell lysate. The precipitates were washed with minimal volume of 0.20 M triethylammonium bicarbonate buffer (TEAB, pH 8.0) and combined with the lysate. The protein solution was filtered with 0.45 µm filter using Ultrafree MC (Millipore, Billerica, MA, USA). The flow-through was ultrafiltrated by Amicon Ultra (10 kDa MWCO; Millipore), and buffer was exchanged into 0.20 M TEAB buffer (pH 8.0).

Trypsin digestion: The solution of 0.20 M Tris(2-carboxyethyl)phosphine was added to each sample up to 9.5 mM, and incubated at 55°C for 60 min. Then, 375 mM iodoacetamide solutions were added up to 17.9 mM, and incubated at room temperature. After incubation for 30 min, 2- to 4-fold volume of ice-cold acetone were added to each sample, and incubated at -20°C for 3 h. Proteins were harvested by centrifugation at $13,000 \times g$ for 10 min at 4°C and the residual acetone was removed at 37°C for 2 min. The precipitated proteins were resuspended with 0.20 M TEAB buffer and digested by appropriate amount of Sequence Grade Modified Trypsin (Promega, Madison, WI, USA) at 37°C for overnight. The tryptic digests were purified using Pierce detergent removal spin columns (Thermo Fisher Scientific).

LC-MS/MS analysis: Proteome analyses were performed by a Prominence nano flow system (Shimadzu) connected with an LTQ Velos orbitrap mass spectrometer (Thermo Fisher Scientific). Tryptic digests (20 µL) were injected and separated on long monolithic silica capillary columns (200 cm long, 0.1 mm ID) at a flow rate of 500 nL/min. The eluents A was 0.1% (v/v) formic acid, and eluents B was acetonitrile containing 0.1% (v/v) formic acid. The gradient was started with 5% B and increased to 45% B for 600 min. The separated peptides were detected on the mass spectrometer with full scan range 350–1500 *m/z*. For data-dependent acquisition, the method was set to automatically analyze the top 10 most intense ions observed in the MS scan. An ESI voltage of 2.4 kV was applied directly to the LC buffer distal to the chromatography column using a microtee. The ion transfer tube temperature on the LTQ Velos

ion trap was set to 300°C.

Protein identification: The mass spectrometry data were used for protein identification by Proteome Discover 1.4 (Thermo Scientific). Protein identification was performed using Mascot and Sequest HT against the protein database from strain A9-2-3 genome database.

Cloning of candidate genes encoding NDI-hydrolyzing imidase

Target genes encoding subunits encoding 4611/4610 and 4631/4630 of *Mesorhizobium* sp. A9-2-3 were amplified using PrimeSTAR MAX DNA polymerase (Takara Bio) with two sets of following primers: 5' GAATTCATTAAAGAGGAGAAATTAACCATGAAGACGATCGGCGTCGA 3' and 5' CAACAGGAGTCCAAGCTCAGCTAATTATCAGGCCCTCCTCGCCCGCT 3', or 5' GAATTCATTAAAGAGGAGAAATTAACCATGATCCGGGTCGGGGTCGA 3' and 5' CAACAGGAGTCCAAGCTCAGCTAATTACTATGAATGCACCAGTTCCG 3', respectively. By NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Hitchin, UK), the PCR product was inserted into pQE-80L vector (Qiagen), which was previously digested with *Hind*III and *Nco*I. Each plasmid was transformed into *E. coli* JM109.

Activity assay for NDI-hydrolyzing imidase

The *E. coli* transformants were cultivated in 2 mL of LB medium supplemented with 50 µg/mL ampicillin at 37°C with shaking at 300 rpm. When the OD₆₀₀ of the cultures reached 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures at final concentration of 0.5 mM, and incubated at 28°C with the shaking at 300 rpm overnight. After the cultivation, cells were harvested by centrifugation and washed twice using 0.85% NaCl and used for the reaction. The harvested cells were resuspended in 200 µL of reaction mixture containing 10 mM NDI and 100 mM potassium phosphate buffer (pH 7.5). The reaction was carried out at 28°C with the shaking at 300 rpm overnight.

Results and discussions

Screening of NDI-hydrolyzing microorganisms

From soil and plant samples, more than 1,300 microorganisms were isolated to assimilate a cyclic imide, NDI. These microorganisms were screened for hydrolyzing activity towards NDI. In reaction mixture using wet cells of these microorganisms, two isolates, A9-2-3 and E2-4-2, produced the corresponding half amide, NDM, as observed in the HPLC analysis. According to phylogenetic analysis of 16S ribosomal DNA sequence, strain A9-2-3 was

identified as *Mesorhizobium* sp. and strain E2-4-2 was identified as *Arthrobacter oxydans* (Fig. 4-2 and 4-3).

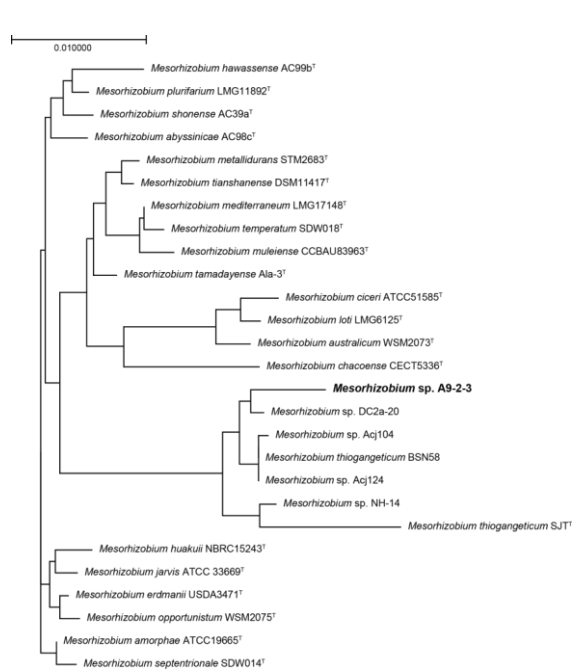


Fig. 4-2. Phylogenetic analysis of strain A9-2-3 and *Mesorhizobium* species by using their 16S rDNA sequences

All data of 16S rDNA sequences of *Mesorhizobium* species were obtained from GenBank, and used for phylogenetic analysis with the 16S rDNA sequence of strain A9-2-3.

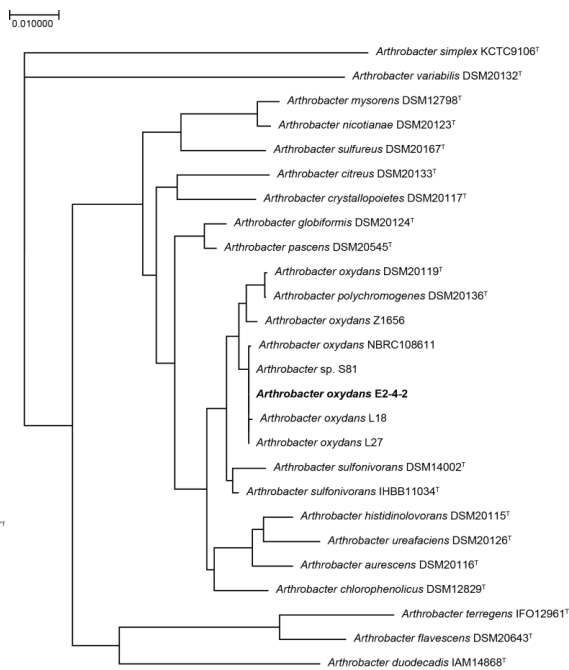


Fig. 4-3. Phylogenetic analysis of strain E2-4-2 and *Arthrobacter* species by using their 16S rDNA sequences

All data of 16S rDNA sequences of *Arthrobacter* species were obtained from GenBank, and used for phylogenetic analysis with the 16S rDNA sequence of strain E2-4-2.

Microbial production of NDM by the NDI-hydrolyzing strains

In the reaction mixture with cells of *Mesorhizobium* sp. A9-2-3 or *Arthrobacter oxydans* E2-4-2 as biocatalyst, NDI was regio-selectively hydrolyzed into NDM (Table 4-1). When NDI was reacted with *Mesorhizobium* sp. A9-2-3, an enantiomer of NDM was produced with production yield of 4.5% and enantiomeric excess of 96.6% e.e. for 24-h reaction. At 48 h, the yield and enantiomeric excess of NDM production decreased to 3.4% and 85.7% e.e., respectively. It was suggested that NDM would be unstable and spontaneously hydrolyzed to the corresponding dicarboxylic acid in aqueous solution. In the case of *Arthrobacter oxydans*

E2-4-2, the yield and enantiomeric excess of the NDM production reached up to 1.9% and 75.7% e.e. at 48 h, respectively. As the result, *Mesorhizobium* sp. A9-2-3 showed the higher regio-selectivity in the hydrolysis reaction towards NDI than *Arthrobacter oxydans* E2-4-2, and then was selected to be used in the further investigation.

Table 4-1. Hydrolysis activity towards NDI and enantiomeric excess of NDM as the reaction products

Reaction time (h)	<i>Mesorhizobium</i> sp. A9-2-3		<i>Arthrobacter oxydans</i> E2-4-2	
	Production yield (%)	Enantiomeric excess (% e.e.)	Production yield (%)	Enantiomeric excess (% e.e.)
5	0.8	<i>n. d.</i>	0.3	<i>n. d.</i>
24	4.5	96.6	1.1	<i>n. d.</i>
48	3.4	85.7	1.9	75.7

Relative activity was defined as the percent decrease in mass amount of NDI. *n. d.*, not determines.

Identification of NDI-hydrolyzing enzyme of *Mesorhizobium* sp. A9-2-3

According to the comparative proteomic analysis, a total of 3,675 proteins were obtained as expressed in both cells grown with and without glutarimide. Among them, 561 proteins were specifically induced by the addition of glutarimide, and 621 proteins were only detected in the non-induced sample. Moreover, 36 out of the 561 proteins specifically induced with glutarimide were annotated as hydrolyzing enzymes involved in cleavage of C-N bonds (Table 4-2). Remarkably, there were several proteins annotated as *N*-methylhydantoinase (*N*-MH). It was reported that the *N*-MH of *Pseudomonas putida* 77 consisted of two hetero subunits and catalyzed the regio-selective hydrolysis of cyclic imides such as glutarimide (73-75). Importantly, two sets of *N*-MH subunits, 4611/4610 and 4631/4630, were found to be simultaneously induced with glutarimide in *Mesorhizobium* sp. A9-2-3. Then, these *N*-MHs comprised of 4611/4610 and 4631/4630 were heterologously expressed in *E. coli* JM109, and assayed for the NDI-hydrolyzing activity. As the result, *E. coli* cells expressing 4631/4630 showed the hydrolysis activity towards NDI to produce NDM (Fig. 4-4). Thus, 4631/4630 was identified to the responsible enzyme hydrolyzing NDI and termed as imidase of *Mesorhizobium* sp. A9-2-3 (*MeIH*). Although *N*-MH of *P. putida* 77 was reported to require ATP for the reactions, the hydrolytic activity of *MeIH* was not affected by the addition of ATP. Thus, *MeIH* would be a novel type of microbial imidase useful for the production of optically active half amides.

Table 4-2. Glutarimide-induced proteins involved in cleavage of C-N bonds

Protein ID	Putative function	Σ # PSMs	Σ #Unique Peptides	Score Sequest HT	Score Mascot
1335	tRNA-specific adenosine-34 deaminase	55	2	73	386
3706	HtrA protease	50	7	75	323
540	Conservative hypothetical protein probably involved in hydantoin, pyrimidine utilization	32	5	31	178
4540	<i>N</i> -methylhydantoinase B	25	7	52	429
1196	<i>N</i> -formylglutamate deformylase	21	4	30	86
4631	<i>N</i> -methylhydantoinase A	20	5	34	212
3230	Aminopeptidase YpdF	19	5	23	77
7406	Formiminoglutamic iminohydrolase	14	4	21	98
194	Imidazolonepropionase	11	3	22	94
4299	D-alanyl-D-alanine carboxypeptidase	11	4	18	72
1927	ATP-dependent nuclease subunit A	9	4	14	62
4611	<i>N</i> -methylhydantoinase A	9	4	15	35
488	Allophanate hydrolase 2 subunit 2	8	2	9	117
468	<i>S</i> -adenosylhomocysteine deaminase, Methylthioadenosine deaminase	6	2	8	49
4988	<i>N</i> -acetylmuramoyl-L-alanine amidase	6	1	13	37
6122	Inosine-uridine preferring nucleoside hydrolase	6	2	7	26
3110	Urease alpha subunit	5	3	10	28
538	Allantoinase	5	2	10	96
638	Hippurate hydrolase	5	3	5	26
1288	6-Aminohexanoate-dimer hydrolase	4	1	8	93
2098	Probable hydrolase	4	1	2	25
2390	Amidase	4	1	5	46
2968	Guanine deaminase	4	2	6	51
4630	<i>N</i> -methylhydantoinase B	4	1	11	103
5564	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	4	1	8	56
6590	D-alanyl-D-alanine carboxypeptidase	4	2	5	31
7611	Peptide deformylase	4	2	6	23
3103	Urease gamma subunit	3	1	6	54
1828	Predicted Zn peptidase	2	1	3	43
1879	Arginine deiminase	2	1	3	16
2118	Hydrolase, alpha/beta hydrolase fold family	2	1	3	39
3126	Nicotinamidase	2	1	4	21
4365	Metal-dependent hydrolases of the beta-lactamase superfamily I; PhnP protein	2	1	3	36
4610	<i>N</i> -methylhydantoinase B	2	1	3	31
7839	<i>N</i> -carbamoylsarcosine amidase	2	1	3	50
7943	Amidases related to nicotinamidase	2	1	3	47

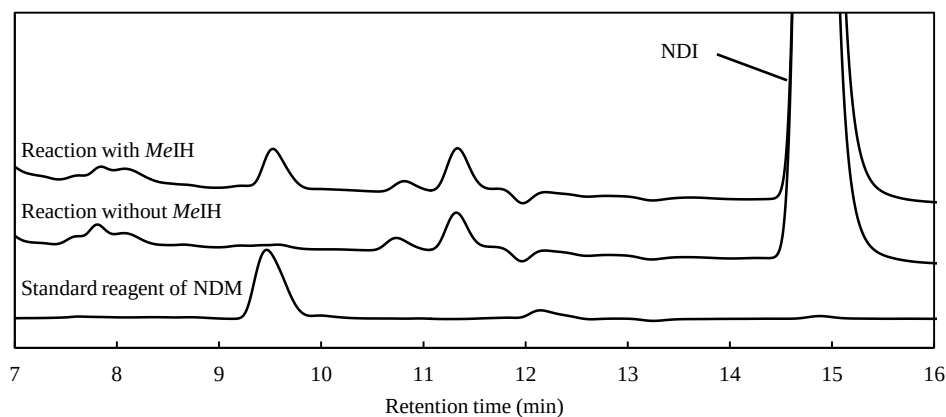


Fig. 4-4. HPLC analysis of the reaction mixture with the *N*-MH of 4631/4630 (*MeIH*)

Chromatograms of the standard reagent of NDM and reaction mixtures containing NDI with *E. coli* cells expressing *N*-MH of 4631/4630 (*MeIH*) or not. The peaks detected at 9.5 min and 15 min corresponded to the standard reagents of NDM and NDI, respectively.

Summary

MeIH, an imidase obtained from *Mesorhizobium* sp. A9-2-3 was used for region-selective hydrolysis of a cyclic imide, NDI, and production of optically active half amide, NDM, which is a valuable pharmaceutical intermediate easily converted to optically active amino acids via the Hofmann rearrangement. *MeIH* consisted of two kinds of hetero subunits and showed a homology with *N*-methylhydantoinase, but ATP was not required for the NDI-hydrolyzing reaction.

CONCLUSIONS

CHAPTER I

In this chapter, a novel nucleoside hydrolase, *LbNH*, of *Lactobacillus buchneri* LBK78 was found to be the first enzyme catalyzing the hydrolysis towards 2'-*O*-methylribonucleosides (2'-OMe-NRs). 2'-OMe-NRs are promising raw materials for nucleic acid drugs because of their high thermal stability and nuclease tolerance. In the course of microbial screening for metabolic activity towards 2'-OMe-NRs, *Lactobacillus buchneri* LBK78 was found to decompose 2'-*O*-methyluridine (2'-OMe-UR). The enzyme responsible was partially purified from *L. buchneri* LBK78 cells by a four-step purification procedure, and identified as a novel nucleoside hydrolase. This enzyme, *LbNH*, belongs to the nucleoside hydrolase superfamily, and formed a homotetrameric structure composed of subunits with a molecular mass of around 34,000. *LbNH* hydrolyzed 2'-OMe-UR to 2'-*O*-methylribose and uracil, and the kinetic constants were K_m of 0.040 mM, k_{cat} of 0.49 s⁻¹, and k_{cat}/K_m of 12 mM⁻¹·s⁻¹. In a substrate specificity analysis, *LbNH* preferred ribonucleosides and 2'-OMe-NRs as its hydrolytic substrates, but reacted weakly with 2'-deoxyribonucleosides. In a phylogenetic analysis, *LbNH* showed a close relationship with purine-specific nucleoside hydrolases from trypanosomes.

CHAPTER II

In this chapter, *LbNH* was found to be a bi-functional enzyme catalyzing the hydrolysis and the transribosylation towards 2'-*O*-methylribonucleosides (2'-OMe-NRs), and the enzymatic synthesis of 2'-*O*-methyladenosine (2'-OMe-AR) was accomplished by the transribosylation between 2'-*O*-methyluridine (2'-OMe-UR) and adenine. 2'-OMe-NRs are promising raw materials for the production of nucleic acid drugs. We previously reported that *LbNH*, a nucleoside hydrolase from *Lactobacillus buchneri* LBK78 (NITE P-01581), was the first enzyme found to act on 2'-OMe-NRs. In the present study, we determined that *LbNH* also has the transribosylation activity between 2'-OMe-NRs and nucleobases, in addition to the hydrolyzing activity towards 2'-OMe-NRs. When 2'-OMe-UR and adenine were reacted with *LbNH*, 2'-OMe-AR was produced. *LbNH* preferred purine nucleobases as its acceptor substrates for the transribosylation with 2'-OMe-UR as a donor substrate. Kinetic analysis of *LbNH* revealed that adenine behaved as a mixed inhibitor of the hydrolysis of 2'-OMe-UR. Under the optimal reaction conditions, the maximum molar yield of enzymatic 2'-OMe-AR produced reached 0.97% towards 2'-OMe-UR, corresponding to 0.16 g/L.

CHAPTER III

In this chapter, a screening for microorganisms producing 2'-O-methyladenosine (2'-OMe-AR) via the transribosylation was conducted, and an actinomycete nucleoside hydrolase, AgNH, of *Agromyces* sp. was applied for 2'-O-methylribonucleosides (2'-OMe-NRs) production. Microorganisms were screened for the transribosylation activity between 2'-OMe-UR and nucleobases, for the purpose to develop biotransformation process into various 2'-OMe-NRs, which are raw materials for nucleic acid drugs. Then, an actinomycete, *Agromyces* sp. MM-1 was found to produce 2'-O-methyladenosine (2'-OMe-AR) with its whole cells in the reaction mixture containing 2'-OMe-UR and adenine. The responsible enzyme for the transribosylation was partially purified from *Agromyces* sp. MM-1 cells through a six-step separation procedure, and identified to a nucleoside hydrolase family enzyme termed AgNH. AgNH was a bi-functional enzyme catalyzing the hydrolysis towards 2'-OMe-NRs and the transribosylation between 2'-OMe-UR and various nucleobases as well as adenine. In the hydrolysis, AgNH especially preferred guanosine analogues as its substrates. As for the transribosylation, AgNH showed the remarkable activity towards 6-chloroguanine with 520% relative activity to adenine as the acceptor substrate. The transribosylation reaction product from 2'-OMe-UR and 6-chloroguanine was determined to 2'-O-methyl-6-chloroguanosine (2'-OMe-6ClGR). Under the optimal conditions, the maximum molar yield of 2'-OMe-6ClGR reached at 0.18% in 216-h reaction, corresponding to 34 mg/L.

CHAPTER IV

In this chapter, a screening of microorganisms assimilating a cyclic imide, *cis*-5-norbornene-*exo*-2,3-dicarboximide (NDI), for the dissymmetric synthesis of *cis*-5-norbornene-*exo*-2,3-dicarboxylic acid monoamide (NDM) was conducted, and *Mesorhizobium* sp. A9-2-3 was found to catalyze the regio-selective hydrolysis towards NDI to produce NDM. MeIH, an imidase obtained from *Mesorhizobium* sp. A9-2-3 was used for region-selective hydrolysis of a cyclic imide, NDI, and production of optically active half amide, NDM, which is a valuable pharmaceutical intermediate easily converted to optically active amino acids via the Hofmann rearrangement. MeIH consisted of two kinds of hetero subunits and showed a homology with *N*-methylhydantoinase, but ATP was not required for the NDI-hydrolyzing reaction.

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- 1) Mitsukawa, Y., Hibi, M., Matsutani, N., Horinouchi, N., Takahashi, S., and Ogawa, J.: A novel nucleoside hydrolase from *Lactobacillus buchneri* LBK78 catalyzing hydrolysis of 2'-O-methylribonucleosides. *Biosci. Biotechnol. Biochem.*, **80** (8), 1568-1576 (2016).
- 2) Mitsukawa, Y., Hibi, M., Matsutani, N., Horinouchi, N., Takahashi, S., and Ogawa, J.: Enzymatic synthesis of 2'-O-methylribonucleosides with a nucleoside hydrolase family enzyme from *Lactobacillus buchneri* LBK78. in preparation.
- 3) Mitsukawa, Y., Hibi, M., Matsutani, N., Horinouchi, N., Takahashi, S., and Ogawa, J.: A new nucleoside hydrolase with transribosylation activity from *Agromyces* sp. MM-1 and its application for enzymatic synthesis of 2'-O-methylribonucleosides. in preparation.
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