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EM2487, a Novel Anti-HIV-1 Antibiotic, Produced by *Streptomyces* sp. Mer-2487:

Taxonomy, Fermentation, Biological Properties, Isolation and Structure Elucidation

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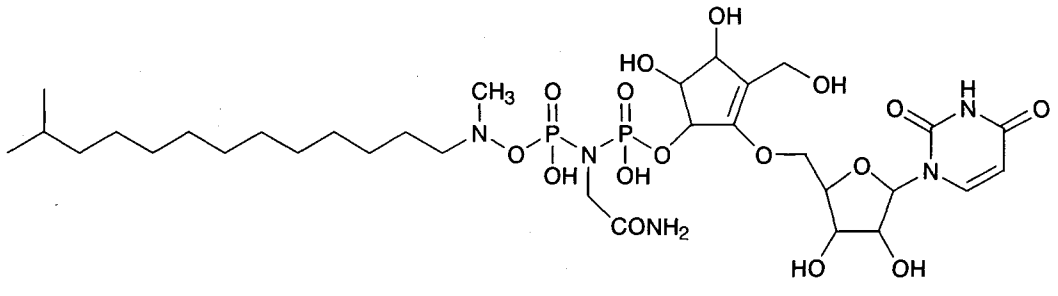
For the purpose of discovering novel agents that inhibit HIV-1 replication at the transcriptional level, we have established cell lines reflecting the HIV-1 long terminal repeat-driven gene expression. Using these cell lines, we have screened approximately 10,000 microorganism products and found that the culture supernatant of *Streptomyces* sp. Mer-2487 suppresses the HIV-1 Tat-induced gene expression without affecting the basal or tumor necrosis factor-α-induced transcription. The purified active component has a unique structure, as shown in Fig. 1. This compound has an inhibitory effect on HIV-1 replication in chronically infected cells as well as acutely infected cells, suggesting that the inhibition occurs at a postintegration step of HIV-1 proviral DNA in the HIV-1 replication cycle.

Combination chemotherapy with reverse transcriptase inhibitors and protease inhibitors for the treatment of HIV-1 infection has considerably improved the prognosis of AIDS patients. However, the discovery of novel anti-HIV-1 agents with a different mode of action is still necessary because of the problems of drug-resistance,

Fig. 1. Structure of EM2487.



high cost, and low compliance associated with drug combinations. Furthermore, the HIV-1 genome, which is integrated into the host genome after infection, exists over a long period in resting T cells and maintains its ability to replicate[1,2]. Among the strategies to overcome such limitations of current anti-HIV-1 chemotherapy, we have chosen the inhibition of HIV-1 transcription by small molecules. The benzodiazepine derivatives Ro 5-3335 and Ro 24-7429 were shown to inhibit HIV-1 replication in certain cell cultures by interfering with Tat functions[3,4]. ALX40-4C was designed to inhibit the Tat-TAR interaction, an essential step of Tat-induced transactivation, yet its inhibitory effect on HIV-1 replication through an anti-Tat mechanism was not demonstrated.

To discover a novel compound that inhibits HIV-1 transcription, we have established human T-cell lines that are able to produce a secretory form of alkaline phosphatase under the control of HIV-1 long terminal repeat (LTR). Using these cell lines, we have screened our compound libraries, plant extracts, and culture supernatants of microorganisms. Here, we report that EM2487, a product of *Streptomyces* sp. Mer-2487, has a unique structure and inhibits Tat-induced gene expression without affecting the basal or tumor necrosis factor (TNF)-α-induced transcription. We also report the anti-HIV-1 activities of EM2487 in acutely and chronically infected cell cultures.

Materials and Methods

Analytical Measurement

General Procedures; Ultraviolet spectra were measured on a SHIMADZU UV-2400PC spectrophotometer. Infrared spectra were recorded on a JASCO FT/IR-620 spectrometer. Specific rotations were determined at 22°C with a JASCO DIP 1000 digital polarimeter. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a JEOL JNM-α600 or a Varian UNITY INOVA-500 spectrometer. Chemical shifts were referenced to internal solvent peaks: δ_H 3.35 for CHD_2OD and δ_C 49.0 for CD_3OD; δ_H 4.70 for HOD; δ_H 7.27 for $CHCl_3$ and δ_C 77.0 for $CDCl_3$; and external standard δ_P 0.00 for H_3PO_4. FAB mass spectra were measured on a JEOL JMS-SX102 mass spectrometer using glycerol, *m*-nitrobenzylalcohol, or triethanolamine as matrix.

EM2487 (**1**); Physico-chemical data, see Table 1; HRFAB-MS of fragment ions *m/z* 603.0748 ($C_{17}H_{25}N_4O_{16}P_2$, Δ+0.7 mmu), 458.2169 ($C_{17}H_{38}N_3O_7P_2$, Δ−1.6 mmu), 214.9618 ($C_2H_5N_2O_6P_2$, Δ−0.5 mmu); (−)-FAB-MS *m/z* 828, 603, 585, 480, 458, 215, 197, 79; (+)-FAB-MS *m/z* 872 $(M+2Na-H)^+$, 850 $(M+Na)^+$, 504, 482, 226, 115; ^{1}H and ^{13}C NMR (CD_3OD) see Table 2; ^{31}P NMR (243 MHz, D_2O, 60°C) δ_P 3.61 (d, J=17 Hz), 1.48 (d, J=17 Hz).

Hydrolysis of EM2487 (**1**); A solution of EM2487 (10.2 mg) in 0.1 N HCl (2.0 ml) was stirred at room temperature for 0.5 hour. After neutralization with 1 N $NaHCO_3$, the reaction mixture was passed through a C_{18} cartridge (Sep-Pak Plus PS-2), washed with water, and then eluted with MeOH. The fraction eluted with water was subjected to ODS-HPLC (YMC-pak AM-324, MeOH/H_2O 5 : 95) to yield uridine (**2**). The physico-chemical data of **2** were identical to those of the authentic sample. On the other hand, the MeOH eluate from the C_{18} cartridge was dried under a N_2 flow to give **3** (7.6 mg) as a white powder. **3**: IR (KBr) ν_{max} 3388, 2925, 2854, 1718, 1672, 1466, 1242, 1103, 1073, 1040, 981, 877, 553 cm^{-1}; HRFAB-MS $(M-H)^-$ *m/z* 584.2516 ($C_{23}H_{44}N_3O_{10}P_2$, Δ+1.4 mmu); (−)-FAB-MS *m/z* 584 $(M-H)^-$, 458, 197, 79; (+)-FABMS *m/z* 608 $(M+Na)^+$, 586 $(M+H)^+$, 244, 226; ^{1}H NMR (500 MHz, CD_3OD, 50°C) 7.35 (1H, br s, b4), 4.88 (1H, m, b2), 4.88 (1H, m, b3), 4.28 (2H, s, b6), 4.05 (2H, t, $^3J_{PH}$=13.1 Hz, c2), 2.95 (2H, br m, d1), 2.79 (3H, s, *N*-CH_3) 1.59 (2H, br m, d2), 1.56 (1H, sept-t, J=6.6 Hz, d12), 1.40~1.28 (16H, br m, d3~d10), 1.21 (2H, m, d11), 0.91 (6H, d, J=6.6 Hz, d13, 14); ^{13}C NMR (150 MHz, CD_3OD, 30°C) 202.6 (b1), 178.6 (c1), 154.9 (b4), 146.2 (b5), 84.2 (b2), 75.7 (b3), 63.3 (d1), 56.9 (b6), 51.8 (c2), 46.8 (*N*-CH_3), 40.2 (d11), 31.5~27.3 (d3~d10, 8C), 23.0(d13, d14, 2C).

Hydrolysis of **3**; To a sample of **3** (5.0 mg) 1.0 N HCl (1.0 ml) was added and heated at 100°C for 1 hour. The reaction mixture was then cooled in ice and neutralized with 1.0 N $NaHCO_3$. The resulting suspension was passed through a C_{18} cartridge and washed with water to obtain hydrophilic components **5** and **6** as an inseparable mixture. Then alkylhydroxylamine (**4**) (3.0 mg) was eluted from the resin with MeOH.

N, 12-Dimethyltridecyl hydroxylamine (**4**); IR (KBr) ν_{max} 3164, 2921, 2849, 1466 cm^{-1}; HRFAB-MS *m/z* $(M+H)^+$ 244.2648 ($C_{15}H_{34}NO$, Δ+0.7 mmu); (+)-FAB-MS *m/z* 244, 226; ^{1}H NMR (500 MHz, $CDCl_3$) 2.86 (2H, t, J=7.5 Hz, H-1), 2.80 (3H, s, *N*-CH_3), 1.67 (2H, m, H-2), 1.53 (1H, sept-t, J=6.6, 6.6 Hz, H-12), 1.38~1.24 (16H, br m, H-3~H-10), 1.16 (2H, m, H-11), 0.87 (6H, d, J=6.6 Hz, H-13, 14); ^{13}C NMR (125 MHz, $CDCl_3$) 61.6 (C-2), 47.4 (*N*-CH_3), 39.1 (C-12), 29.9, 29.7, 29.6, 29.6, 29.5, 29.4, 28.0, 27.4, 27.0, 25.8, 22.7 (C-13, 14).

5-Hydroxy-3-hydroxymethyl-2-oxocyclopent-3-enyl dihydrogen phosphate (**5**); (−)-FAB-MS *m/z* 223 $(M-H)^-$,

Table 1. Physico-chemical properties of EM2487 (**1**).

Formula	$C_{32}H_{57}N_5O_{16}P_2$
HRFABMS	[*m/z*, (M-H)$^-$]
Found	828.3216
Calcd	828.3197
Appearance	White powder
$[\alpha]_D$ (22°C, MeOH)	-7.9 (*c* 1.3)
UV λ_{max}	262 (ε 34200)
(MeOH, nm)	
IR (KBr) ν_{max} cm^{-1}	3357, 2925, 2853, 1685, 1467, 1239, 1101, 1070, 875, 551

79 (PO_3^-); ^{1}H NMR (600 MHz, D_2O) 7.40 (1H, br m, H-4), 4.88 (1H, br m, H-3), 4.62 (1H, dd, J=2.6 Hz, $^3J_{PH}$= 10.7 Hz, H-2), 4.30 (2H, br s, H-6); ^{13}C NMR (150 MHz) 203.5 ($^3J_{PC}$=4.9 Hz, C-1), 157.2 (C-4), 145.8 (C-5), 85.0 ($^2J_{PC}$=5.9 Hz, C-2), 75.7 (C-3), 57.2 (C-6); ^{31}P NMR (243 MHz, D_2O, 30°C) −0.70 (s).

Identification of glycinamide (**6**) by PITC method; To a 1/10 portion of the hydrolysis product containing **6** was added 20 μl of solvent mixture (EtOH - H_2O - TEA, 2 : 2 : 1). After concentration under reduced pressure, the residue was dissolved in a mixture of EtOH - H_2O - TEA - PITC (7 : 1 : 1 : 1, 20 μl). The reaction mixture was allowed to stand for 20 minutes at room temperature and then concentrated under reduced pressure to obtain the PTC (phenylthiocarbamoyl) derivative, which was then subjected to HPLC analysis. Authentic glycinamide (*ca.* 50 μg) was converted to the PTC derivative employing the same procedure. The retention time of the natural PTC-glycinamide was the same as that of authentic PTC-glycinamide.

Cells and Plasmids

The T cell line CEM was obtained from the American Type Culture Collection (CCL-119) and was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The plasmids, pUC-BENN-CAT and pSV2*tat*72, were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious disease.

Table 2. ^{13}C and ^{1}H NMR spectral data for EM2487 (**1**) in CD_3OD.

position	δC ppm (multiplicity)[a]	δH ppm (multiplicity, J = Hz)[b]
a2	152.5 (s)	
a4	166.2 (s)	
a5	103.0 (d)	5.84 (d, 7.9)
a6	142.4 (d)	7.91 (d, 7.9)
a1'	90.4 (d)	5.98 (d, 4.6)
a2'	75.7 (d)	4.24 (dd, 5.2, 4.6)
a3'	70.3 (d)	4.42 (dd, 5.2, 5.2)
a4'	84.8 (d)	4.22 (ddd, 5.2, 2.7, 2.4)
a5'	69.5 (t)	4.51 (dd, 11.6, 2.7) , 4.27 (dd, 11.6, 2.4)
b1	155.2 (s)	
b2	81.0 (d)	5.48 (br m)
b3	77.0 (d)	4.38 (ddd, 5.8, 4.0, $^4J_{PH}$=2.5)
b4	71.9 (d)	4.69 (dd, 5.8, 1.0)
b5	121.4 (s)	
b6	55.4 (t)	4.31(d, 12.2), 4.20 (dd 12.2, 1.0)
c1	178.7 (s)	
c2	51.7 (t)	4.10-3.90 (br m)
d1	63.2 (t)	2.98 (br m)
d2	27.3 (t)	1.61 (br m)
d3-d10	31.1-28.6	1.39-1.28 (br m)
d11	40.3 (t)	1.21 (br m)
d12	29.2 (d)	1.56 (sept, t, 6.5, 6.5)
d13	23.1(q)	0.91 (d, 6.5)
d14	23.1(q)	0.91 (d, 6.5)
N-CH_3	46.7 (q)	2.81 (br s)

[a] ^{13}C NMR data were collected at 125 MHz, 20°C.

[b] ^{1}H NMR data were collected at 500 MHz, 50°C.

DNA Constructions

Construction of Cell-HIV-1-LTR-PLAP-PGK-neo: The HIV-1-LTR reporter gene was constructed from an NF-κB reporter gene, TNF-α-PLAP-PGK-neo[6], which contained a promoter region of the TNF-α gene, a secretory form of placental alkaline phosphatase (PLAP) gene and a neomycin-resistant gene driven by a phosphoglycerokinase (PKG) promoter. A promoter region of the TNF-α gene (*Xba*I-*Hind*III fragment) of TNF-α-PLAP-PGK-neo was substituted for the *Xba*I-*Hind*III fragment of a pUC-BENN-CAT containing HIV-1-LTR and a cellular fragment

(Cell-HIV-1-LTR-PLAP-PGK-neo). In some cases, the HIV-1-LTR region was amplified by PCR from pUC-BENN-CAT and mut-LTR-CAT, which had a mutated NF-κB responsive element (TCTACTTTCC), using a primer pair (CGCCAAGCTC TATTGAGGCT TAAGC and CGTCTAGATG GAAGGGCTAA TTCACTCC), digested by *Xba*I and *Hind*III, and inserted into the *Xba*I-*Hind*III site of TNF-α-PLAP-PGK-neo (HIV-1-LTR-PLAP-PGK-neo and HIV-1-mut-LTR-PLAP-PGK-neo).

Construction of pSV2-*tat*-long: The Tat expression vector containing the 1st and 2nd exons of *tat* was constructed from pSV2-*tat*, since the 2nd exon of *tat* was necessary for the transactivation from integrated HIV-1-LTR[7]. Two oligonucleotides (AATTCGGATC CCAGACCCAC CAGGTTTCTC TGTCTAAACA ACCCACCTCC CAATCC and AGATCTTTAT TCCTTCGGGC CTGTCGGGTC CCCTCGGGAT TGGGAGGTGG GTTGT) coding the 2nd exon of *tat* were annealed at their complementary region and elongated with Vent polymerase (New England Biochemistry) to form a double strand DNA. This DNA was digested by *Bam*HI and *Bgl*II and inserted into the *Bam*HI-*Bgl*II site of pSV2-*tat* (pSV2-*tat*-long).

Alkaline Phosphatase Reporter Assay

Assay for alkaline phosphatase activity: To inactivate the alkaline phosphatase of FCS, culture supernatants were incubated at 65°C for 20 minutes. Ten μl of test sample, 50 μl of buffer (0.28 M Na_2CO_3, 8 mM $MgSO_4$, pH 10.0) and 50 μl of Lumistain were mixed in a 96-well microplate for the chemiluminescence assay. After a 60-minute incubation, chemiluminescence was measured with a microplate luminometer LB96P (Berthold).

Establishment of reporter cell lines: Three to ten million CEM cells were suspended in 1 ml of serum free RPMI 1640 medium and mixed with 10 to 40 μg of either Cell-HIV-1-LTR-PLAP-PGK-neo, HIV-1-LTR-PLAP-PGK-neo, or HIV-1-mut-LTR-PLAP-PGK-neo DNA. They were transferred to an electroporation cuvette and incubated at 4°C for 10 minutes. Each DNA was introduced to the CEM cells by electroporation at 300 V, 1000 μF with Gene Pulser (BioRad).

The transfected cells were diluted in 10% FCS RPMI 1640 medium, incubated overnight, and further cultured in 10% FCS RPMI 1640 medium containing 0.8 mg/ml geneticine in a 24-well microplate for 2 weeks. The CEM transformants that constitutively produced alkaline phosphatase (AP) were cloned by limiting dilution.

The selected clones were examined to determine whether their AP level was enhanced by Tat or TNF-α. These clones were transfected or mock transfected by pSV2-*tat*-long, and cultured in the presence or absence of 2 ng/ml TNF-α for 2 days. Alkaline phosphatase activities of the supernatants were determined and the clones having a high ratio of the basal AP level to the Tat or TNF-α-enhanced AP level were chosen. We selected 3A5 (a clone transfected with Cell-HIV-1-LTR-PLAP-PGK-neo), W-3 (a clone transfected with HIV-1-LTR-PLAP-PGK-neo), and KM-3 (a clone transfected with HIV-1-mut-LTR-PLAP-PGK-neo), as reporter cell lines. It was confirmed that the KM-3 clone did not respond to TNF-α stimulation.

Assay of Tat enhanced transcription (**1**): The test compound was dissolved in dimethylsulfoxide (DMSO) and diluted serially with RPMI 1640 containing 5% FCS. The reporter cells were transfected or mock transfected with pSV2-*tat*-long and cultured overnight. The transfected or mock transfected reporter cells (2×10^4) were cultured in 5% FCS RPMI 1640 medium containing serially diluted test compounds and 2 ng/ml TNF-α in a 96-well microplate for 2 days. The culture supernatant (10 μl) was collected and assayed for the alkaline phosphatase activity. The number of viable cells was determined by the MTT method, as described by PAUWELS *et al.*[8]

Assay of Tat enhanced transcription (**2**): The test compound was dissolved in DMSO and diluted serially in RPMI 1640 containing 5% FCS. Reporter cells were suspended in serum free RPMI 1640 containing 2 ng/ml Tat, 100 μM chloroquine, and 1 mM DTT and cultured for 2 hours. The Tat treated reporter cells were centrifuged and resuspended in 5% FCS RPMI 1640 medium containing serially diluted test compounds and 2 ng/ml TNF-α and cultured in a 96-well microplate overnight. The culture supernatant (10 μl) was collected and assayed for the alkaline phosphatase activity. The number of viable cells was determined by the MTT method[8].

Anti-HIV-1 Assay

Anti-HIV-1 assay in MOLT-4 cell: MOLT-4 cells (1×10^5 cells/ml) were infected with HIV-1 (III_B strain) at a multiplicity of infection (MOI) of 0.1, and cultured in the presence of various concentrations of compounds at 37°C. After a 4-day incubation, the infected MOLT-4 cells were subcultured at a ratio of 1 : 5 in the culture medium containing the same concentration of the compounds. On day 7, the anti-HIV-1 activity and cytotoxicity were determined by the MTT method[8].

Anti-HIV-1 assay in OM-10.1 cell: OM-10.1 cells (1×10^5 cells/ml) were incubated in the presence of various concentrations of compounds for 2 hours. They were stimulated with 1 ng/ml TNF-α and further incubated. After 3 days, the amounts of p24 antigen were measured by

ELISA (Cellular Products. Buffalo. N.Y.), and the viable cell numbers were determined by the MTT method.

Result

Fermentation

A sample of the slant culture of *Streptomyces* sp. Mer-2487 was inoculated into 500 ml Erlenmeyer flasks, each containing 50 ml of seed medium composed of glycerol 2.0%, glucose 2.0%, soybean meal 2.0%, yeast extract 0.5%, NaCl 0.25%, $CaCO_3$ 0.32%, and 0.2% of metal salt solution containing 0.25% $CuSO_4 \cdot 5H_2O$, 0.25% $MnCl_2 \cdot 4H_2O$, and $ZnSO_4 \cdot 7H_2O$, pH adjusted to 7.4 before sterilization. The seed flasks were shaken on a rotary shaker for 48 hours. at 28°C. One hundred and 50 ml of the seed culture was transferred to 30-liter jar fermenters containing 15 liter of producing medium. The producing medium contained dextrin 3.0%, glucose 0.5%, soybean meal 1.5%, C.S.L. 0.5%, $CaCO_3$ 0.5% and adecanol 0.05% (pH 7.2 before sterilization).

Fermentations were carried out at 28°C with an aeration of 7.5 liters/minute and agitation at 100~250 rpm. The concentration of the active component reached maximum levels at 64 hours after inoculation.

Taxonomy of the Producing Strain

Strain Mer-2487 was isolated from a soil sample collected in the Shiga-Kogen, Nagano Prefecture, Japan.

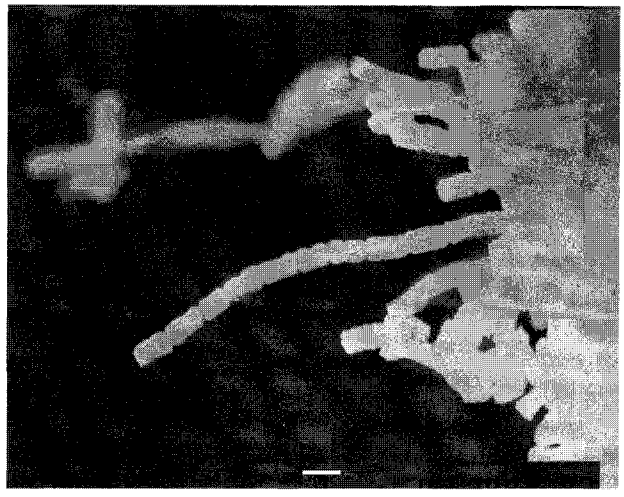
As morphological characteristics, the aerial mycelium of the strain Mer-2487 was well branched and the top of the aerial mycelium was straight or flexuous. After growing, it became divided and formed straight spore chains. The size of the elliptical spore was about 0.6~0.8×1.0~1.2 μm. The surface of the spore was smooth. No sclerotic granules, sporangia or zoospores were observed (Fig. 2).

The strain had the following cultural characteristics when grown on media as described below at 28°C. The color of the surface of colony is indicated according to the symbols described in Color Harmony Manual[9]. The results of cultural characteristics are shown in Table 3. The color of the aerial mycelium was white to red on ISP media Nos. 2, 3, 4. No melanoid pigment was observed in tyrosine agar (ISP media No. 7).

The carbon source utilizing pattern according to Pridham-Gottlieb medium[10] are summarized in Table 4. L,L-Diaminopimelic acid was observed as one of the components of cell wall when the whole-cell hydrolysate of the strain was analyzed by cellulose thin layer chromatography.

Fig. 2. Aerial mycelium of strain Mer-2487.

Observation after incubation at 28°C for 14 days on ISP-3 medium. Bar represents 1 μm.



From the foregoing taxonomic characteristics, the strain Mer-2487 was found to belong to the genus *Streptomyces*. The strain has been deposited at the National Institute of Bioscience and Human-Technology in Japan with the accession number FERM P-16718.

Isolation of EM2487

The isolation procedure for EM2487 is outlined in Fig. 3. The fermentation broth (24 liter) was separated to the mycelia cake and the supernatant by centrifugation. The mycelia cake was extracted with methanol (20 liter), then the methanol layer was concentrated under reduced pressure until methanol was removed. The concentrated solution was added to the supernatant, and the mixed solution was applied to a Diaion HP-20 (2.4 liter, Mitsubishi Chemical Corp.) column. The column was washed with 20% methanol (6 liter), then eluted with 80% acetone (41 liter). The eluate was concentrated *in vacuo* to give a crude EM2487 powder.

The crude powder was subjected to an ODS column (ODS-AM 120-S50, Yamamura Chemical Institute), eluted with 35% acetonitrile in buffer (20 mM phosphate buffer pH 7.0). The eluate including EM2487 was desalted using a Diaion HP-20 column and then concentrated *in vacuo*.

Finally, EM2487 (432 mg) was isolated by preparative HPLC (mobile phase: 35% acetonitrile/20 mM phosphate buffer, pH 7.0) using an ODS column (J'sphere ODS-AM

Table 3. Cultural characteristics of strain Mer-2487.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract - malt extract agar (ISP No.2)	Moderate	Light yellowish pink [5ca, Flesh Pink]~ Grayish yellowish pink [6ec, Powder Rose]	Dark brown	Light brown
Oatmeal agar (ISP No.3)	Good	Grayish yellowish pink [5dc, Pussywillow gray]	Grayish olive green	Light brown
Inorganic salts - starch agar (ISP No.4)	Good	Light yellowish pink [5ca, Flesh Pink]~ Grayish yellowish pink [6ec, Powder Rose]	Dark gray	None
Glycerol asparagine agar (ISP No.5)	Moderate	None	None	None
Tyrosine agar (ISP No.7)	Moderate	None	Grayish olive green	None

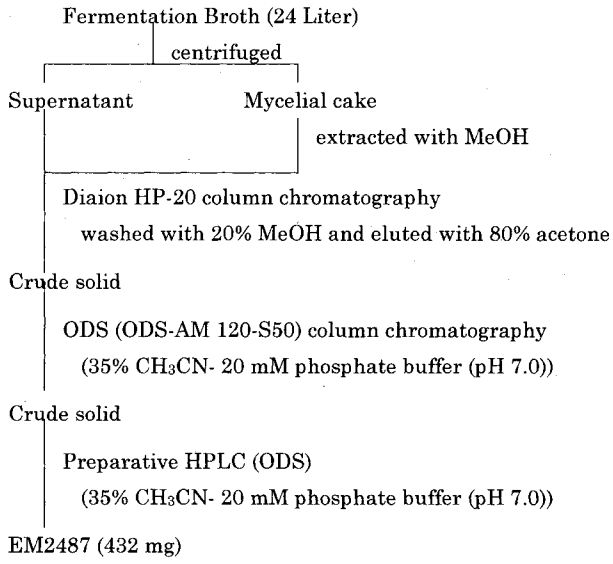
Observation after incubation at 28°C for 14 to 21 days.

Table 4. Carbon utilization of strain Mer-2487.

Carbon source	Growth
L-Arabinose	−
D-Xylose	+
D-Glucose	+
D-Fructose	±
Sucrose	+
Inositol	−
L-Rhamnose	−
Raffinose	−
D-Mannitol	−

+; positive, ±; slightly positive, −; negative.

Fig. 3. Purification procedure of EM2487.



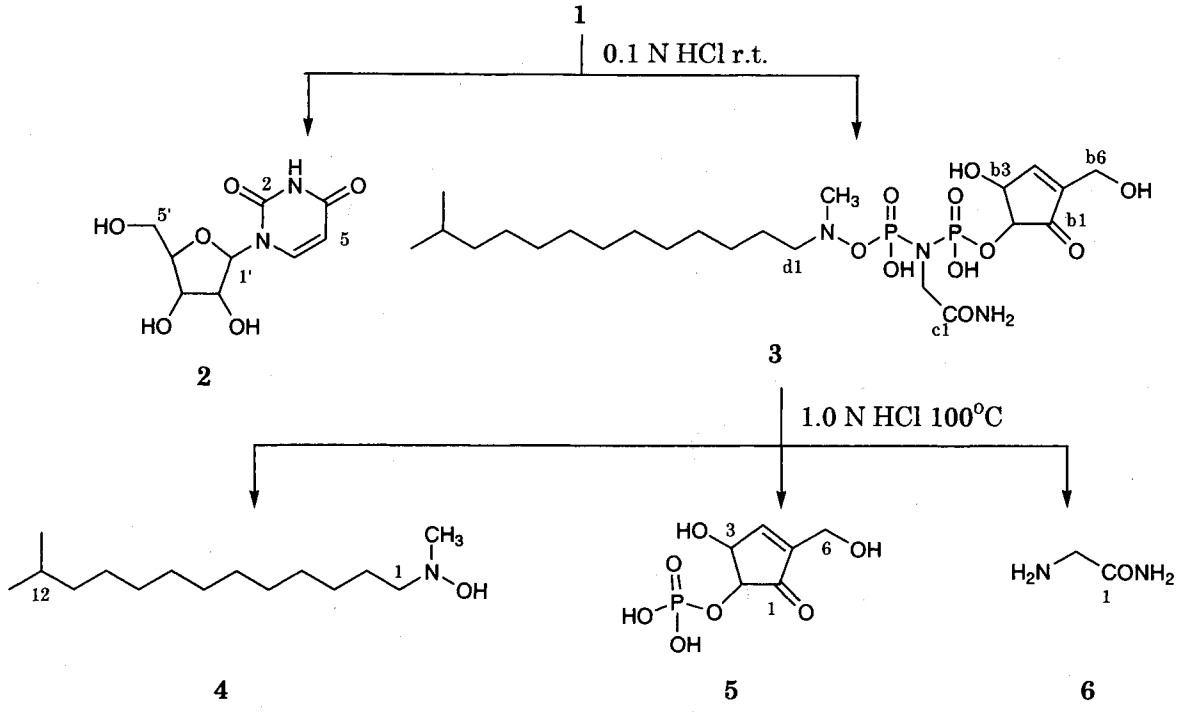
H80, 2 cm i.d. ×25 cm length, Yamamura Chemical Institute).

Structure Elucidation

The physico-chemical properties of EM2487 (**1**) are summarized in Table 1. The molecular formula of EM2487 (**1**) was established as $C_{32}H_{57}N_5O_{16}P_2$ by high resolution FAB mass spectrometry (HRFAB-MS). The presence of phosphorus was also supported by ^{31}P NMR (δ_P 3.61 and 1.48) and negative ion FAB-MS (*m/z* 79, PO_3^-) experiments. The UV spectrum (λ_{max} 262) was indicative of a uracil chromophore, which was also supported by NMR data (δ_H 7.91, 5.84, δ_C 166.2, 152.5, 142.4, 103.0). IR absorption bands at ν_{max} 3357, 1685, and 1239 cm^{-1} evidenced the presence of hydroxy, amide carbonyl, and phosphate groups, respectively.

The ^1H and ^{13}C NMR data are summarized in Table 2. Concomitant analysis of ^1H, ^{13}C, and HMQC (heteronuclear multiple quantum coherence) spectra indicated that this compound contains 3 methyl, 14 methylene, 7 sp^2 methine, and 8 sp^3 methine groups. The presence of a long alkyl chain was readily inferred from a broad signal from δ_H 1.39 to 1.28 ppm observed in the ^1H NMR spectrum. In ^1H-^1H COSY and TOCSY (total correlation spectroscopy) spectra, a spin system of ribose including a characteristic signal of anomeric proton (δ_H 5.98) was clearly demonstrated. In addition to ribose, **1**

Fig. 4. Hydrolysis products of EM2487 (**1**).



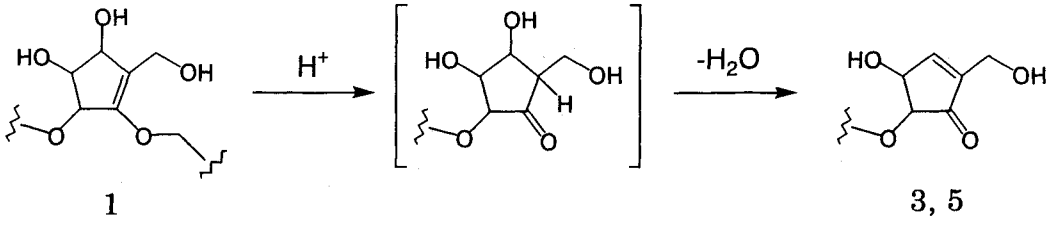
contains a highly oxygenated C_6 unit [–CH(O)–CH(O)–CH(O)–C(CH_2O)=C(O)–], which was determined from ^{1}H-^{1}H COSY, TOCSY, HMQC, and HMBC (heteronuclear multiple bond correlation) data. However, further correlative information could not be obtained due to significant line broadening of the NMR spectra.

Treatment of **1** with 0.1 N HCl afforded uridine (**2**) and a degradation product **3** (Fig. 4). Compound **3** had a molecular formula of $C_{23}H_{45}N_3O_{10}P_2$, which was defined as the base for HRFAB mass measurements. Because uridine ($C_9H_{12}N_2O_6$) and **3** were derived from **1** ($C_{32}H_{57}N_5O_{16}P_2$), dehydration occurred during the degradation step. The ^{1}H and ^{13}C NMR spectra (δ_H 7.35, δ_C 202.6, 154.9, and 146.2) and also the IR spectrum (ν_{max} 1718) of **3** indicated the presence of an α,β-unsaturated ketone which was absent in **1**. Since complete structural determination of **3** by an spectroscopic procedure was not feasible, **3** was further hydrolyzed with 1.0 N HCl at 100°C to yield the degradation products **4**, **5**, and **6** (Fig. 4).

The molecular formula of **4** was deduced as $C_{15}H_{33}NO$ by HRFAB-MS. The ^{1}H and ^{13}C NMR spectra were indicative of *N*-CH_3 (δ_H 2.80, δ_C 47.4), *N*-CH_2 (δ_H 2.86, δ_C 61.6), long alkyl chain (δ_H 1.67~1.16, δ_C 39.1~25.8) and terminal dimethyl groups (δ_H 0.87, δ_C 22.7). The existence of a hydroxylamine group was indicated by the IR absorption band at ν_{max} 3164 cm^{-1}. Therefore, the structure of **4** was determined as *N*-methyl-*N*-(12-methyltridecyl)hydroxylamine.

The molecular formula of **5** was determined as $C_6H_9O_7P$ by FAB mass spectrometry in conjunction with ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra of **5** exhibited signals corresponding to α,β-unsaturated ketone (δ_H 7.40, δ_C 203.5, 157.2, and 145.8), an oxygenated methylene (δ_H 4.30, δ_C 57.2), and two oxygenated methine (δ_H 4.88, 4.62, δ_C 85.0, 75.7) groups. Existence of a phosphate group was indicated by the ^{31}P NMR spectrum (δ_P −0.70) and a diagnostic fragment ion at *m/z* 79 (PO_3^-) observed in the negative mode FAB mass spectrum. The above mentioned structural units fully explained the molecular formula. The methine groups were adjacent ($^3J_{HH}$=2.6 Hz) and one of them was also coupled with the phosphorus (δ_H 4.62, $^3J_{PH}$=10.7 Hz) to define a phosphate ester of a glycol as a partial structure. In the HMBC spectrum, long range C–H couplings were observed between H2 and C1/C3, H3 and C2/C4/C5, H4 and C1/C2/C3/C5/C6, H-6 and C1/C4/C5, respectively. On the basis of these results, the structure of **5** was established as 5-hydroxy-3-hydroxymethyl-2-oxocyclopent-3-enyl

Fig. 5. Formation of α,β-unsaturated ketone in **3** and **5** by acid hydrolysis.



dihydrogen phosphate.

Apparently, compound **5** originated from the C_6 unit [–CH(O)–CH(O)–CH(O)–C(CH_2O)=C(O)–] of **1** described above. The *de novo* formation of an α,β-unsaturated ketone in **3** and **5** was explained by assuming cleavage of the enol ether linkage by acid hydrolysis, and subsequent dehydration as depicted in Fig. 5. On the basis of these data, the structure of the original C_6 unit was unambiguously determined as shown in Fig. 1.

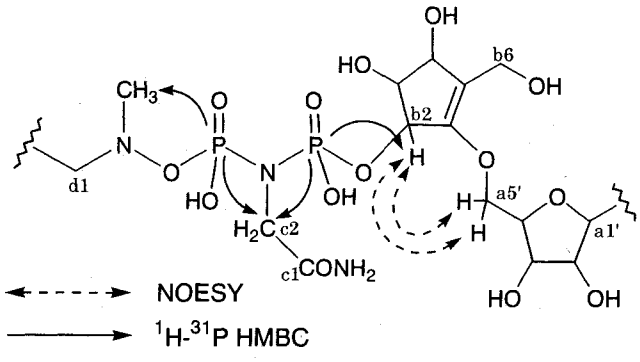
The degradation product **6** was identified as glycinamide. The presence of glycinamide was first suspected when ^{1}H and ^{13}C NMR spectra of **1** showed signals of methylene (δ_H 4.10~3.90, δ_C 51.7) and carbonyl groups (δ_C 178.7), which were isolated from the other spin systems. TLC analysis of the degradation products showed a ninhydrin positive spot whose Rf value was identical with that of glycinamide. Finally, the presence of glycinamide was substantiated by conventional PITC (phenylisothiocyanate) method.

At this stage, all six substructures were established, *i.e.*, uridine, C_6 cyclopentene unit, alkylhydroxylamine, glycinamide, and two phosphate groups. Assembly of these substructures was accomplished by a combination of spectroscopic techniques particularly by ^{1}H-^{31}P HMBC and ^{31}P decoupled ^{1}H NMR experiments.

The key correlations connecting the partial structures are summarized in Fig.6. The NOESY cross peaks between H-a5′ and H-b2 (Fig. 6) indicated that a5′ of the uridine was the linking position to the C_6 unit. Relatively low chemical shifts of the a5′ protons (δ_H 4.51 and 4.27) also supported this connection.

The two phosphorus were coupled with each other by 17 Hz, which is a typical value of $^2J_{PP}$[11]. The ^{1}H-^{31}P HMBC spectrum demonstrated long range coupling between one of the phosphorus signals and the H-b2 of the C_6 unit, while the other phosphorus showed ^4J correlation with *N*-CH_3 protons of the alkylhydroxylamine. Furthermore, cross peaks were detected between both

Fig. 6. Selected ^{1}H-^{31}P HMBC and NOESY data for **1**.



phosphorus signals and methylene protons of the glycinamide. These data strongly suggested that glycinamide was situated between both phosphorus.

In order to confirm the unique P-N-P link and also to determine the linking position of glycinamide, ^{31}P selective decoupling experiments were performed. When either of the phosphorus was irradiated, about 13 Hz of scalar coupling disappeared from the glycinamide CH_2 proton resonances. This value was regarded as that of a three bond coupling when compared with a model compound reported in the literature;[12] thus it was determined that the linking position was an amine nitrogen. A negative result of **1** with ninhydrin reagent was consistent with these results.

The mass spectral data also supported the above mentioned link. The negative ion FAB mass spectrum of **1** showed several diagnostic fragment ion peaks at *m/z* 603, 458, and 215 (Fig. 7). The assignments shown in Fig. 7 were confirmed by the accurate mass of the fragment ions such as $C_{17}H_{25}N_4O_{16}P_2$ for *m/z* 603, $C_{17}H_{38}N_3O_7P_2$ for *m/z* 458, and $C_2H_5N_2O_6P_2$ for *m/z* 215 by HRFAB-MS.

From all these results, the structure of EM2487 (**1**) was

Fig. 7. The negative ion FAB mass spectrum of EM2487.

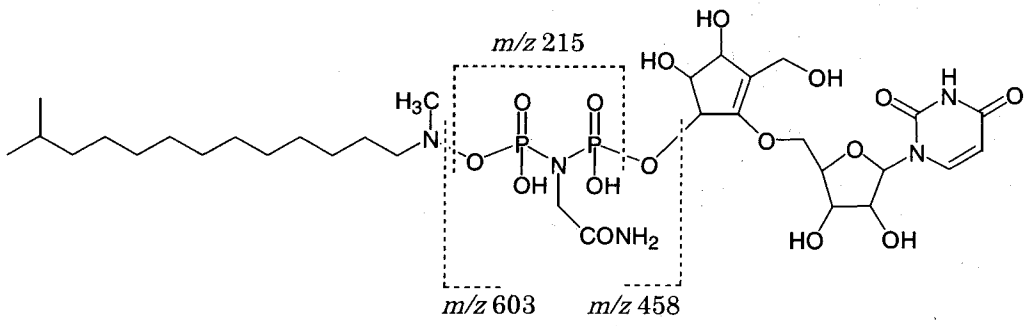
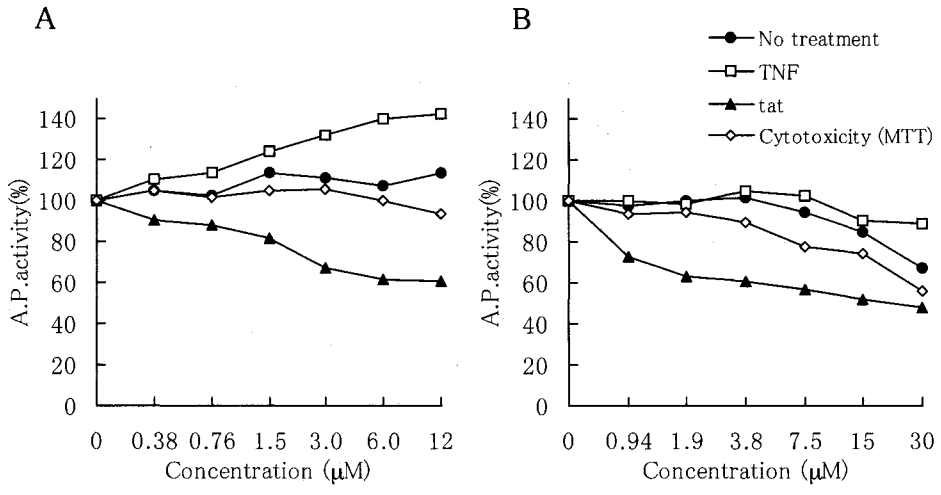


Fig. 8. Inhibitory effect of EM2487 (A) and Ro 24-7429 (B) on HIV-1 LTR-driven gene expression.

3A5 cells were either stimulated with 2 ng/ml TNF-α (■), or were transfected with Tat-expression vector (▲), or remained unstimulated (●), and were cultured in the presence of various concentrations of the test compounds for 2 days. The effects were determined by the alkaline phosphatase activity in culture supernatants, and the viability of cells was assayed by the MTT method (◇).



determined as shown in Fig. 1.

Biological Properties

Inhibitory effect on Tat-induced gene expression: *Streptomyces* sp. Mer-2487 product was found to suppress the Tat-induced but not the basal and TNF-α-induced gene expression, as determined by the production of PLAP (data not shown). Its purified active component was designated as EM2487. In 3A5 cells, the production of PLAP was enhanced approximately 20-fold by transfection with the Tat-expression plasmid and 5-fold by addition of TNF-α into the culture medium. EM2487 suppressed the Tat-induced gene expression in a dose-dependent fashion. It reduced the PLAP level to 60% of the control at a concentration of 12 μM (Fig. 8A). Ro 24-7429 displayed a similar inhibitory effect on Tat-induced gene expression in 3A5 cells (Fig. 8B). In contrast, both EM2487 and Ro 24-7429 slightly enhanced the TNF-α-induced gene expression at their nontoxic concentrations (Fig. 8). Exogenous addition of Tat protein also increased the level of PLAP approximately 50-fold in 3A5 cells, and this effect was suppressed by the presence of EM2487 (data not shown).

Fig. 9. Anti-HIV-1 activity of EM2487 (A) and Ro 24-7429 (B) in MOLT-4 cells.

MOLT-4 cells (1×10^5 cells/ml) were infected with HIV-1 (III_B strain) at a MOI of 0.1, and cultured in the presence of various concentrations of the test compounds. After a 4-day incubation at 37°C, MOLT-4 cells were subcultured at ratio of 1 : 5 with fresh culture medium containing the same concentration of the compounds. On day 7, anti-HIV-1 activity and cytotoxicity were determined by the MTT method.

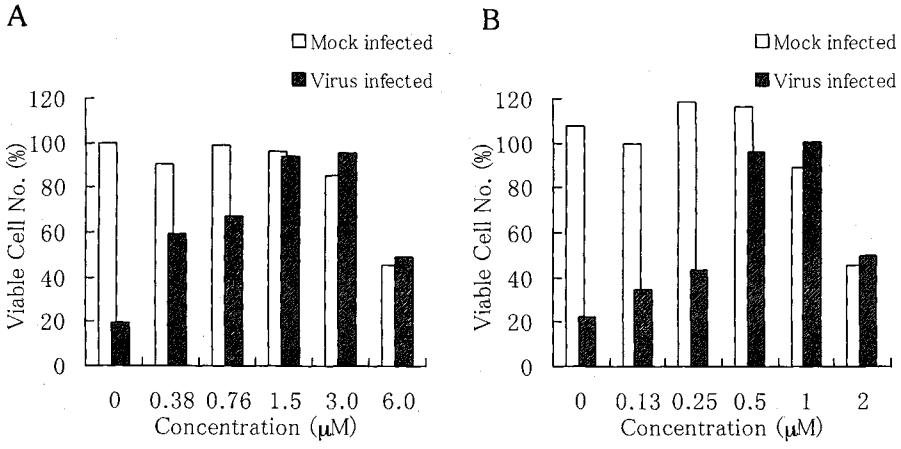
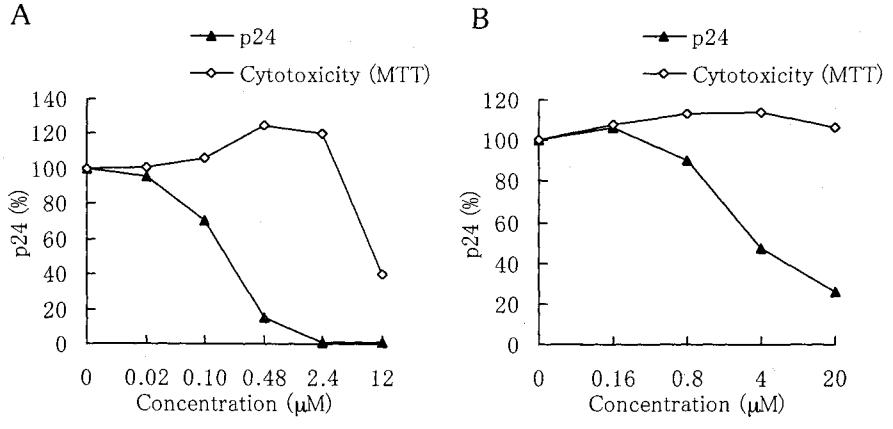


Fig. 10. Anti-HIV-1 activity of EM2487 (A) and Ro 24-7429 (B) in OM-10.1 cells.

OM-10.1 cells (1×10^5 cells/ml) were incubated in the presence of various concentrations of the test compounds for 2 hours, stimulated with 1 ng/ml TNF-α, and further incubated. After a 3-day incubation at 37°C, the culture supernatants were collected nd examined for their p24 antigen levels (●). The cytotoxicity of the test compounds were also determined by the MTT method (△).



Anti-HIV-1 activity: When EM2487 was examined for its inhibitory effect on HIV-1 replication in acutely infected MOLT-4 cells, it protected the cells almost completely against HIV-1-induced cytopathicity at a concentration of 1.5 μM (Fig. 9A). This inhibition was dose-dependent. The inhibitory effect on HIV-1 replication was also observed with Ro 24-7429 at a concentration of 0.5 and 1 μM (Fig. 9B). The 50% effective concentrations (EC_{50}s) of EM2487 and Ro 24-7429 were 0.4 and 0.3 μM (data not shown). In the chronically infected cell line OM-10.1, EM2487 inhibited HIV-1 replication, as determined by the production of p24 antigen in culture supernatants. EM2487 reduced the p24 antigen level in a dose-dependent fashion and completely suppressed the antigen production at a concentration of 2.4 μM (Fig. 10A). At this concentration, the compound did not inhibit the proliferation and viability

of OM-10.1 cells, as determined by the MTT method. On the other hand, complete inhibition was not achieved by Ro 24-7429 at concentrations up to 20 μM (Fig. 10B). These results indicate that EM2487 is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells.

Discussion

HIV-1 gene expression triggered by viral and host cellular factors is an attractive target for inhibition of viral replication[13]. Although the cellular transcription factor nuclear factor κB (NFκB), which is strongly activated by TNF-α, is a potent enhancer of HIV-1 gene expression, the viral transactivator protein Tat seems to play a more important role in sustaining a high level of viral replication. In fact, Tat enhanced the HIV-1 LTR driven gene expression 20 to 100-fold in our reporter cell lines, whereas TNF-α achieved only a 5-fold enhancement (data not shown). The therapeutic potential of Tat inhibitors as anti-HIV-1 agents has not been proven yet, since Ro 24-7429 failed to demonstrate its clinical efficacy *in vivo*[14]. It has been reported that Ro 24-7429 did not show any selective inhibition of HIV-1 replication in MT-4 cells[4]. Furthermore, the compound is assumed to target a host cellular factor that binds to TAR[15]. Such nonspecificity of Ro 24-7429 might have resulted in the failure of clinical trials. In contrast, EM2487 showed inhibitory activity against HIV-1 replication in acutely infected MT-4 cells as well as peripheral blood mononuclear cells and MOLT-4 cells (data not shown).

Using the 3A5 cell line, we have screened compounds libraries, plant extracts and culture supernatants of fungi and actinomycetes, and found some compounds which could suppress HIV-1 LTR-driven transcription with similar potency to EM2487 at their nontoxic concentrations. However, they could not inhibit HIV-1 replication in MOLT-4 cells and OM-10.1 cells. It is thought that these compounds failed to show anti-HIV-1 activity because the transcription control mechanisms of the integrated HIV-1 genome are more complicated than those of the simple LTR-driven transcription and our reporter system is not capable of reflecting such complex mechanisms. A more elaborate system would be necessary for the more efficient discovery of new anti-HIV-1 compounds.

EM2487 is a new class of secondary metabolites of microbial origin whose structure is completely different from that of known nucleoside antibiotics[16]. Some antibiotics such as septacidine[17], tunicamycins[18], and liposidomycins[19,20] can be related to EM2487 as they possess both a nucleoside unit and long alkyl groups. However, none of them contain the unique structural constituents of EM2487, namely, alkylhydroxylamine, phosphate imide of glycinamide, and highly oxygenated cyclopentene substructures.

Since the unique structure of EM2487 makes its chemical modification extremely difficult, we have not performed structure-activity relationship studies of EM2487 derivatives. EM2487 has a enol-ether linkage, which is susceptible to acid hydrolysis (Fig. 1). Both of the hydrolysis products, uridine and product **3** (Fig. 4), did not show any inhibitory effects on the Tat-induced gene expression or HIV-1-replication (data not shown). Further studies are required to pinpoint the target and determine the mode of action of EM2487.

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