independent experiments. (**G**) Schematic structure of the plasmid, pEF Rluc EMCV IRES Feo. The bicistronic gene is transcribed under the control of elongation factor 1α (EF1α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r), and is translated under the control of the EMCV IRES. (**H**) Huh7 cell line transfected with pEF Rluc EMCV IRES Feo was established in the presence of G418. The cells were incubated for 72 h without (control) and with 50 μg/mL of SG1-23-1. Firefly or *Renilla* luciferase activity was measured by the method described in Materials and Methods and was normalized by the protein concentration. F/R: Relative ratio of Firefly luciferase activity to *Renilla* luciferase activity. F/R is presented as a percentage of the control condition. Error bars indicate standard deviation. The data represent three independent experiments.

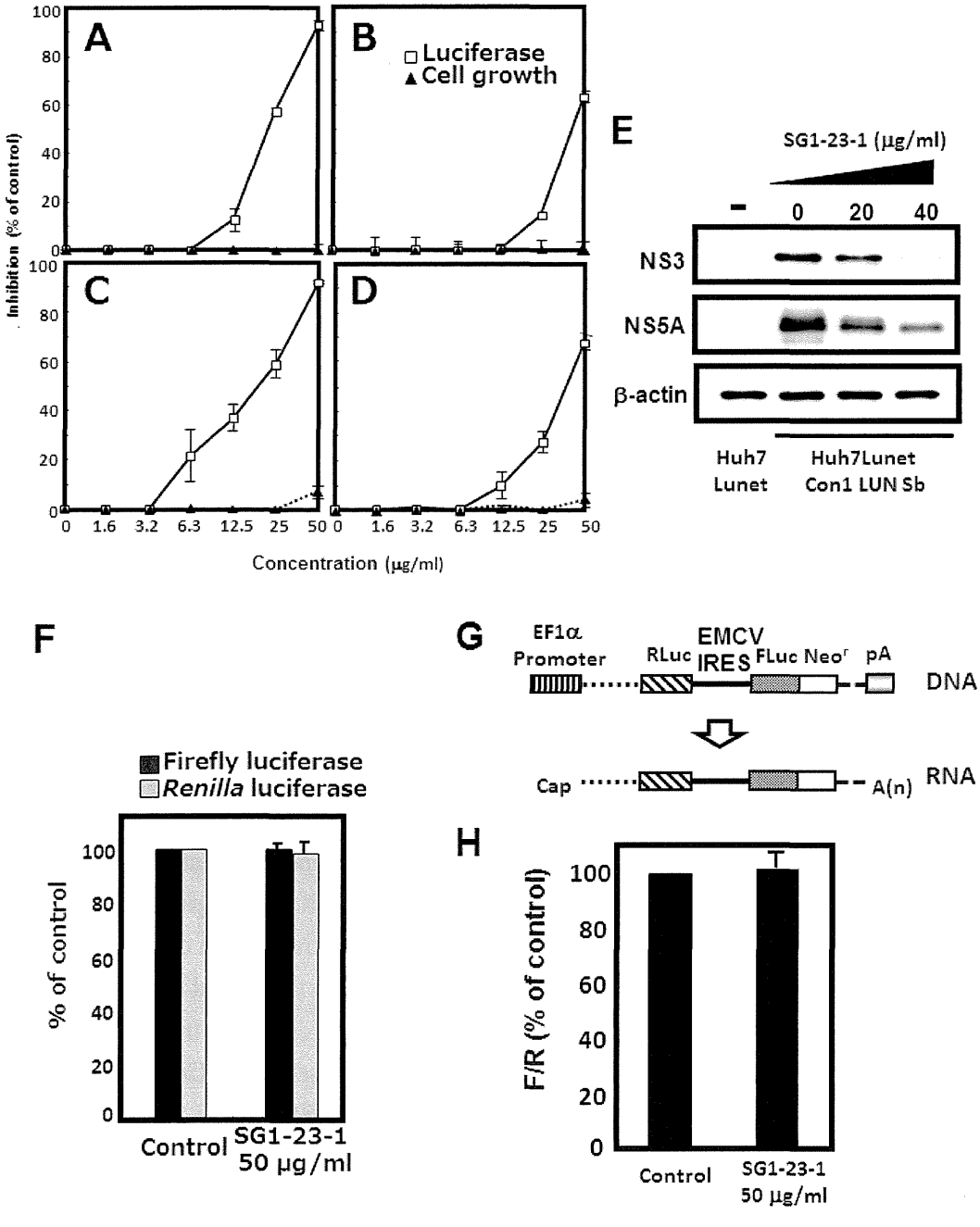


Table 2. Anti-HCV activity of SG1-23-1 in different replicon cell lines of genotype 1b.

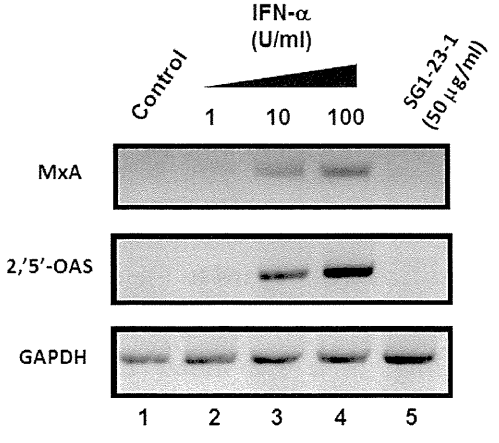
Replicon Cell Line	Virus Strain (Genotype 1b)	EC_{50} [a] (μg/mL)	EC_{90} [b] (μg/mL)	CC_{50} [c] (μg/mL)
Subgenome				
Huh7 Lunet/ Con1 LUN Sb #26	Con1	22.9 ± 0.4	48.1 ± 1.5	>50
Huh7 rep Feo	N	44.2 ± 1.5	>50	>50
Hu7#94/ORN3-5B#24	O	19.9 ± 1.8	48.8 ± 0.3	>50
Full genome				
OR6	O	39.5 ± 0.8	>50	>50

All data represent means ± standard deviation for three independent experiments; [a] Fifty percent effective concentration based on the inhibition of HCV replication; [b] Ninety percent effective concentration based on the inhibition of HCV replication; [c] Fifty percent cytotoxicity concentration based on the reduction of cell viability.

2.4. Effect of SG1-23-1 on the Interferon (IFN) Signaling Pathway

It has been reported that the HCV replication in cultured cells is potently inhibited by interferon (IFN) [36,37]. We examined whether or not treatment with SG1-23-1 induces interferon from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 50 μg of SG1-23-1 per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible genes, MxA and 2',5'-OAS, were induced with IFN-alpha 2b but not with SG1-23-1 (Figure 6). These results suggest that the inhibitory effect of SG1-23-1 on the replication of the HCV replicon is independent of the IFN signaling pathway.

Figure 6. Effect of SG1-23-1 on interferon signaling pathway. Huh7 Lunet/Con1 LUN Sb #26 cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNa-2b (lanes 2–4), and 50 μg/mL SG1-23-1 (lane 5) for 48 h. The mRNAs of MxA, 2',5'-OAS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse-transcription polymerase chain reaction (RT-PCR). Error bars indicate standard deviation. The data represent three independent experiments.



Treatment with SG1-23-1 suppressed the helicase activity of NS3 in a dose-dependent manner and exhibited an IC_{50} of 11.7 μg/mL. Interestingly, treatment with SG1-23-1 inhibited the RNA binding activity of the helicase but not the ATPase activity of NS3. Treatment with SG1-23-1 inhibited the luciferase activity corresponding to the HCV replication in the replicon cell lines, but not the enzymatic activity of luciferase or the translational activity of EMCV IRES, suggesting that treatment with SG1-23-1 decreases HCV replication. Figure 4 shows that the viral proteins NS3 and NS5A in replicon cells were decreased by treatment with SG1-23-1, supporting the notion that SG1-23-1 inhibits HCV replication but not the enzymatic activity of luciferase. The inhibition of cell growth would not contribute to the inhibition of HCV replication by SG1-23-1 (Figure 3 and Table 2). Treatment with SG1-23-1 did not induce the interferon-stimulated genes in the replicon cell lines (Figure 6), suggesting that inhibition of HCV replication by treatment with SG1-23-1 is not due to interferon induction or interferon signaling. The extract SG1-23-1 inhibited the HCV replicon with an EC_{50} of 22 to 44 μg/mL, which is similar to the value of IC_{50}. These results suggest that the anti-HCV compound(s) included in *A. polycladia* can suppress viral replication by inhibiting NS3 helicase activity.

3. Experimental Section

3.1. Preparation of Extracts from Marine Organisms

All marine organisms used in this study were collected by hand during scuba diving off Shimoji, Okinawa, Chibishi, Kuro, Kume, and Tokashiki Islands in Okinawa Prefecture, Japan. In the case of OK-99-tagged extract, a specimen was soaked in ethanol. The ethanol-soluble fraction was concentrated, and the resulting aqueous material was suspended in ethyl acetate (EtOAc). The organic fraction was used for screening.

Each specimen from Kume was soaked in ethanol. The ethanol-soluble fraction was concentrated. The resulting material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG1 and the last digit of "1". The water layer was concentrated to dryness and suspended in methanol (MeOH). The MeOH-soluble fraction was used for screening and tagged with SG1 and the last digit of "2".

Each specimen from Tokashiki was extracted three times with acetone. After removal of acetone from the solution, the residual material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG3.

All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

3.2. High-Throughput Screening of NS3 Helicase Inhibitors

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [38] and was slightly modified with regard to the reaction mixture. A schematic diagram of the PET assay for HCV NS3 helicase activity is shown in Figure 1. The double-strand RNA was prepared as a substrate by annealing, at a 1:2 molar ratio, a 5' BODIPY FL-labeled 37-mer (5'-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUU-3') to a 23-mer (GGUUAUGAG GGUGGAGGUAAUAG). When unwound by HCV NS3 helicase, the unlabeled ssRNA was captured by a DNA capture strand (5'-CTATTACCTCCACCCTCATAACC-3'). A fluorescent-dye-labeled

oligonucleotide was purchased from J-Bio 21 Corporation. BODIPY FL was attached to the 5'-end via an aminohexylphosphate linker with a six-carbon spacer. Unlabeled oligonucleotides were purchased from Japan Bio Services Co., Ltd. The PET NS3 helicase assay was carried out in 22 μL of 25 mM MOPS-NaOH (pH 6.5) containing 3 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 4 U RNasin, 50 nM of the double-strand RNA described above, 100 nM DNA capture strand, 5 mM ATP, and the extract (25 μg/mL) and 240 nM HCV NS3 helicase. The reaction was started by the addition of HCV NS3 helicase. The reaction mixture was incubated at 37 °C for 30 min. The fluorescence intensity was recorded every 5 s until 5 min post-reaction, and then every 30 s between 5 and 30 min post-reaction by using a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The initial reaction velocity was calculated and represented as NS3 helicase activity.

3.3. ATPase Assay

NS3 ATPase activity was determined by the method of Gallinari *et al.* [39] with slight modifications. The reaction was carried out at 37 °C for 10 min in 10 μL of the reaction mixture containing 25 mM MOPS-NaOH (pH 7.0), 1 mM DTT, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM [γ-^{32}P] ATP (Muromachi, Tokyo, Japan), 300 nM NS3, and 0.1 μg poly (U) per microliter and an indicated concentration of SG1-23-1, and then was terminated by the addition of 15 microliters of 10 mM EDTA. Two microliters of the reaction mixture were spotted onto a polyethyleneimine cellulose sheet (Merck, Darmstadt, Germany) and then developed in 0.75 M LiCl/1 M formic acid solution at room temperature for 20 min. The sheet was air-dried completely and then exposed to an image plate. Radioactive bands were visualized with an Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software (version 3.11; Fujifilm: Tokyo, Japan, 2008).

3.4. RNA Helicase Assay

NS3 RNA helicase assay was carried out by the method of Gallinari *et al.* [39] with slight modifications. The substrate for annealing two complementary RNA oligonucleotides, 5'-AGAGAGAGAGGUUGAGAGAGAGAGUUUGAGAGAGAGAG-3' (40-mer, template strand) and 5'-CAAACUCUCUCUCUCAACAAAAAA-3' (26-mer, release strand) was purchased from Shanghai GenePharma Co., Ltd. The release strand was labeled at the 5'-end with [γ-^{32}P] ATP (Muromachi, Tokyo, Japan) using the T4 polynucleotide kinase (Toyobo, Osaka, Japan) at 37 °C for 60 min and then purified by phenol chloroform extraction. The template and the labeled release strands were annealed at a molar ratio of 3:1 (template: release), denatured at 80 °C for 5 min, and slowly renatured at 23 °C for 30 min in an annealing buffer consisting of 20 mM Tris-HCl (pH 8), 0.5 M NaCl, and 1 mM EDTA. The partial duplex RNA substrate was purified on a G-50 micro column (GE Healthcare, Uppsala, Sweden) and stored at −20 °C in H_2O containing 0.25 U of RNasin Plus (Promega, Madison, WI, USA) per microliter.

SG1-23-1 extract was added at various concentrations to a helicase reaction mixture consisting of 25 mM MOPS-NaOH (pH 7.0), 2.5 mM DTT, 2.5 U of RNasin Plus (Promega), 100 μg of BSA per milliliter, and 3 mM $MgCl_2$. The mixture was supplemented with 300 nM NS3 protein and 5 fM ^{32}P-labeled partial duplex RNA substrate. It was then preincubated at 23 °C for 15 min. After adding ATP at a final concentration of 5 mM, the reaction mixture (20 μL) was incubated at 37 °C for 30 min

and stopped by adding 5 μL helicase termination buffer consisting of 0.1 M Tris-HCl (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 25% glycerol. The terminated reaction mixture was subjected to native TBE 10% polyacrylamide gel electrophoresis. The radioactive RNAs in the gel were visualized with an Image Reader FLA-9000 (Fujifilm) and quantified by Multi Gauge V 3.11 software.

3.5. RNA Binding Assay

RNA binding to NS3 helicase was analyzed by gel mobility shift assay [40]. First, let-7 single-strand RNA (5'-UGAGGUAGUAGGUUGUAUAGU-3') was incubated with [γ-^{32}P] ATP (Muromachi, Tokyo, Japan) and T4 polynucleotide kinase (Toyobo) at 37 °C for 60 min for labeling at the 5'-end of the single-strand RNA. The reaction mixture was subjected to phenol chloroform extraction for purification of labeled RNA. The reaction was carried out at room temperature for 15 min in 20 μL of the mixture consisting of 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 1 unit of RNasin Plus (Promega) per microliter, 300 nM NS3, 5 fmol let-7-labeled ssRNA, and an indicated concentration of SG1-23-1. The reaction was stopped by adding an equal volume of dye solution consisting of 0.025% bromophenol blue, 10% glycerol, and 0.5× Tris/borate/EDTA (TBE). The resulting mixture was subjected to native 6% polyacrylamide gel electrophoresis (acrylamide: bis acrylamide = 19:1). The radioactive RNA was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

3.6. Cell Lines

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/mL G418: The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b) [34]; the Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) [35]; the Huh7 Rep Feo cell line, which harbors the subgenomic replicon RNA of the N strain (genotype 1b) [33]; and the OR6 cell line, which harbors the full genomic RNA of the O strain (genotype 1b) [35].

3.7. Determination of Luciferase Activity in HCV Replicon Cells

HCV replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. The extract SG1-23-1 was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon.

3.8. Determination of Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at a density of 1×10^4 cells per well in a 96-well plate and incubated at 37 °C for 24 h. The extract fraction of the sample code SG1-23-1 was added to the culture medium at various concentrations. These cells were treated with an indicated concentration of the extract fraction and then were harvested 72 h post-treatment. Cell viability was measured by dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

3.9. Effects on Activities of Luciferase and Internal Ribosome Entry Site (IRES)

The plasmid pEF Fluc IN and pEF Rluc EMCV IRES Feo were described previously [41]. The firefly luciferase gene was replaced with the *Renilla* luciferase gene in the plasmid pEF Fluc IN. The resulting plasmid was designated as pEF RlucIN in this study. The Huh7 cells were transfected with the pEF Fluc IN, pEF Rluc IN, or pEF Rluc EMCV IRES Feo and then were established in a medium containing 0.25 mg/mL G418 as described previously [41]. These cell lines were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with 50 μg/mL extract SG1-23-1, and then harvested at 72 h post-treatment. Activities of firefly and *Renilla* luciferases in pEF Rluc EMCV IRES Feo were measured with the dual luciferase reporter assay system (Promega). Total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

3.10. Western Blotting

The cells were lysed in lysis buffer containing Cell Culture Lysis Reagent (Promega). The cell lysate was subjected to SDS-10% polyacrylamide gel (SDS-PAGE). The proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane. The resulting membrane was incubated with the primary antibodies at 4 °C overnight and then was washed three times with PBS containing 0.02% Tween 20 (PBS-T). The resulting membrane was reacted with a horseradish peroxidase-labeled anti-IgG antibody at room temperature for 2 h and then was washed three times with PBS-T. The reacted proteins were visualized with ImmunoStar LD (Wako Pure Chemical, Osaka, Japan). The antibodies to NS3 (Abcam, Cambridge, UK), NS5A (ViroGen, Watertown, MA, USA) and beta-actin were purchased from New England Biolabs (Beverly, MA, USA) and were used as the primary antibodies in this study.

3.11. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The previously described method of RT-PCR [41] was slightly modified, as described below. Total RNA was isolated from cultured cells with the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) and then was reverse-transcribed with a Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The transcribed mRNA was amplified with PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and an appropriate primer pair. Primer sequences targeting the genes encoding 2',5'-oligoadenylate synthetase (2',5'-OAS), myxovirus resistance protein A (MxA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [41].

4. Conclusions

In conclusion, we showed that the ethyl acetate extract from *Alloeocomatella polycladia* significantly inhibits HCV replication by suppressing viral helicase activity. The purification of an inhibitory compound from the extract of *Alloeocomatella polycladia* will be required in order to improve the efficacy of chemical modification of the compound(s).

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Tuning efficiency of the 4-*exo-trig* cyclization by the electronic effect: ring closure of 3,3-difluoro-4-pentenyl carbon radicals and synthesis of a *gem*-difluorocyclobutane nucleoside†

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4-*exo-trig* Cyclization reaction of a 4-pentenyl carbon radical containing the *gem*-difluoromethylene moiety adjacent to a radical accepting α,β-unsaturated ester was found to proceed efficiently to furnish a novel *gem*-difluorocyclobutane derivative. The cyclized product could be transformed into a *gem*-difluoromethylene analogue of oxetanocin T.

Oxetanocin A (**1**)[1] is a nucleoside antibiotic isolated from *Bacillus megaterium* (Fig. 1). Due to the unique four-membered oxetanose moiety conferring promising anti-viral properties,[2] considerable efforts have been devoted to the synthesis of **1**, and its base- or sugar-modified derivatives.[3] Interestingly, the thymine analogue of oxetanocin T **2**[4] also exhibits anti-HIV activity. As a sugar-modified derivative, cyclobutane nucleoside **3**[5] has been synthesized and reported to possess promising anti-HBV activity. In this context, we have envisaged that a novel nucleoside **4**, in which the oxetanose ring oxygen of **2** is replaced with a geminal-difluoromethylidene (CF_2) group, would show promising antiviral activities, as the CF_2 group has been suggested as an isopolar and isosteric substituent for oxygen.[6]

Intramolecular cyclization of carbon-centered radicals to unsaturated bonds has been widely used for the construction of cyclopentanes or cyclohexanes *via* 5-*exo*, 6-*exo* or 6-*endo-trig* mode according to Baldwin's rule.[7,8] In contrast, there have been only a few examples of 4-*exo-trig* cyclization reactions of 4-pentenyl carbon radicals leading to cyclobutane rings.[9] Successful ring closures have relied upon the *gem*-dialkyl effect.[9,10] During the course of our synthetic studies of **4** by radical cyclization, we found that the electron-withdrawing effect of the fluorine atom facilitates the 4-*exo-trig* cyclization. In this communication we report these results and their application to the synthesis of **4**.

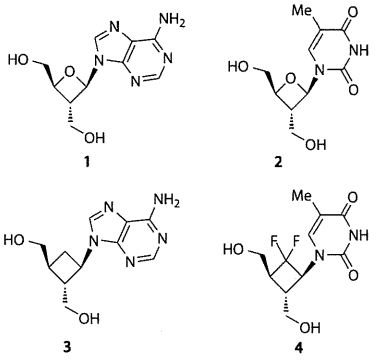

Fig. 1 Structure of compounds **1–4**.

There are three possible 4-pentenyl carbon radicals shown as **A–C** leading to the *gem*-difluorocyclobutane structure *via* 4-*exo-trig* cyclization reaction (Fig. 2). Initially, **5a**, which is a precursor of the 1,1-difluoro-4-pentenyl carbon radical **A**, was reacted with Bu_3SnH in the presence of AIBN in refluxing toluene. This reaction gave target **6a** in 29% yield (*cis*/*trans* = 3/1). However, a major product was the reduced product **7a** (32%). 2,2-Difluoro-carbon-radical **B** generated from **5b** failed to cyclize and gave a complex mixture.

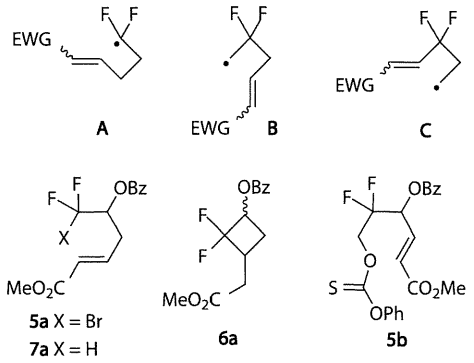

Fig. 2 Plausible radical intermediates **A–C** and their model compounds.

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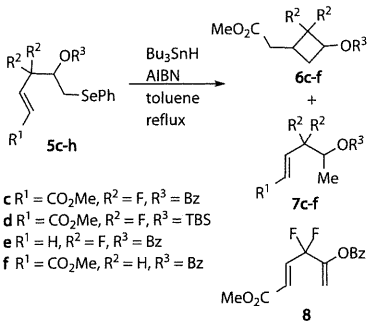
† Electronic supplementary information (ESI) available: Experimental procedures for all newly synthesized products. CCDC 895081. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2cc35876j

Table 1 Radical reaction of **5c–5f**[a]

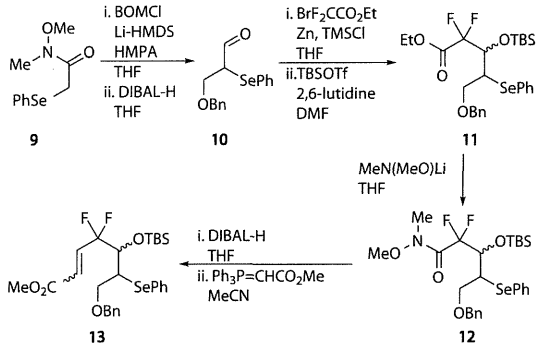


Entry	Precursor	Yield [%] of **6** (ratio; *cis*/*trans*)	Yield [%] of **7**
1	**5c**	72 (2/1)	12[b]
2	**5d**	91 (2/1)[c]	—
3	**5e**	—	72
4	**5f**	22[d]	61

[a] Reaction was carried out in refluxing toluene using 2.0 equiv. of Bu_3SnH and 0.2 equiv. of AIBN (dropwise addition over 4 h). [b] Compound **8** was obtained in 7% yield. [c] The ratio was calculated by the integration of ^{1}HNMR. [d] The stereochemistries of **6f** were not determined.

In contrast to these results, when **5c** was subjected to the same reaction conditions for the ring-closure of **5a**, intramolecular 4-*exo*-*trig* cyclization of the resulting 3,3-difluoro-4-pentenyl carbon radical **C** efficiently proceeded to give the target difluorocyclobutane **6a** in 72% yield (*cis*/*trans* = 2 : 1), along with the reduction product **7c** (12%) and diene **8** (7%) (Table 1, entry 1). As shown in entry 2, TBS-protected **5d** gave **6d** (*cis*/*trans* = 2 : 1) in 91% isolated yield. As can be seen in entry 3, the simple alkene **5e** lacking the electron-withdrawing group gave reduced product **7e** as a sole product in 72% yield (entry 3). A similar result was obtained in the case of the de-fluorinated analogue **5f** (entry 4). These results suggested that the efficiency of the 4-*exo*-*trig* cyclization was dependent upon the electron density of the double bond although the influence of the conformational change of each radical intermediate cannot be ruled out. To validate our assumption, SOMO and LUMO levels of the model intermediates were calculated based on Natural Bind Orbital (NBO) theory Table S1, see ESI.† As anticipated, the energy level of LUMO of **5c′** having both two fluorine atoms and unsaturated ester is lower than those of **5e′** and **5f′** lacking ester or two fluorine atoms. By taking the nucleophilic character of the carbon centered radical into consideration,[11] it would be reasonable that increasing electrophilicity of the radical-accepting unsaturated bond by fluorine substituents and ester facilitated the 4-*exo*-*trig* cyclization.

After optimization of the substrate for the radical cyclization, we have turned our attention to the synthesis of the difluoromethylene analogue **4** of oxetanocin T **2**. For the synthesis of the target molecule, we needed to prepare the radical precursor **13** and the synthetic route is illustrated in Scheme 1. Initially, Weinreb amide **9**[12] was treated with Li-HMDS and subsequently BOMCl in one pot to give the α-benzyloxymethylated product, which was then reduced by DIBAL-H to furnish the aldehyde **10** in 47% yield. Compound **10** was subjected to Reformatsky reaction utilizing BrF_2CCO_2Et and activated zinc[13] followed by silylation of the resulting secondary alcohol to give **11** in 77% yield.



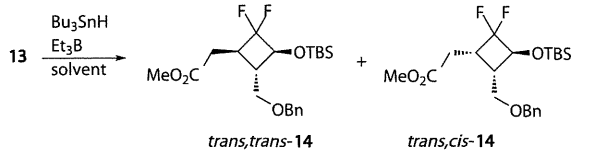
Scheme 1 Preparation of radical precursor **13**.

The ester **11** was then converted into the Weinreb amide **12** in excellent yield (90% yield). DIBAL-H reduction of **12** and subsequent Wittig reaction gave the desired substrate **13** [a mixture of three stereoisomers; *ca.* major-(*E*)-**13** : minor-(*E*)-**13** : (*Z*)-**13** = 1 : 0.22 : 0.14].

With the substrate **13** in hand, we next carried out the radical cyclization and the results are summarized in Table 2. As shown in entry 1, when **13** was reacted with Bu_3SnH under the conditions described in Table 1, the yield of the desired difluorocyclobutane derivative **14** was unexpectedly decreased to 38% yield (diastereomeric mixture: *ca.* 2 : 1). On the basis of NOE experiments, the major isomer was found to be *trans*,-*trans*-**14** with the minor isomer being *trans*,*cis*-**14**, respectively (see ESI†). To improve the yield of **14**, the reaction was carried out at ambient temperature using Et_3B as an initiator under an O_2 atmosphere. As can be seen in entries 2 to 5, the longer the duration time of the dropwise addition of Bu_3SnH, the better the isolated yield of **14** although the ratio of *trans*,*trans*-**14**/*trans*,*cis*-**14** was unchanged.[14] The best result was obtained when Bu_3SnH was added dropwise over 24 h to give **14** in 78% yield (entry 5). No improvement was observed at −20 °C due to the recovery of **13** (47%) (entry 6).

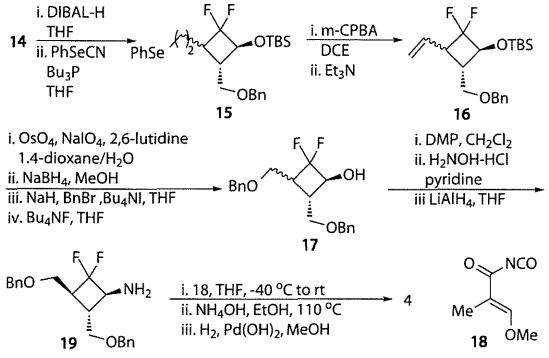
Finally, conversion of **14** to the target **4** was performed (Scheme 2). DIBAL-H reduction of an epimeric mixture of **14** and subsequent phenylselenylation of the resulting hydroxyethyl derivative by using PhSeCN and Bu_3P[15] gave **15** in 97% yield in two steps. The selenide **15** was converted to terminal olefin **16**

Table 2 Radical cyclization of **13**



Entry	Solvent	Temp (°C)	Time[a] (h)	Yield [%] of **14**	Ratio[b] (*trans*,*trans*/*trans*,*cis*)
1	Toluene	110[c]	4	38	2.0/1
2	Benzene	rt	2	72	2.7/1
3	Benzene	rt	4	70	2.7/1
4	Benzene	rt	8	77	2.6/1
5	Benzene	rt	24	78	2.6/1
6	Toluene	−20	48	48[d]	3.0/1

[a] Bu_3SnH was dropwise added at an indicated time. [b] The ratio (*trans*,*trans*/*trans*,*cis*) was calculated by integration of ^{1}HNMR. [c] AIBN was used as an initiator. [d] Compound **13** was recovered in 47%.



Scheme 2 Synthesis of **4**.

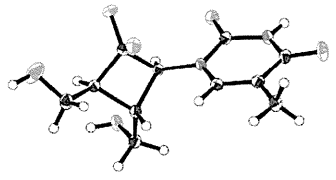


Fig. 3 ORTEP drawing of compound **4**.

(86% yield) by oxidation with *m*-CPBA and subsequent *syn*-elimination of the respective selenoxide. Next, **16** was transformed into **17** (diastereomeric mixture; *ca.* 3 : 1) in 65% yield through the following four steps: (1) Lemieux–Johnson oxidation in the presence of 2,6-lutidine,[16] (2) $NaBH_4$ reduction of the resulting aldehyde, (3) benzylation of the primary alcohol, (4) removal of the TBS group by using Bu_4NF. Next, the cyclobutylamine **19** was synthesized through (1) Dess–Martin periodinane (DMP) oxidation of **17**, (2) oximation of the ketone, (3) $LiAlH_4$ reduction of the oxime.[17] Although, the yield was not satisfied (32%), amine **19** was obtained as a single stereoisomer. Finally, **19** was transformed into the corresponding thymine nucleoside by a reported procedure[18] using isocyanate **18**. Debenzylation with $Pd(OH)_2/C$ gave the title compound **4** in 56% yield. The relative stereochemistry of **4** was assigned by NOE experiments (see ESI†) and confirmed by X-ray crystallographic analysis (Fig. 3).

It was found that the 4-*exo*-*trig* cyclization reaction of the 3,3-difluoro-4-pentenyl carbon radical efficiently proceeded to furnish the novel *gem*-difluorocyclobutane. The electron withdrawing effect of the two fluorine atoms adjacent to the radical accepting double bond accelerated the cyclization reaction. As a synthetic application of this radical ring closure, the synthesis of difluoromethylene oxetanocin T **4**, a potential anti-viral agent, was achieved. Anti-viral assay revealed that compound **4** did not show any activity against HIV-1, VZV and HCMV.

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From the Chemistry of Epoxy-Sugar Nucleosides to the Discovery of Anti-HIV Agent 4′-ethynylstavudine-Festinavir

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Abstract: Branched sugar nucleosides have attracted much attention due to their biological activities. We have demonstrated that epoxy-sugar nucleosides serve as versatile precursor for the stereo-defined synthesis of these nucleoside derivatives on the basis of its ring opening with organoaluminum or organosilicon reagents. In this review article, novel methods for the synthesis of nucleoside analogues branched at the 1′ and 4′-position will be described. During this study, we could discover an anti-HIV agent, 4′-ethynylstavudine (Festinavir).

Festinavir showed more potent anti-HIV activity than the parent compound stavudine (d4T). Other significant properties of Festinavir are as follows: 1) much less toxic to various cells and also to mitochondorial DNA synthesis than d4T, 2) better substrate for human thymidine kinase than d4T, 3) resistant not only to chemical glycosidic bond cleavage but also to catabolism by thymidine phosphorylase, 4) the activity improves in the presence of a major mutation, K103N, associated with resistance to non-nucleoside reverse transcriptase inhibitors. Detailed profile of the antiviral activities, biology and pharmacology of Festinavir are also described.

Keywords: Epoxide, sugar, nucleoside, organoaluminum reagent, NRTIs, stavudine, anti-HIV-1 agent.

(I) CHEMISTRY OF EPOXYSUGAR NUCLEOSIDES AND SYNTHESIS OF 2′,3′-DIDEHYDRO-3′-DEOXY-4′-ETHYNYLTHYMIDINE (4′-ETHYNYLSTAVUDINE)

(1) Background

Human Immunodeficiency virus (HIV) is causative agent of acquired immunodificiency syndrome (AIDS) and it is estimated that more than 33.3 million individuals worldwide are infected with this pathogen [1]. HIV is RNA virus and belongs to retrovirus family. Retro-virus has characteristic key enzyme, reverse transcriptase (RT), which catalyzes reverse transcription of viral RNA into provirus DNA. The enzyme is essential for lifecycle of HIV. Because the activity of RT in host-cell is quite low, it is rationale that RT is a target enzyme for developing anti-HIV agent.

In 1987, AZT was approved as the first anti-HIV drug, which is nucleoside reverse transcriptase inhibitor (NRTI) (Fig. (**1**)). The discovery of AZT as antiretroviral agent stimulated the synthesis of sugar-modified nucleosides and the evaluation of their anti-HIV activity. These studies provided other clinically used anti-HIV derivatives such as zalcitabine (ddC), didanosine (ddI) and stavudine (d4T). However, the long-term usage of these anti-viral drugs lead to delayed toxicity in patients and/or emergence of drug resistant HIV variants. These circumstances necessitate novel anti-HIV agent with lower toxicity and wider anti-viral spectrum [2], and extensive synthetic research for developing novel anti-viral sugar-modified nucleosides has continued [3-6].

Among sugar-modified nucleosides, compounds substituted with carbon-substituent at the 1′-, 2′-, 3′-, 4′- or 5′-position of the pentose moiety are called branched-sugar nucleosides. Biologically active branched-sugar analogues are shown in Fig. (**2**). Anti-tumor nucleoside antibiotic angustmycin C, which is structurally-unique in being branched at the anomeric position of adenosine [6]. 2′-Methyladenosine has been reported to possess anti-HCV activity [5]. On the other hand, 3′-ethynylcytidine exhibits anti-tumor activity [8]. Furthermore, 4′-cyanothymidine shows significant inhibitory activity to HIV [9].

2′-Branched ribonucleosides such as 2′-methyladenosine have been synthesized by means of addition of carbon nucleophile to 2′-keto derivative available from oxidation of 2′-hydroxyl group of naturally occurring ribonucleosides (Fig. (**3**)). Likewise, 3′-keto-nucleosides have been utilized for the synthesis of 3′-branched nucleosides exemplified by 3′-ethynylcytidine.

On the contrary, the 1′ or 4′-position of nucleosides are inert for introducing the carbon-substituent because no functional groups are present at the position. Therefore, synthesis of 1′- or 4′-branched nucleosides such as angustmycin C and 4′-cyanothymidine has been difficult. Therefore, development of efficient method leading to the synthesis of these nucleosides has been challenging and arduous task in the nucleoside chemistry.

The first synthesis of 1′-branched derivatives starting from naturally occurring nucleoside has been carried out on the basis of nucleophilic substitution of **1**, which was prepared from bromopivaloyloxylation of 1′,2′-unsaturated nucleosides, with organosilicon or organoaluminum reagent (Scheme **1**) [10]. In this transformation, the desired 1′-α-carbon-substituted nucleoside **2** was obtained *via* neighboring group participation exerted by 2′-β-bromine atom of nucleoside anomeric carbenium ion **I**. Although **2** was converted into 2′-deoxy- and arabinofuranosyl nucleosides **3**, the corresponding ribofuranosyl counterpart could not be synthesized.

To overcome this problem, reaction of samarium enolate **II**, generated by SmI_2-mediated reduction of α-ketophenylselenide **4**, with carbon electrophile has been reported (Scheme **2**) [11]. Thus, reaction of **4** with aldehyde gave aldol **5**, which was subjected to β-face-selective hydride reduction to provide 1′-carbon-subjected ribonucleoside **6**.

In the meantime, nucleoside anomeric radical has also been utilized as intermediate for the synthesis of 1′-branched nucleoside. The first report on the basis of this strategy is shown in Scheme **3**

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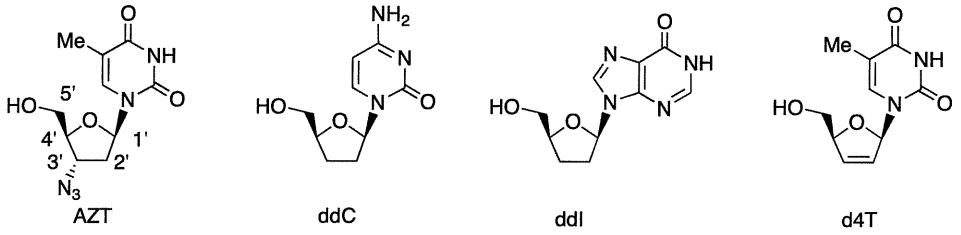


Fig. (1). Anti- HIV Drugs: Nucleoside Reverse Transcriptase Inhibitors (NRTIs).

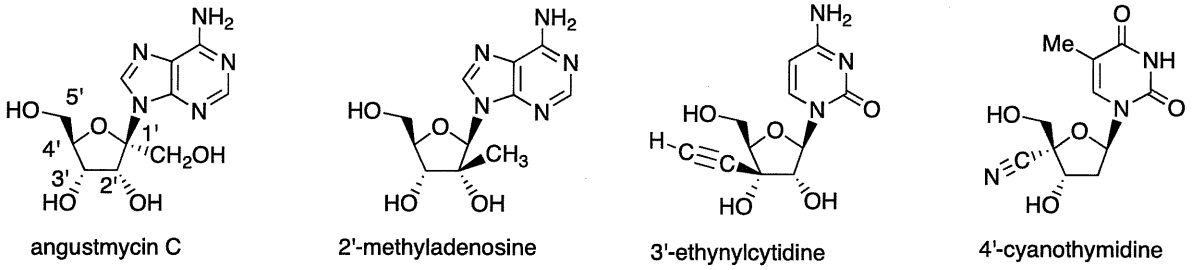


Fig. (2). Biologically-active Branched-Sugar Nucleosides.

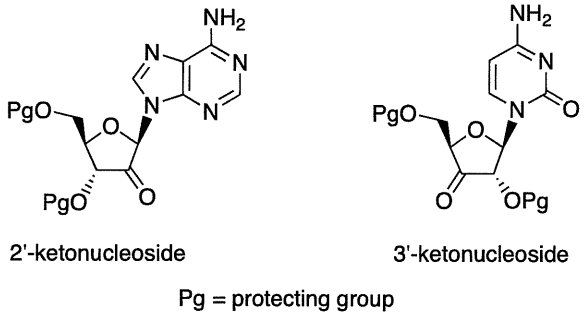
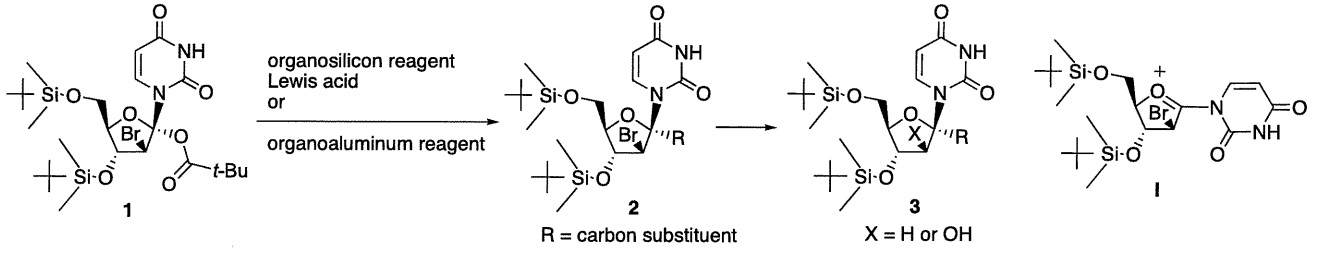
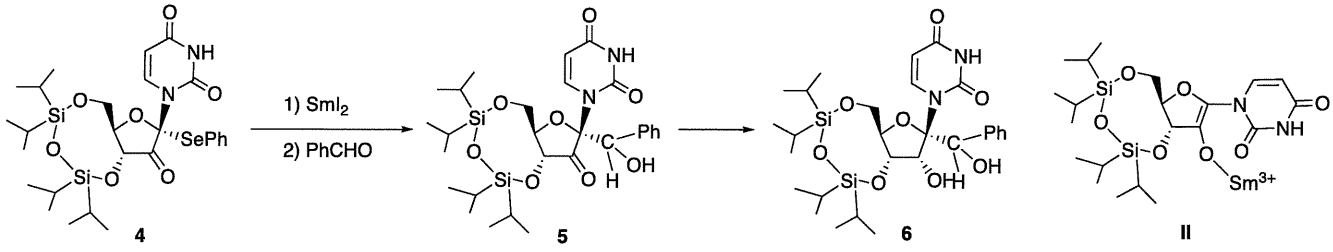


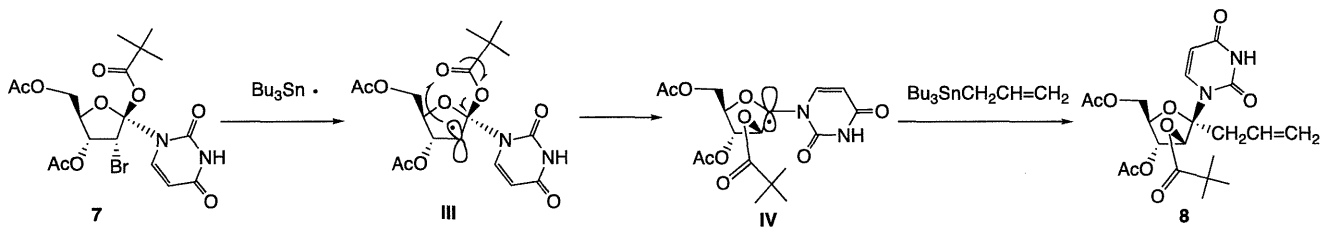
Fig. (3). 2'-Keto and 3'-Ketonucleosides Utilizing for the Synthesis of 2'-and 3'-Branched Derivatives.



Scheme 1. Synthesis of 1'-Branched Nucleosides **3** *via* **2** obtained on thde basis of Nucleophilc Substitution of **1**.



Scheme 2. Synthesis of 1'-Branched Nucleosides on the basis of Electrophilic Substitution.



Scheme 3. Nucleoside Anomeric Radical based Synthesis of 1'-Branched Nucleosides.

[12]. Thus, when bromo-pivaloyloxy derivative **7** was treated with tributyltin radical, the incipient 2'-carbon radical **III** was rearranged to anomeric radical **IV** through 1,2-acyloxy migration and subsequent reaction of **IV** with allyltributyltin gave 1'-allyl-arabinofuranosyluracil **8**. In this case, sp^2 hybridized anomeric radical reacted at α-face to give β-anomer as a sole product.

The other anomeric radical-based methods are shown in Schemes **4** and **5**. The method shown in Scheme **4** is intermolecular radical reaction of 1'-phenylsulfanyldeoxyuridine **9** with allyltributyltin to give 1'-allyl-2'-deoxynucleoside (**10**) [13]. The other method depicted in Scheme 5 is intramolecular radical cyclization of **11** (Scheme **5**) [14]. The cyclized product **12** could be transformed into 1'-vinyluridine (**13**) by means of fluoride ion-mediated E2-elimination. These above-mentioned novel methods for the synthesis of 1'-branched nucleosides have some drawbacks such as limited substituent to be introduced and difficulty of selective synthesis of the β-anomers.

On the other hand, 4'-branched nucleosides such as 4'-cyanothymidine have been synthesized from common key intermediate 4'-hydroxymethylnucleoside **16** (Scheme **6**) [15]. Thus, aldol-Cannizzaro reaction of thymidine 5'-aldehyde **14** gave **15**. Selective tritylation of α-hydroxyl group, silylation of β-hydroxyl group of **15** and subsequent removal of the trityl group provide 4'-α-hydroxymethyl nucleoside **16**. Oxidation of the primary hydroxyl group of **16** gave the corresponding aldehyde. Finally, reaction of the aldehyde with hydroxylamine and E2 elimination of the mesylate furnished **17** after deprotection. Although the method has been utilized most frequently, it required tedious steps for the synthesis of hydroxymethyl derivative **16** and the introduction of 4'-substituents has been limited.

(2) Synthetic use of epoxy-sugar nucleoside

Ring opening of epoxides with carbon nucleophile have been recognized as one of the important synthetic operation for construction of carbon-carbon bond [16]. Epoxy-sugar nucleosides consist of four possible derivatives; 1',2'-, 2',3'-, 3',4'-, and 4',5'-epoxides (Fig. (**4**)). Among these epoxy-sugar nucleosides, ring opening of 2',3'-epoxy derivative with carbon nucleophile has been only precedent. Thus, Walker *et al.* has reported that 2',3'-lyxo-epoxyuracil nucleoside **18** underwent regioselective ring opening by the reaction with LiC≡CH to give 3'-ethynylnucleoside **19** (Scheme **7**) [17].

The other three epoxy-sugar nucleosides are structurally unique in that the carbon bonded to the furanose ring oxygen is directly attached to the epoxide ring. These epoxides, therefore, are expected to readily undergo regioselective nucleophilic ring opening because of concomitant formation of oxonium ion. By the same reason, the preparation of these epoxides from the corresponding unsaturated-sugar nucleosides has to be carried out under non-nucleophilic conditions. In fact, 1',2'-epoxynucleoside **20** exists as an equilibrium mixture with oxonium ion **21**. Under aqueous conditions, **20** is readily converted into uracil and ribonolactone via hemiacetal **22** (Scheme **8**).

We have envisioned that dimethyldioxirane (DMDO) would be suitable oxidizing reagent for the preparation of the above unstable 1',2'-, 3',4'-, and 4',5'-epoxides because DMDO is able to transform an alkene into the respective epoxide in aprotic solvent under neutral conditions. DMDO is prepared by oxidation of acetone by potassium peroxomonosulfate (Oxone) (Scheme **9**) [18-20].

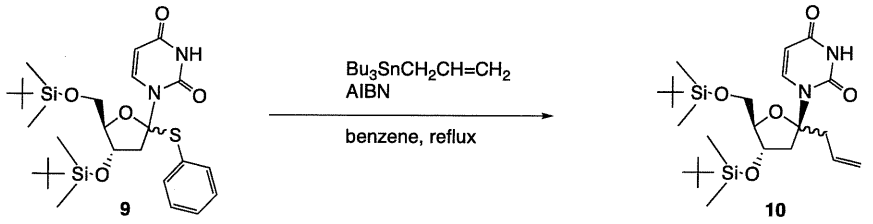
As expected, 1',2'-, 3',4'- and 4',5'-epoxynucleosides could be prepared by DMDO-meditade oxidation of the corresponding unsaturated nucleosides (Scheme **10**). Reaction of 1',2'-epoxynucleoside with organoaluminum reagent gave "*syn*-opened" product stereoselectively. On the contrary, "*anti*-opened" product was obtained as a major product from the reaction of 3',4'-epoxynucleoside with trimethylaluminum reagent. Interestingly, stereoselective 4'-α-carbon-carbon bond formation occurred in the $SnCl_4$-initiated ring opening of 4',5'-epoxynucleoside with organosilicon reagent.

In this review article, we describe stereo-defined synthesis of 1'-α- and 4'-α-branched nucleosides on the basis of ring opening of epoxy-sugar nucleosides. During this study, we have discovered an anti-HIV agent, Festinavir (4'-ethynylstavudine or 4'-Ed4T). Structure–activity relationships, detailed profile anti-HIV activity, biology and pharmacology of Festinavir are dicussed.

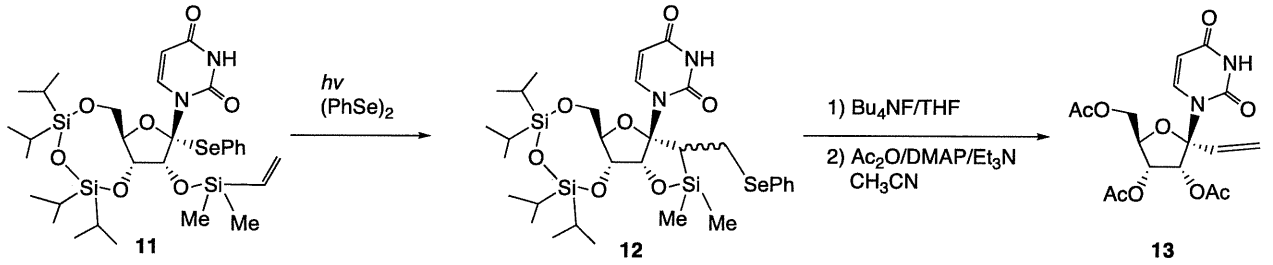
(3) Ring opening of nucleoside 1',2'-epoxides with organoaluminum reagents: stereoselective entry to ribonucleosides branched at the anomeric position [21]

When TBDMS-protected 1',2'-unsaturated uridine (**23**) was epoxidized with DMDO in CH_2Cl_2 at −30 °C, the reaction was completed for 30 min (Scheme **11**). Because of its instability, the resulting epoxide could not be characterized by ^1H NMR. Therefore, the reaction mixture was subsequently treated with Me_3Al to give 1'-methyluracil nucleoside (**25α**) as a sole product. The depicted structure of **25α** was determined on the basis of NOE correlation between H-6/H-4', CH_3-1'/H-3 and H-2/H-4'. The configuration of 2'-OH of **25α** revealed that the epoxidation of **23** gave 1',2'-"up"-epoxide **24** selectively. Similarly, Me_3Al-mediated ring opening of the epoxide formed from the epoxidation of 1,1,3,3-(tetraisopropyldisiloxane-1,3-diyl) (TIPDS)-protected **26** provided **27α** as a major isomer. These stereochemical outcome indicated that the epoxidation of TBDMS- and TIPDS-protected 1',2'-unsaturated uracil nucleosides proceeded at the β-face predominantly.

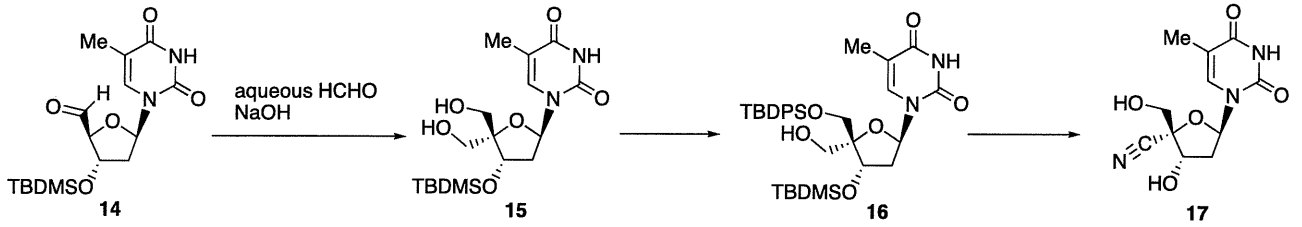
On the other hand, di-*tert*-butylsilylene (DTBS)-protected epoxide **29** derived from **28** was found to be stable for ^1H NMR assignment of its structure (Scheme **12**). The NOE experiment suggested that the epoxide **29** has 1',2'-"down"-configuration. When the epoxide **29** was reacted with Me_3Al, the desired *syn*-adduct **30β** and its epimer *anti*-adduct **30α** was obtained in a ratio of 5 : 1 in 86% isolated yield. On the basis of the molecular modeling study and the value of $J_{3',4'}$ (11.0 Hz) of **29**, its Newman projection formulae of sugar portion was proposed (Fig. (**5**)) [22]. As can be seen in the Figure, the hydrogen atom at the 3'-position occupies pseudo-axial position. When DMDO is approaching from the β-face of the enol ether, steric repulsion between the 3'-hydrogen and



Scheme 4. Radical Reaction of 1'-Phenylsulfanyl Nucleoside **9** under Radical Reaction leading to1'-Allyldeoxyuridine **10**.



Scheme 5. Intramolecular Radical Cyclization of **11** and Subsequent Transformation of **12** to 1'-Vinyl Uridine **13**.



Scheme 6. Synthetic Sequence for 4'-Branched Nucleosides by means of Aldol-Cannizzaro Reaction.

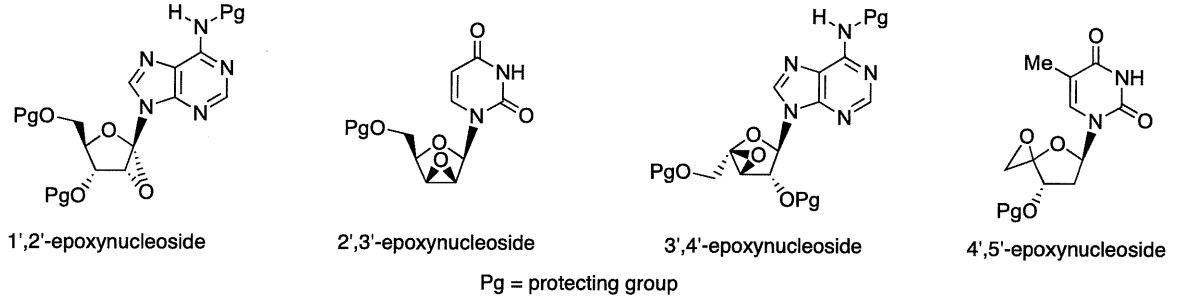
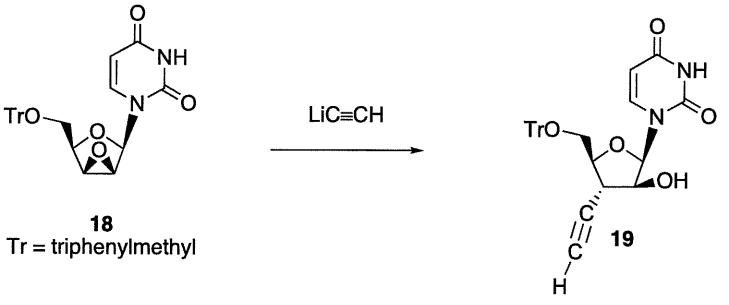
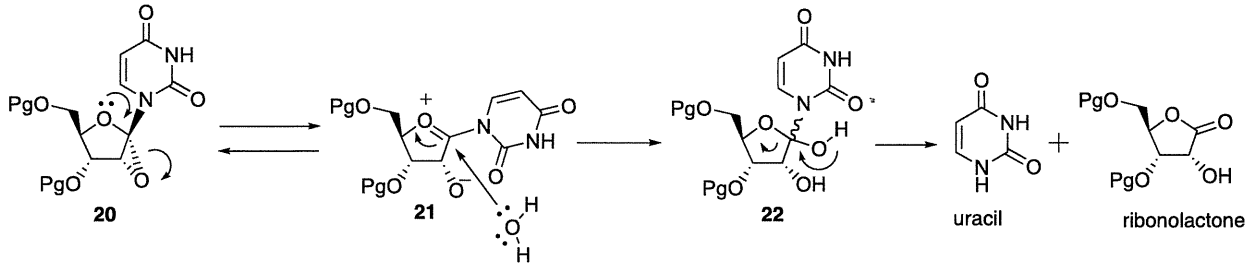


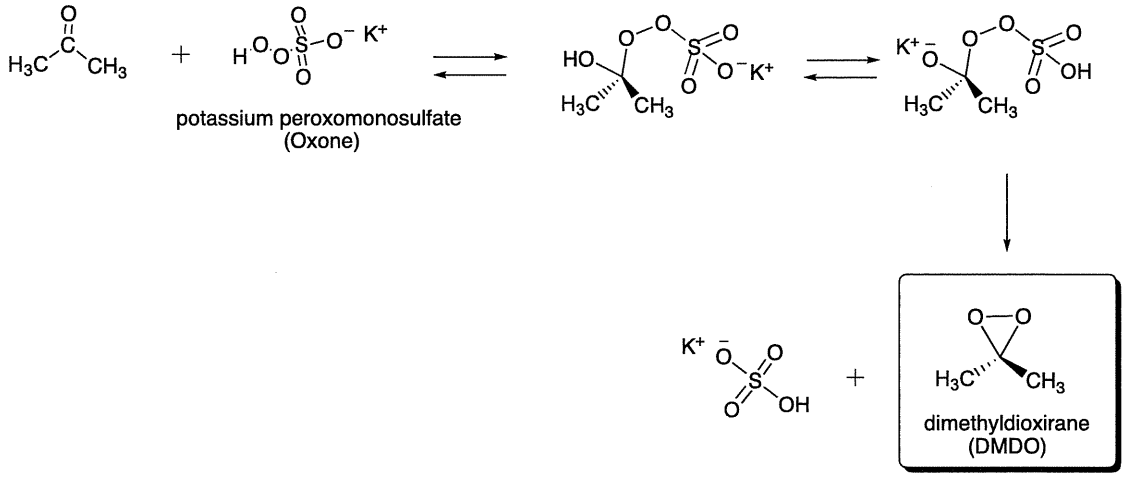
Fig. (4). Structures of Epoxy-Sugar Nucleosides.



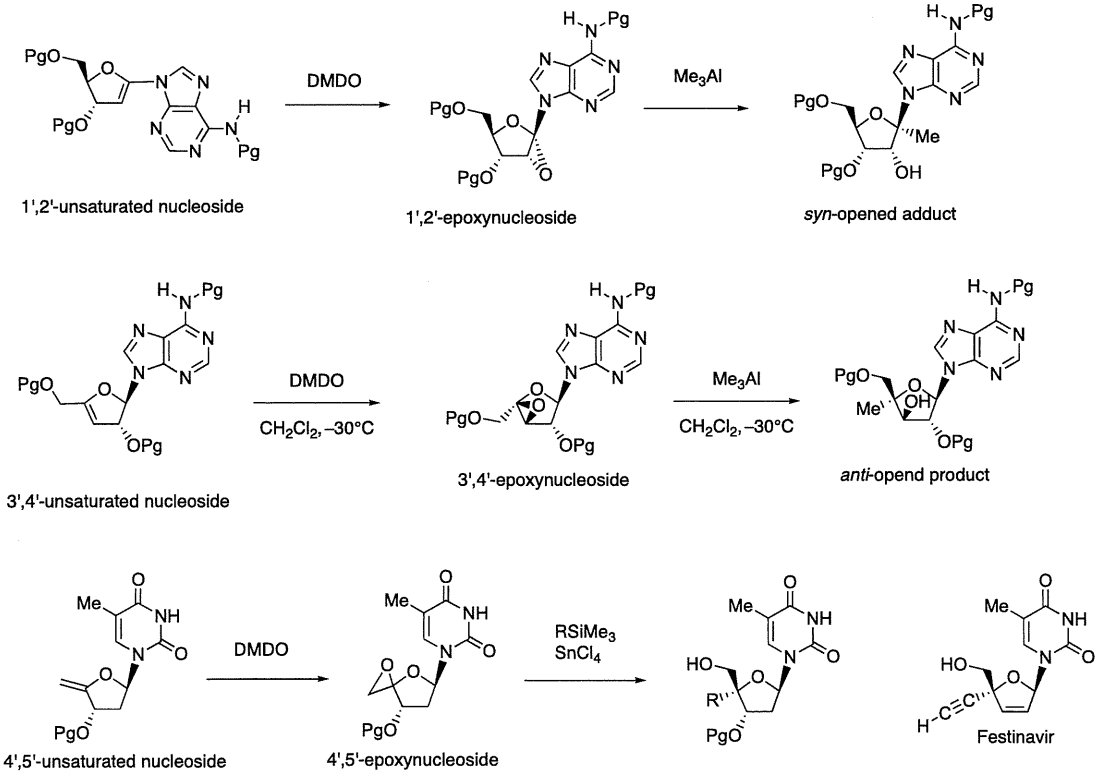
Scheme 7. Synthesis of 3'-Branched Uracil Nucleoside **19** by Means of Ring Opening of 2',3'-Lyxo-epoxide **18** with LiC≡CH.



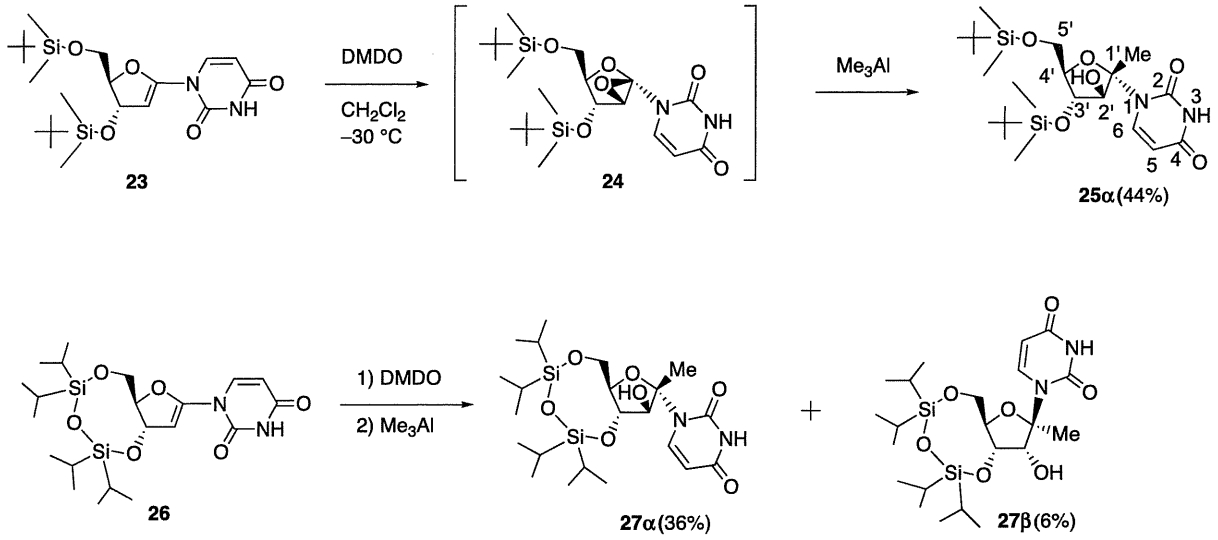
Scheme 8. Characteristic Behaviour of 1',2'-Epoxynucleoside **20**.



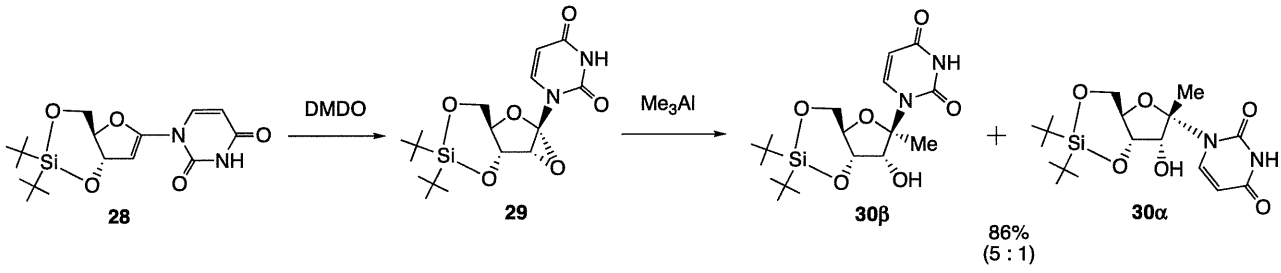
Scheme 9. Preparation of Dimethyldioxirane (DMDO).



Scheme 10. Preparation and Ring-Openig of Epoxy-Sugar Nucleosides (Pg = protecting group).



Scheme 11. Epoxidation of 1',2'-Unsaturated Uracil Nucleosides **23** and **26**: Ring Opening of 1',2'-Epoxynucleosides with Me_3Al.



Scheme 12. DMDO-mediated Epoxidation of DTBS-protected 1',2'-Unsaturated Uracil Nucleoside **28** and Ring Opening of 1',2'-"Down"-Epoxynucleoside **29** leading to **30**.

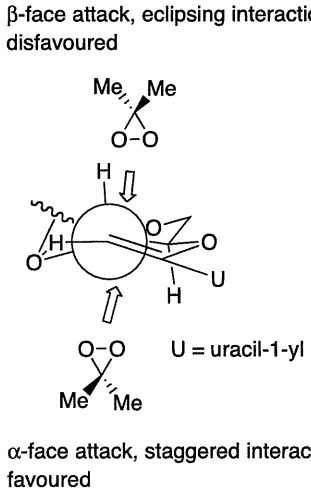
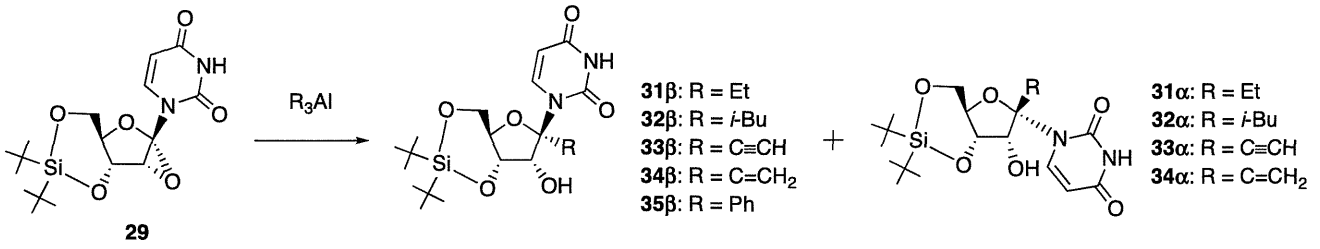


Fig. (5). Plausible Elucidation for α-Face-Selectivity of DMDO-Epoxidation of DTBS-protected **28**.

DMDO is seen. Therefore, the epoxidation of the double bond proceeded at the α-face to furnish **29** as a sole product. In the case of TBDMS-protected **23** and TIPDS-protected **26**, these $J_{3',4'}$ values are 2.6 and 4.4 Hz, which suggest these 3'-hydrogen occupy the pseudo-equatorial position. In these cases, DMDO approaches from the α-face due to predominant steric hindrance of 3'-silyloxy-substituent leading to the formation of 1',2'-"up"-epoxide **24** as a major product.

To examine the scope and limitations, the ring-opening of **29** with other organoaluminum reagents was examined (Scheme **13**) and these results are summarized in Table **1**, which includes the results of the reaction with Me_3Al in entry 1. Except for the result with triisobutylaluminum shown in entry 3, in which the isolated yield of 1'-isobutyl derivative (**32**) decreased to 32% due to concomitant hydride reduction, 1'-ethyl- (**31**), 1'-ethynyl- (**33**), 1'-vinyl- (**34**) and 1'-phenyluridine derivative (**35**) could be obtained in moderate to good yields (entries 2 and 4-6). As can be seen in the ratio of β- and α-anomers, the expected *syn*-ring-opened β-anomer was always accompanied with the *anti*-ring-opened α-uridine derivatives, except for the reaction of Ph_3Al.

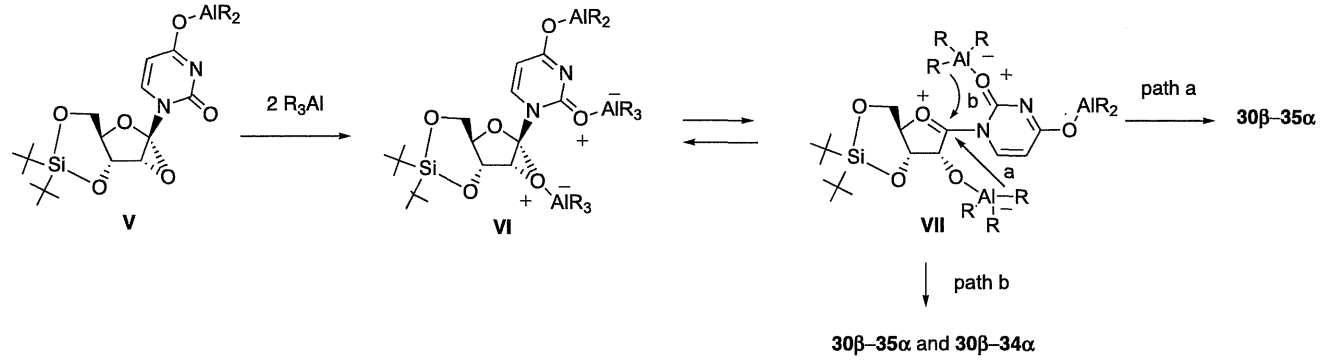
To explain these results, we have proposed a plausible mechanism for the reaction of **29** with R_3Al (Scheme **14**). Dissociation of an acidic N^3-H of **29** with R_3Al give **V** and subsequent coordination to the oxygen atom of the epoxide ring as well as to that of the C2-carbonyl of **V** would furnish **VI**, which in turn forms the oxonium intermediate **VII**. At this stage, if nucleophilic transfer of the aluminum ligand R takes place from the 2'-*O*-aluminate (path a), β-uridine derivatives **30β-35β** should be formed, whereas such attack from the base moiety (path b) results in the formation of α-uridine derivatives **30α-34α** or β-uridine derivatives depending upon the comformation about the *N'-C'* pivot bond. The observed sole formation of **35β** in the reaction of Ph_3Al could be explicable in terms of inability of this bulky reagent to coordinate to the C^2-carbonyl oxygen.



Scheme 13. Reaction of 1',2'-Epoxynucleoside **29** with Organoaluminum Reagents.

Table 1. Reaction of 1',2'-epoxynucleoside 28 with Organoaluminum Reagents

entry	R_3Al (equiv)	products	isolated yield (%)	ratio of β/α
1	Me_3Al (3)	**30β/30α**	86	5/1
2	Et_3Al (3)	**31β/31α**	90	4/1
3	*i*-Bu_3Al (6)	**32β/32α**	35	9/1
4	$(HC{\equiv}C)_3Al$ (6)	**33β/33α**	64	4/1
5	$(H_2C{=}CH)_3Al$ (6)	**34β/34α**	90	32/1
6	Ph_3Al (6)	**35β**	55	–



Scheme 14. Plausible Mechanism for the Reaction of 1',2'-Epoxynucleoside **29** with Organoaluminum Reagents.

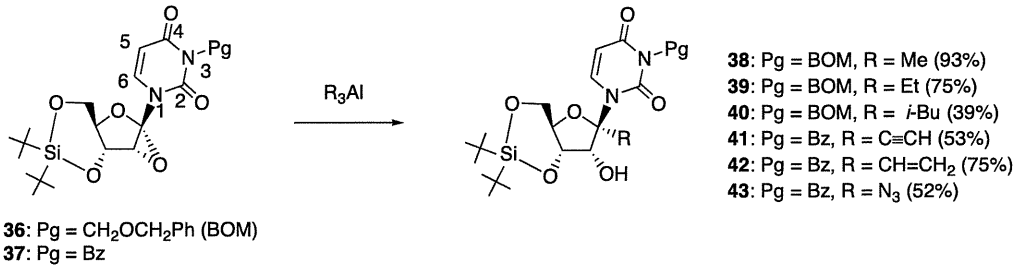
Based on this mechanism, it would be reasonable to expect that the presence of a bulky protecting group at the N^3-position will prevent coordination of R_3Al to the C^2-carbonyl oxygen. Reactions carried out along this line by employing the N^3-protected substrates **36** and **37** uniformly gave the *syn*-ring-opened β-uridine derivatives **38-43** as a sole product (Scheme **15**). Removal of N^3-BOM group of **38-40** and N^3-benzoyl group of **41-43** could be carried out by catalytic hydrogenolysis or treatment by methanolic ammonia, respectively.

With the above successful results in hand, next, we have examined ring-opening of 1',2'-epoxyadenosine derivative with organoaluminun reagents (Scheme **16**). When 1',2'-"down"-epoxide **44** was reacted with Me_3Al, 1'-methyladenosine derivative **45** was obtained in 80% isolated yield. Likewise, 1'-ethyl- (**46**), 1'-isobutyl- (**47**), 1'-ethynyl- (**48**) and 1'-vinyl- (**49**) adenine nucleosides could be synthesized as a sole stereoisomer. 1'-Vinyl-adenosine **49** was transformed into protected angustmycin C **50** through OsO_4-mediatde oxidative cleavage of vinyl group and subsequent hydride reduction of the resulting aldehyde. This is the first example that the nucleoside antibiotic was synthesized from adenosine.

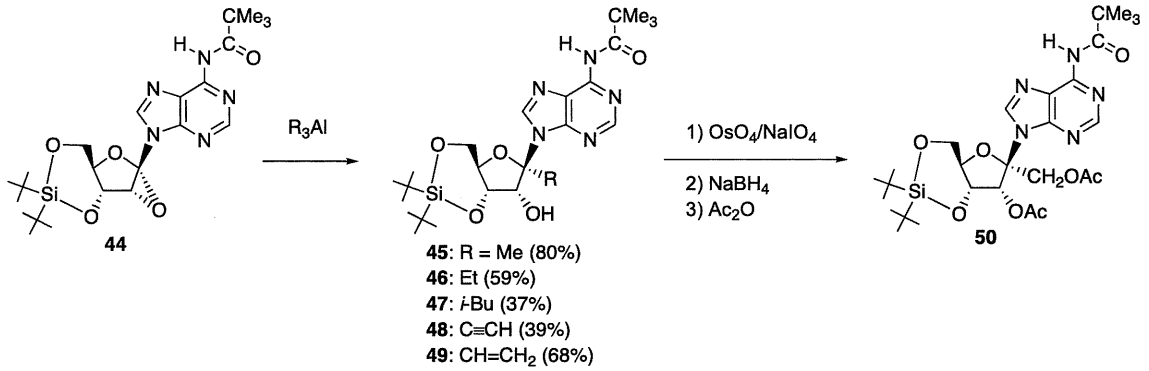
As mentioned above, we have developed a novel method for the synthesis of 1'-branched uridine and adenosine derivatives by α-face-selective-epoxidation of 1',2'-unsaturated nucleosides with DMDO and subsequent *syn*-ring-opening of the resulting 1',2'-α-epoxides with organoaluminum reagents. These novel 1'-branched nucleosides did not show any anti-viral activities.

(4) *Anti* versus *syn* opening of epoxides derived from 9-(3-deoxy-β-D-*glycero*-pento-3-enofuranosyl)adenine with Me_3Al: factors controlling the stereoselectivity [23]

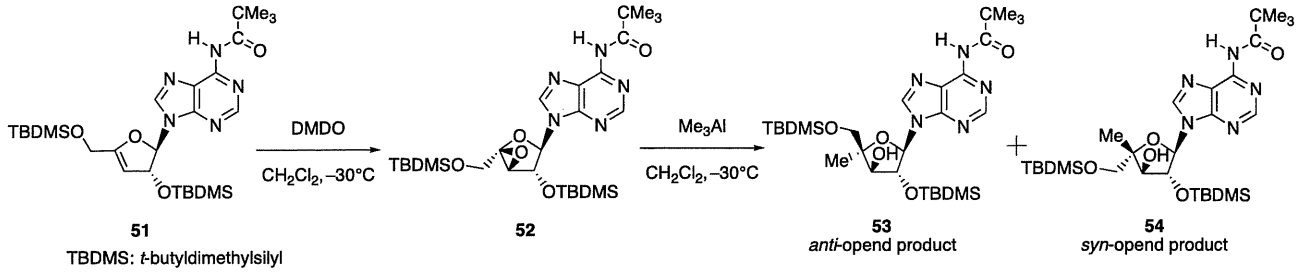
Simple epoxides are known to react with Me_3Al in a manner of *anti*-ring-opening, but no clear explanation is available for this stereochemical outcome. On the other hand, the epoxides derived from glycal, cyclic enol ethers, and 3,4-dihydro-2*H*-pyran give *syn*-ring-opened products. As shown in Scheme **17**, by employing 3',4'-β-epoxy nucleoside **52** derived from the DMDO-mediated epoxidation of 3',4'-unsaturated adenine nucleoside **51**, we investigated factors governing the stereoselectivity of its epoxy- ring-opening (*anti*- vs. *syn*-opening) with Me_3Al.



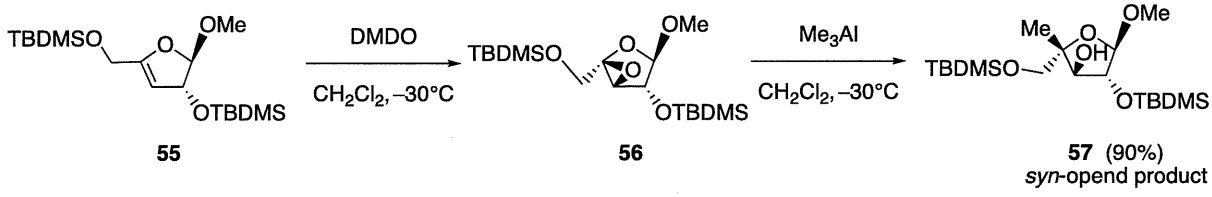
Scheme 15. Reaction of N^3-Protected 1',2'-Epoxyuridine Derivatives **36** and **37** with Organoaluminum Reagents.



Scheme 16. Reaction of 1',2'-α-Epoxyadenosine Derivative **44** with Organoaluminum Reagents and Synthesis of Protected Angustmycin C.



Scheme 17. Epoxidation of 3',4'-Unsaturated Adenine Nucleoside **51** and Ring-Opening of the Epoxy Nucleoside **52** with Me_3Al.



Scheme 18. Epoxidation of **55** and Ring-Opening of the Sugar Epoxide **56** with Me_3Al leading to **57**.

Although **52** is a kind of glycal-derived epoxide, preferential formation of the *anti*-opened **53** was observed when the reaction was carried out in CH_2Cl_2. Also, it was found that the ratio of **53** (*anti*-opened)/**54** (*syn*-opened) varied significantly (from 2/1 to 6/1) by increasing the amount of Me_3Al (from 1.0 equiv. to 10 equiv.). In contrast to this, the same reaction carried out in THF, Et_2O, or 1,4-dioxane by using 6.0 equiv. of Me_3Al uniformly led to the exclusive formation of the *syn*-opened product **54**. To see if the presence of the N^6-pivaloyladenine base has any influence on the stereochemistry, the corresponding sugar epoxide **56** was prepared from **55** and reacted with Me_3Al (6.0 equiv.). As shown in Scheme **18**, although this reaction was carried out in CH_2Cl_2, the sole formation of the *syn*-opened product **57** was observed.

These experimental results enable us to propose a possible reaction mechanism between **52** and Me_3Al depicted in Scheme **19** (N^6-pivaloyladenine moiety is omitted for simplicity). Highly oxygenophilic Me_3Al would prefer coordination to the 3',4'-epoxy structure of **52** to give **VIII**, which subsequently undergoes epoxide ring opening to form an oxonium ion that carries an alkoxyaluminate at the 3'-position. Two extreme conformers can be depicted for the oxonium ions as a result of rotation of the 3'-*O*-Al bond. In one conformer **IX**, Al is located above the furanose ring, and in the