Synthesis and anti-dengue virus activity of 5-ethynylimidazole-4-carboxamide (EICA) nucleotide prodrugs

Motoki Nakamura\textsuperscript{a}, Kentaro Uemura\textsuperscript{b,c,d}, Noriko Saito-Tarashima\textsuperscript{a}, Akihiko Sato\textsuperscript{b,c}, Yasuko Orba\textsuperscript{c,e}, Hirofumi Sawa\textsuperscript{c,e,f}, Akira Matsuda\textsuperscript{g}, Katsumi Maenaka\textsuperscript{d,g,h} and Noriaki Minakawa\textsuperscript{a,*}

\textsuperscript{a}Graduate School of Pharmaceutical Science, Tokushima University, Tokushima, Japan
\textsuperscript{b}Drug Discovery and Disease Research Laboratory, Shionogi & Co., Ltd., Osaka, Japan
\textsuperscript{c}Division of Molecular Pathobiology, International Institute for Zoonosis Control, Hokkaido University, Sapporo, Japan
\textsuperscript{d}Laboratory of Biomolecular Science, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan
\textsuperscript{e}International Collaboration Unit, International Institute for Zoonosis Control, Hokkaido University, Sapporo, Japan
\textsuperscript{f}One Health Research Center, Hokkaido University, Sapporo, Japan
\textsuperscript{g}Center for Research and Education on Drug Discovery, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan
\textsuperscript{h}Global Station for Biosurfaces and Drug Discovery, Hokkaido University, Sapporo, Japan

*Correspondence e-mail: minakawa@tokushima-u.ac.jp
Summary

We previously showed that 5-ethynyl-(1-β-D-ribofuranosyl)imidazole-4-carboxamide (1; EICAR) is a potent anti-dengue virus (DENV) compound but is cytotoxic to some cell lines, while its 4-thio derivative, 5-ethynyl-(4-thio-1-β-D-ribofuranosyl)imidazole-4-carboxamide (2; 4′-thioEICAR), has less cytotoxicity but also less anti-DENV activity. Based on the hypothesis that the lower anti-DENV activity of 2 is due to reduced susceptibility to phosphorylation by cellular kinase(s), we investigated whether a monophosphate prodrug of 2 can improve its activity. Here, we first prepared two types of prodrug of 1, which revealed that the S-acyl-2-thioethyl (SATE) prodrug had stronger anti-DENV activity than the aryloxyphosphoramidate (so-called ProTide) prodrug. Based on these findings, we next prepared the SATE prodrug of 4′-thioEICAR 18. As expected, the resulting 18 showed potent anti-DENV activity, which was comparable to that of 1; however, its cytotoxicity was also increased relative to 2. Our findings suggest that prodrugs of 4′-thioribonucleoside derivatives such as EICAR (1) represent an effective approach to developing potent biologically active compounds; however, the balance between antiviral activity and cytotoxicity remains to be addressed.

Keywords

imidazole nucleoside, 4′-thio-modification, prodrug, dengue virus, antiviral activity
Introduction

Dengue virus (DENV) is an enveloped positive-sense single-stranded RNA virus belonging to the family Flaviviridae, which includes members such as Yellow fever virus, West Nile virus, and Zika virus. DENV is transmitted to humans by Aedes mosquitoes and is the etiologic agent of both dengue fever and dengue hemorrhagic fever. Each year, 100 million people are infected with DENV and 10,000 die from dengue hemorrhagic fever. Due to global warming and increased global migration of humans, infection with DENV is no longer a threat only in the tropics and subtropical regions: at present, 40% of the world’s population is faced with the risk of viral infection. Owing to these reasons and the fact that no drug or broadly applicable vaccine has yet been approved, development of an anti-DENV agent is a key priority for human health.

A common strategy used to inhibit DENV proliferation effectively in a host’s cells is to inhibit proteins that are uniquely encoded by the virus, and intense efforts to develop a safe and effective anti-DENV agent are ongoing. Among proteins specifically encoded by DENV, RNA-dependent RNA polymerase (RdRp) is an attractive drug target because it catalyzes viral RNA replication; currently, a number of nucleoside antimetabolites including their prodrugs have been reported to be inhibitors of RdRp.

Our group is also interested in developing RdRp inhibitors, and we previously screened compounds in a nucleoside library prepared in our laboratory. Among more than 150 compounds tested, 5-ethynyl-(1-β-D-ribofuranosyl)imidazole-4-carboxamide (I; EICAR) was identified as a potent anti-DENV agent. At the concentration needed to inhibit DENV virus, however, this compound showed cytotoxicity to some human (tumor) cell lines. In an attempt to develop a
less-cytotoxic agent, we subsequently prepared its 4’-thio and 4’-seleno derivatives and evaluated their anti-DENV activity. Using ribavirin as a positive control to evaluate anti-DENV activity, we showed that 4’-thioEICAR (2) showed moderate anti-DENV activity without cytotoxicity (Fig. 1). \(^8\)

Collectively, these studies showed that 4’-thio-modification of nucleosides is an effective approach for reducing cytotoxicity; however, the antiviral activity is reduced at the same time. We hypothesized that the reason for the reduced anti-DENV activity of 2 is that it is less susceptible to phosphorylation by cellular kinase(s), which is necessary to convert the drug to its active form. To test this hypothesis, we prepared prodrugs of 2. Furthermore, we revalidated the anti-DENV activity of the compounds with a more accurate method based on cells infected by DENV virus, rather than the transient reporter replicon assay utilized in our previous study. In this paper, we describe the chemical synthesis and biological evaluation of a prodrug of 4’-thioEICAR (2).

![Chemical Structures](attachment:image.png)

|        | IC\(_{50}\) (µM) | CC\(_{50}\) (µM) |
|--------|------------------|-----------------|
| EICAR  | 0.27             | 1.24            |
| 4’-thioEICAR | 14.63           | > 50            |
| Ribavirin | 6.66            | 48.98           |

Fig. 1. Anti-DENV activity in reporter replicon assay

**Results and discussion**

In order to inhibit RdRp, a nucleoside antimetabolite must be phosphorylated by cellular kinase(s) to its corresponding triphosphate, which is the active form. The first phosphorylation to the monophosphate is often the rate-determining step that determines antiviral activity in host cells.
To increase metabolic activation, therefore, a strategy to develop prodrugs for the nucleoside monophosphate has been widely adopted. Since the successful discovery of sofosbuvir\(^9\) for treatment of hepatitis C virus infection, and remdesivir\(^{10,11}\) for treatment of Ebola virus and novel severe acute respiratory syndrome coronavirus-2 infection, various prodrug skeletons have been developed, and derivatization of nucleoside antimetabolites into these prodrugs is being actively studied.\(^{12}\) Based on this research background, we attempted to prepare a prodrug of \(\mathbf{2}\). Because biological activity often varies with the prodrug skeleton, we first prepared two prodrugs of \(\mathbf{1}\), namely \(S\)-acyl-2-thioethyl (SATE) and aryloxyphosphoramidate (so-called ProTide) to identify a suitable skeleton for our assay system.

The SATE prodrug was first reported by Imbach’s group.\(^{13}\) Generally, three approaches are adopted for its synthesis: 1) coupling of a nucleoside \(H\)-phosphonate with a hydroxythioester reagent; 2) coupling of a nucleoside 5'-monophosphate with a hydroxythioester derivative; and 3) coupling of a \(N,N\)-diisopropylphosphoramidite reagent to nucleoside followed by oxidation of the phosphorus atom.\(^{12}\) The two former methods necessitate the treatment of highly polar phosphorylated nucleosides as intermediates; therefore, we modified the third approach to synthesize the SATE prodrug of \(\mathbf{1}\) by a convenient one-pot method, as illustrated in Chart 1. Because the 4-carboxamide group of EICAR derivative \(\mathbf{3}\) would be expected to react with the phosphitylation reagent used for prodrug synthesis,\(^{14}\) the 4-cyanoimidazole derivative \(\mathbf{4}\)\(^{15}\) was used as the starting material. In brief, a \(\text{CH}_2\text{Cl}_2\) solution of \(\mathbf{4}\) containing \(N,N\)-diisopropylethylamine (DIPEA) was first phosphorylated with bis(diisopropylamino)chlorophosphine at \(-78 \text{ °C}\) to give \(\mathbf{5}\), and then a mixture of the \(S\)-pivaloylthioethanol \(\mathbf{6}\)\(^{16}\) and 5-(benzylthio)-1\(H\)-tetrazole (BTT) in
CH$_3$CN was added dropwise to the reaction mixture at the same temperature to give 7. The reaction mixture was then warmed to room temperature, and tert-butyl hydroperoxide (TBHP) in toluene was added to carry out oxidation of the phosphorus atom and give the desired SATE prodrug 8 in 67% yield in the one-pot reaction. We then examined deprotection of isopropylidene group and hydrolysis of 4-cyano group of 8 under acidic conditions. When 8 was heated in 60% aqueous AcOH, deprotection of the isopropylidene group was observed to give 9 as the only product. No hydrolysis of the 4-cyano group even under the conditions of aqueous trifluoroacetic acid or aqueous HCl occurred. After several attempts, the desired product 10 was obtained in 52% yield when 8 was treated with a mixture of HCl-AcOH (2:1) at room temperature. In the $^1$H-NMR spectrum of 10, two broad singlets at 7.34 and 7.26 ppm (exchangeable with D$_2$O) corresponding to the 4-carboxamide and no decomposition at SATE skeleton were observed. The mass spectrum also supported the structure of 10.

Chart 1. Synthesis of SATE prodrug of EICAR and 4’-thioEICAR.
We also explored the synthesis of a ProTide prodrug of 1 (Chart 2). This skeleton was developed by McGuigan’s group and has been successfully incorporated in the structures of sofosbuvir and remdesivir. Therefore, the synthetic approach of ProTide prodrugs has been well studied, and we attempted the synthesis of 15 in accordance with a published method. Initially, we treated 4 with phenyl-(iso-propyl-L-alanyl)-phosphorochloridate (11) in the presence of tBuMgCl to give 12 as a diastereomixture. In the same manner as described for 8, the resulting 12 was treated with a mixture of HCl-AcOH (2:1). However, decomposition of 12 occurred and no desired 15 was obtained. Other acidic conditions, such as 60% aqueous AcOH at 95 °C, afforded only the 4-cyano product without the isopropylidene group 14 (data not shown). Accordingly, we attempted the reaction of the 4-carboxamide derivative 3 with 11, resulting in two main spots on TLC analysis. After purification by a silica-gel column, the structure corresponding to the more polar spot (32% yield) was determined to be a 7:3 of diastereomixture of 13 from the 1H-NMR and mass spectra. Although the 1H-NMR spectrum of the less polar spot was too complex to determine the structure, the mass spectrum suggested that it was the diphosphoramidate compound with phosphoramidate attached on both the 5'-hydroxyl and 4-carboxamide groups (data not shown). Despite the low yield of 13, we attempted its deprotection by treatment with 60% aqueous AcOH at 95 °C, which gave 15, the desired ProTide prodrug of 1, in 45% yield. The resulting 15 was further purified by HPLC to give pure 15a (fast moving isomer) and 15b (slow moving isomer) (the configuration at the phosphorus center of 15a and 15b has not been determined because crystallization of both isomers failed).
Next, we evaluated the anti-DENV activity of these three prodrugs of 1 – namely, 10, 15a, and 15b – by a MTT assay using DENV2-infected BHK-21 cells, in contrast to our previous study, which used a transient reporter replicon assay. As summarized in Table 1, EICAR (1) exhibited potent anti-DENV2 activity (EC50 = 0.95 μM) at a much lower concentration as compared with ribavirin (EC50 = 32.24 μM), as well as moderate cytotoxicity (CC50 = 42.19 μM) under our test conditions. The anti-DENV2 activity of the SATE prodrug 10 was comparable to that of 1 (0.95 vs. 2.70 μM), while that of the ProTide prodrugs 15a and 15b was both negligible. In general, the Sp diastereomer is known to have more potent antiviral activity than the Rp diastereomer. Therefore, our results suggest that neither 15a nor 15b is metabolized to the corresponding monophosphate, while the SATE prodrug 10 is effectively converted to the monophosphate, which would be
successively metabolized to the active triphosphate, in our assay system.

The ProTide prodrugs should be hydrolyzed by carboxypeptidase(s) and phosphoramidase(s) to afford the corresponding monophosphates in cells. On the other hand, the SATE prodrugs need activation by (thio)esterases. The difference of activation enzymes required would lead to the difference of antiviral activity mentioned above.

Table 1. Anti-DENV2 activity of synthesized imidazole nucleosides and prodrugs.\textsuperscript{a}

| Compound       | EC\textsubscript{50} (\textmu M) | CC\textsubscript{50} (\textmu M) | SI    |
|----------------|---------------------------------|---------------------------------|-------|
| EICAR (1)      | 0.95                            | 42.19                           | 44.51 |
| 10             | 2.70                            | 54.97                           | 20.43 |
| 15a            | >50                             | >100                            | –     |
| 15b            | >50                             | >100                            | –     |
| 4’-thioEICAR (2)| 32.62                           | >100                            | >3.06 |
| 18             | 1.05                            | 43.26                           | 41.28 |
| Ribavirin      | 32.24                           | >400                            | >12.41|

\textsuperscript{a} Data are the average of three independent experiments.

Based on these results, we attempted to prepare and evaluate the SATE prodrug of 2 (Chart 1). Using the same method described for 4, 16\textsuperscript{a} was treated with bis(diisopropylamino)chlorophosphine in the presence of N,N-diisopropylethylamine, followed by 6, BTT, and TBHP to give 17. The 4-cyano group of 17 was then hydrolyzed under acidic conditions to give the desired 18 in 53\% yield. We compared the anti-DENV2 activity of 18 with that of 4’-thioEICAR (2). As compared with 2, 18 showed much more potent activity (Table 1); indeed, the activity of 18 was almost equal
to that of EICAR (1.05 vs 0.95 μM). However, the cytotoxicity of 18 was also revived and comparable to that of EICAR (43.26 vs 42.19 μM).

To date, a number of 4’-thionucleoside derivatives have been prepared and their biological activities have been evaluated. Among them, 2’-deoxy-4’-thionucleoside derivatives often show potent biological activities,\(^{23-25}\) while 4’-thioribonucleoside derivatives generally have moderate or negligible activity.\(^{26,27}\) One explanation for these observations may be the difference in susceptibility to nucleoside kinase(s) between 2’-deoxy-4’-thionucleoside and 4’-thioribonucleoside derivatives: in short, 2’-deoxy-4’-thionucleoside derivatives are likely to be well phosphorylated by cellular kinases such as deoxycytidine, thymidine, and deoxyadenosine kinases, while 4’-thioribonucleoside derivatives seem to be less susceptible to phosphorylation by cellular kinases such as uridine-cytidine and adenosine kinases. As suggested in our previous study, EICAR is phosphorylated by cellular adenosine kinase to give the corresponding 5’-monophosphate, EICARMP, which inactivate inosine 5’-monophosphate dehydrogenase (IMPDH; an enzyme that convert IMP to xanthosine 5’-monophosphate), and thereby exhibits antitumor activity and cytotoxicity.\(^{5,6,28}\) In addition, further phosphorylation converts the resulting EICARMP to the triphosphate, EICARTP, which inhibits viral RdRp.\(^{29}\)

Regarding the compounds evaluated in this study, we propose the following: 1) similar to other 4’-thioribonucleoside derivatives, 4’-thioEICAR (2) would undergo very slow phosphorylation by adenosine kinase; thus, providing 2 as a SATE prodrug may be an effective tactic to form the corresponding monophosphate 4’-thioEICARMP in cells. 2) The resulting 4’-thioEICARMP would be further phosphorylated to give 4’-thioEICARTP, which would inhibit RdRp of DENV2. 3) At the
same time, however, 4’-thioEICARMP would inhibit IMPDH. Accordingly, compound 2 exhibits biological activity similar to that of EICAR (1). To develop an effective anti-DENV2 compound, further chemical modification that maintains inhibitory activity toward RdRp and decreases that toward IMPDH will be required.

**Conclusion**

In conclusion, we prepared two types of prodrug (SATE and ProTide) of the potent anti-DENV2 compound EICAR (1), and evaluated their antiviral activity in order to identify a suitable prodrug skeleton for our assay system. The SATE prodrug of 1 showed comparable activity to 1, whereas the ProTide prodrug of 1 exhibited less activity. Based on these findings, we prepared the SATE prodrug of 4’-thioEICAR (2) because we speculated that the lower anti-DENV2 of 2 relative to 1 was due to lower phosphorylation efficiency. As we expected, the resulting SATE prodrug 18 showed anti-DENV2 activity comparable to that of 1; however, its cytotoxicity was also revived. From these results, it can be concluded that designing prodrugs of 4’-thioribonucleoside derivatives such as 2 is an effective tactic to developing potent biologically active compounds. However, the balance between antiviral activity and cytotoxicity remains an issue to be resolved, and thus further chemical modifications are underway.

**Experimental**

**General methods**

Physical data were measured as follows: $^1$H, $^{13}$C, and $^{31}$P NMR spectra were recorded at 400 or
500 MHz, 100 or 125 MHz, and 162 or 202 MHz instruments (Bruker FT-NMR AV400 or AV500), respectively in CDCl₃ or DMSO-d₆ as the solvent with tetramethylsilane or H₃PO₄ as an internal or external standard. Chemical shifts are reported in parts per million (ppm), and signals are expressed as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad). All exchangeable protons were detected by addition of D₂O. Mass spectra were measured on a SQD2 (Waters) and BioAccord (Waters). TLC was done on Merck Kieselgel F254 precoated plates. Silica gels used for column chromatography were KANTO Chemical silica gel 60 and KANTO Chemical silica gel 60N (neutral).

5-Ethynyl-1-(2,3-O-isopropylidene-5-O-[bis(S-pivaloyl-2-thioethyl)phosphate]-β-D-ribofuranosyl)imidazole-4-carbonitrile (8). To a solution of 4¹⁵ (170 mg, 0.59 mmol) containing DIPEA (51 μL, 0.30 mmol) in CH₂Cl₂ (5 mL) was dropwisely added a solution of bis(diisopropylamino)chlorophosphine (480 mg, 1.8 mmol) in CH₂Cl₂ (3 mL) using a dropping funnel at −78 °C. After being stirred for 10 min, a solution of 6¹⁶ (763 mg, 4.72 mmol) in CH₂Cl₂ (8 mL) and BTT (0.25 M in CH₃CN, 18.8 mL, 4.7 mmol) were added to the above solution at the same temperature. The reaction temperature was gradually raised to room temperature (about 1 hr) and the whole was stirred for further 3 hr. Then, TBHP (1.0 M in toluene, 0.65 mL, 0.65 mmol) was added to the reaction mixture and the whole was stirred for 20 min at room temperature. The reaction mixture was diluted with CHCl₃ and the organic layer was washed with saturated NaHCO₃ and brine. The separated organic layer was dried and concentrated in vacuo. The residue was purified by a silica gel column, eluted with hexane/AcOEt (2/1–1/2), to give 8 (254 mg, 67% as a
pale yellow oil): ESI-LRMS \textit{m/z} 680 (MNa\(^{+}\)); ESI-HRMS calcd for C\(_{28}\)H\(_{41}\)N\(_{3}\)O\(_{9}\)PS\(_{2}\) 658.2016, found 658.2026; \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 7.81 (1 H, s), 5.90 (1 H, d, \(J = 2.5\) Hz), 4.97–4.93 (2 H, m), 4.46 (1 H, dt, \(J = 4.0\) and 6.5 Hz), 4.31–4.29 (2 H, m), 4.14–4.07 (4 H, m), 3.93 (1 H, s), 3.15–3.10 (4 H, m), 1.60 and 1.38 (each 3 H, each s), 1.24 (9 H, s), 1.23 (9 H, s); \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) 205.70, 205.66, 137.05, 121.35, 120.49, 115.29, 113.25, 92.15, 91.68, 85.15, 84.31, 84.25, 80.30, 68.18, 66.66, 66.62, 66.59, 66.55, 66.51, 66.47, 46.57, 28.45, 28.39, 27.29, 27.16, 25.30; \(^{31}\)P NMR (CDCl\(_3\)) \(\delta\) –1.04.

5-Ethynyl-1-{5-O-[bis(S-pivaloyl-2-thioethyl)phosphate]-\(\beta\)-D-ribofuranosyl}imidazole-4-carbonitrile (9). A solution of 8 (506 mg, 0.77 mmol) in 60% aqueous AcOH (8 mL) was heated for 4.5 h at 95 °C. After being cooled to room temperature, the solvent was removed \textit{in vacuo} and the residue was coevaporated with EtOH and toluene. The residue was purified by a silica gel column, eluted with hexane/AcOEt (2/1–0/1), to give 9 (259 mg, 55% as a colorless oil): ESI-LRMS \textit{m/z} 640 (MNa\(^{+}\)); ESI-HRMS calcd for C\(_{25}\)H\(_{37}\)N\(_{3}\)O\(_{9}\)PS\(_{2}\) 618.1703, found 618.1747; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 8.25 (1 H, s), 5.77 (1 H, d, \(J = 5.8\) Hz, exchangeable with D\(_2\)O), 5.68 (1 H, J = 5.3 Hz), 5.51 (1 H, d, \(J = 5.3\) Hz, exchangeable with D\(_2\)O), 5.38 (1 H, s), 4.37 (1 H, q, \(J = 5.3\) Hz), 4.22–4.15 (2 H, m), 4.12–4.03 (2 H, m), 4.02–3.99 (4 H, m), 3.11–3.07 (4 H, m), 1.18 (9 H, s), 1.17 (9 H, s); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 205.02, 138.59, 122.34, 117.85, 113.83, 94.74, 89.61, 83.00, 74.11, 69.60, 68.08, 66.88, 65.68, 65.64, 45.99, 28.13, 26.85; \(^{31}\)P NMR (DMSO-\(d_6\)) \(\delta\) –1.35.

5-Ethynyl-1-{5-O-[bis(S-pivaloyl-2-thioethyl)phosphate]-\(\beta\)-D-ribofuranosyl}imidazole-4-carbonitrile (9). A solution of 8 (506 mg, 0.77 mmol) in 60% aqueous AcOH (8 mL) was heated for 4.5 h at 95 °C. After being cooled to room temperature, the solvent was removed \textit{in vacuo} and the residue was coevaporated with EtOH and toluene. The residue was purified by a silica gel column, eluted with hexane/AcOEt (2/1–0/1), to give 9 (259 mg, 55% as a colorless oil): ESI-LRMS \textit{m/z} 640 (MNa\(^{+}\)); ESI-HRMS calcd for C\(_{25}\)H\(_{37}\)N\(_{3}\)O\(_{9}\)PS\(_{2}\) 618.1703, found 618.1747; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 8.25 (1 H, s), 5.77 (1 H, d, \(J = 5.8\) Hz, exchangeable with D\(_2\)O), 5.68 (1 H, J = 5.3 Hz), 5.51 (1 H, d, \(J = 5.3\) Hz, exchangeable with D\(_2\)O), 5.38 (1 H, s), 4.37 (1 H, q, \(J = 5.3\) Hz), 4.22–4.15 (2 H, m), 4.12–4.03 (2 H, m), 4.02–3.99 (4 H, m), 3.11–3.07 (4 H, m), 1.18 (9 H, s), 1.17 (9 H, s); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 205.02, 138.59, 122.34, 117.85, 113.83, 94.74, 89.61, 83.00, 74.11, 69.60, 68.08, 66.88, 65.68, 65.64, 45.99, 28.13, 26.85; \(^{31}\)P NMR (DMSO-\(d_6\)) \(\delta\) –1.35.
arboxamide (10). A solution of 8 (56 mg, 0.09 mmol) in a mixture of HCl (1.2 mL) and AcOH (0.6 mL) was stirred for 6 h at room temperature. After being cooled to 0 °C, the reaction mixture was neutralized with 1N NaOH, and the diluted with CHCl₃. The organic layer was washed with H₂O, followed by brine. The separated organic layer was dried and concentrated in vacuo. The residue was purified by a silica gel column, eluted with MeOH in CHCl₃ (0%–7%), to give 10 (28 mg, 52% as a colorless oil): ESI-LRMS m/z 658 (MNa⁺); ESI-HRMS calcd for C₂₅H₃₉N₃O₁₁PS₂ 636.1809, found 636.1841; ¹H NMR (DMSO-d₆) δ 8.03 (1 H, s), 7.34 (1 H, brs, exchangeable with D₂O), 7.26 (1 H, brs, exchangeable with D₂O), 5.70–5.68 (2 H, m, 1 H was exchangeable with D₂O), 5.45 (1 H, J = 5.0 Hz, exchangeable with D₂O), 4.87 (1 H, s), 4.35 (1 H, q, J = 5.0 Hz), 4.23–4.19 (1 H, m), 4.16–4.12 (1 H, m), 4.08–4.06 (2 H, m), 4.04–3.99 (4 H, m), 3.12–3.08 (4 H, m), 1.17 (9 H, s), 1.17 (9 H, s); ¹³CNMR (DMSO-d₆) δ 205.09, 162.54, 139.76, 135.65, 115.55, 91.33, 88.70, 82.49, 74.04, 71.41, 69.75, 66.95, 65.72, 46.01, 28.17, 28.11, 26.88; ³¹P NMR (DMSO-d₆) δ −1.35.

5-Ethynyl-1-(2,3-O-isopropylidene-5-O-[phenyl-(2,2-dimethylisopropyl-L-alaminyl)phosphohate]-β-D-ribofuranosyl)imidazole-4-carbonitrile (12). To a solution of 4 (294 mg, 1.02 mmol) in THF (5 mL) was added a solution of 11 (624 mg, 1.44 mmol) in THF containing t-BuMgCl (1.0 M in THF, 0.85 mL, 0.85 mmol), and the whole was stirred for 2 h at room temperature. The solvent was removed in vacuo and the residue was purified by a silica gel column, eluted with MeOH in CHCl₃ (0%–5%), to give 12 (100 mg, 24% as a yellow oil): ESI-LRMS m/z 581 (MNa⁺); ESI-HRMS calcd for C₂₆H₃₂N₄O₆P 559.1952, found 559.1930; ¹H NMR (CDCl₃) δ 7.80 and 7.79 (each 0.5 H, each s), 7.52–7.16 (5 H, m), 5.88 and 5.82 (each 0.5 H, each d, J = 3.0 Hz), 5.04–4.97
(1 H, m), 4.88 and 4.80 (each 0.5 H, each dd, \( J = 3.0 \) and 6.3 Hz), 4.75 (0.5 H, dd, \( J = 6.3 \) and 2.3 Hz), 4.52–4.51 and 4.47–4.46 (each 0.5 H, each m), 4.37 (0.5 H, dd, \( J = 6.3 \) and 2.3 Hz), 4.35–4.31 (2 H, m), 3.99–3.84 (2 H, m), 3.65–3.52 (1 H, m, exchangeable with D\(_2\)O), 1.59 and 1.55 (each 1.5 H, each s), 1.36 and 1.30 (each 1.5 H, each s), 1.33 and 1.32 (each 1.5 H, each d, \( J = 6.9 \) Hz), 1.25–1.22 (6 H, m); \(^{31}\)P NMR (CDCl\(_3\)) \( \delta \) 2.90, 2.54.

5-Ethynyl-1-(2,3-O-isopropylidene-5-O-[phenyl-(2,2-dimethylisopropyl-L-alaminyl)phosphate]-\( \beta \)-D-ribofuranosyl]imidazole-4-carboxamide (13). In a similar manner as described for 12, 3 (150 mg, 0.5 mmol) was treated with 11 (229 mg, 0.75 mmol) and \( t \)-BuMgCl (1.0 M in THF, 0.85 mL, 0.85 mmol) to give 13 (90 mg, 32% as a yellow oil): ESI-LRMS \( m/z \) 599 (MNa\(^+\)); ESI-HRMS calcd for C\(_{26}\)H\(_{34}\)N\(_4\)O\(_9\)P 577.2058, found 577.2070; \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 7.71 (0.7 H, s), 7.70 (0.3 H, each s), 7.34–7.15 (5 H, m), 6.95 (1 H, brs, exchangeable with D\(_2\)O), 5.96 (0.7 H, d, \( J = 2.8 \) Hz), 5.90 (0.3 H, each d, \( J = 3.0 \) Hz), 5.41 (1 H, brs, exchangeable with D\(_2\)O), 5.04–4.93 (1 H, m), 4.89–4.72 (2 H, m), 4.50–4.27 (3 H, m), 3.98–3.89 (1 H, m), 3.84 (0.7 H, s), 3.83 (0.3 H, s), 3.71–3.61 (1 H, m, exchangeable with D\(_2\)O), 1.60–1.21 (15 H, m); \(^{31}\)P NMR (CDCl\(_3\)) \( \delta \) 3.40, 3.14.

5-Ethynyl-1-(5-O-[phenyl-(2,2-dimethylisopropyl-L-alaminyl)phosphate]-\( \beta \)-D-ribofuranosyl]imidazole-4-carboxamide (15). In a similar manner as described for 9, 13 (90 mg, 0.16 mmol) was treated with 60% aqueous AcOH (3 mL) at 95 °C to give 15 (38 mg, 45% as a yellow foam. The resulting 15 was further purified by reverse-phase HPLC (YMC-Pack R&D ODS-A 250 x 20 mm, 60% MeOH in H\(_2\)O) to give 15a (fast moving isomer; retention time = 20.5 min) and 15b
(slow moving isomer; retention time = 26.3 min).

Data for 15a: ESI-LRMS m/z 559 (MNa\(^+\)); ESI-HRMS calcd for C\(_{23}\)H\(_{29}\)N\(_4\)O\(_9\)P 537.1745, found 537.1787; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 8.02 (1 H, s), 7.37–7.33 (3 H, m, 1H was exchangeable with D\(_2\)O), 7.26 (1 H, br s, exchangeable with D\(_2\)O), 7.18–7.14 (3 H, m), 6.04–5.99 (1 H, m, exchangeable with D\(_2\)O), 5.69 (1 H, d, \(J = 5.8\) Hz), 5.66 (1 H, d, \(J = 6.0\) Hz, exchangeable with D\(_2\)O), 5.44 (1 H, d, \(J = 5.5\) Hz, exchangeable with D\(_2\)O), 4.88 (1 H, s), 4.87–4.82 (1 H, m), 4.35 (1 H, q, \(J = 5.5\) Hz), 4.23–4.08 (4 H, m), 3.78–3.71 (1 H, m), 1.18–1.13 (9 H, m); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 172.73, 172.69, 162.53, 150.64, 150.59, 139.69, 135.59, 129.56, 124.53, 120.14, 120.10, 115.63, 91.29, 88.55, 82.90, 74.15, 71.42, 69.98, 67.97, 65.70, 49.95, 21.39, 21.34, 19.61, 19.53; \(^{31}\)P NMR (DMSO-\(d_6\)) \(\delta\) 4.30.

Data for 15b: ESI-LRMS m/z 559 (MNa\(^+\)); ESI-HRMS calcd for C\(_{23}\)H\(_{29}\)N\(_4\)O\(_9\)P 537.1745, found 537.1741; \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 8.03 (1 H, s), 7.39–7.35 (3 H, m, 1H was exchangeable with D\(_2\)O), 7.26 (1 H, br s, exchangeable with D\(_2\)O), 7.22–7.16 (3 H, m), 6.07–6.01 (1 H, m, exchangeable with D\(_2\)O), 5.67 (1 H, d, \(J = 6.0\) Hz), 5.66 (1 H, d, \(J = 6.3\) Hz, exchangeable with D\(_2\)O), 5.42 (1 H, d, \(J = 5.3\) Hz, exchangeable with D\(_2\)O), 4.88 (1 H, s), 4.87–4.81 (1 H, m), 4.35 (1 H, q, \(J = 5.3\) Hz), 4.22–4.17 (1 H, m), 4.12–4.06 (3 H, m), 3.81–3.75 (1 H, m), 1.20–1.13 (9 H, m); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 172.59, 172.54, 162.52, 150.68, 150.61, 139.67, 135.67, 129.59, 124.54, 120.12, 120.07, 115.65, 91.27, 88.39, 82.89, 74.04, 71.41, 70.03, 67.98, 65.78, 49.81, 21.37, 21.34, 19.73, 19.67; \(^{31}\)P NMR (DMSO-\(d_6\)) \(\delta\) 4.29.
5-Ethynyl-1-(2,3-O-isopropylidene-5-O-[phenyl-(2,2-dimethylisopropyl-L-alaminy]phosphate]-4-thio-β-D-ribofuranosyl)imidazole-4-carbonitrile (17). In a similar manner as described for 8, 16 (210 mg, 0.69 mmol) was treated with bis(diisopropylamino)chlorophosphine (560 mg, 2.1 mmol) and DIPEA (88 μL, 0.52 mmol), followed by 6 (1.12 g, 6.9 mmol) in CH₂Cl₂ and BTT (0.25 M in CH₃CN, 27.6 mL, 6.9 mmol) and then TBHP (1.0 M in toluene, 0.8 mL, 0.8 mmol) to give 17 (416 mg, 90% as a colorless oil): ESI-LRMS m/z 696 (MNa⁺); ESI-HRMS calcd for C₂₈H₄₁N₃O₈PS₃ 674.1788, found 674.1822; ¹H NMR (CDCl₃) δ 8.02 (1 H, s), 5.82 (1 H, d, J = 2.4 Hz), 5.01 (1 H, dd, J = 2.4 and 5.3 Hz), 4.95 (1 H, dd, J = 5.3 and 2.1 Hz), 4.35–4.31 (1 H, m), 4.19–4.09 (5 H, m), 3.96 (1 H, s), 3.92–3.90 (1 H, m), 3.17–3.12 (4 H, m), 1.60 (3 H, s), 1.35 (3 H, s), 1.24 (9 H, s), 1.23 (9 H, s); ¹³C NMR (CDCl₃) δ 205.68, 137.52, 122.66, 120.52, 113.22, 113.02, 92.44, 90.07, 84.85, 69.49, 68.22, 67.94, 67.90, 66.62, 66.57, 66.53, 53.78, 53.71 46.58, 28.50, 28.44, 27.31, 27.28, 25.15; ³¹P NMR (CDCl₃) δ –1.29.

5-Ethynyl-1-(5-O-[phenyl-(2,2-dimethylisopropyl-L-alaminy]phosphate]-4-thio-β-D-ribofuranosyl)imidazole-4-carboxamide (18). In a similar manner as described for 10, 17 (96 mg, 0.14 mmol) was treated with a mixture of HCl (2.0 mL) and AcOH (1.0 mL) to give 18 (49 mg, 53% as a white solid): ESI-LRMS m/z 674 (MNa⁺); ESI-HRMS calcd for C₂₅H₃₉N₃O₉PS₃ 652.1581, found 652.1547; ¹H NMR (DMSO-d₆) δ 8.23 (1 H, s), 7.39 (1 H, brs, exchangeable with D₂O), 7.27 (1 H, brs, exchangeable with D₂O), 5.81 (1 H, d, J = 6.6 Hz, exchangeable with D₂O), 5.65 (1 H, d, J = 7.5 Hz, exchangeable with D₂O), 5.63 (1 H, d, J = 6.6 Hz), 4.95 (1 H, s), 4.54 (1 H, dt, J = 6.6 and 3.5 Hz), 4.39–4.34 (1 H, m), 4.19–4.12 (2 H, m), 4.08–4.04 (4 H, m), 3.48 (1 H, dt, J = 3.4 and 6.7
Hz), 3.14–3.12 (4 H, m), 1.18 (9 H, s), 1.17 (9 H, s); $^{13}$C NMR (DMSO-$d_6$) δ 205.10, 162.56, 139.58, 136.44, 116.37, 91.95, 76.97, 72.68, 71.49, 68.16, 65.75, 62.88, 50.25, 50.20, 46.03, 28.24, 28.17, 26.90; $^{31}$P NMR (DMSO-$d_6$) δ –1.64.

Cytopathic effect-based anti-DENV-2 and cytotoxicity assays.

The MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay was performed as previously described. Briefly, BHK-21 cells (3 × 10$^4$ cells) suspended in EMEM supplemented with 2% FBS were seeded into 96-well plates with diluted compounds in each well. The cells were infected with DENV2 (D2/hu/INDIA/09–74, accession number LC367234) was infected at a multiplicity of infection (MOI) of 0.01. Following incubation for 4 days, the MTT reagent (5 mg/mL; Nacalai tesque) was added to each well, and the plate was incubated at 37 °C for 2 h. After incubation, the culture supernatants were removed, and the cell lysis solution (2-propanol with 10% Triton-X100 and 0.31% HCl) was added. Absorbance at a wavelength of 560 nm and reference wavelength of 660 nm was measured using a microplate reader (model 680; Bio-Rad Laboratories, Hercules, CA, USA), and then the 50% effective concentration (EC$_{50}$) was calculated. Cytotoxicity was assessed under the same conditions as for the measurement of anti-DENV2 activity, and the 50% cytotoxicity concentration (CC$_{50}$) was calculated. These assays were performed in duplicate and repeated thrice.

The EC$_{50}$ value was defined in GraphPad Prism version 8.4.2 (GraphPad Software) with a variable slope (four parameters). Noninfected cells were used as a control for 100% inhibition, whereas for infected cells, DMSO alone was used as a control for 0% inhibition. The CC$_{50}$ value for each cell
line was also measured using the same method. Cell-free samples were used as 100% cytotoxicity control and DMSO-treated cells were used as 0% cytotoxicity control.

• **Acknowledgements**

This work was supported in part by AMED under Grant Number JP20fk0108289 for N.M. and JP21wm0225017 for H.S. N.M also appreciates the Research Cluster program of Tokushima University (Organizer, T. Kagtagiri). N.S-T. appreciates the research program for the development of intelligent Tokushima artificial exosome (iTEX) from Tokushima University.

• **Conflict of Interest**

The authors K.U. and A.S. are employees of Shionogi & Co., Ltd. The other authors declare no competing financial interest.

• **Supplementary Materials**

This article contains supplementary materials.
References and Notes

1. Messina J. P., Brady O. J., Golding N., Kraemer M. U. G., Wint G. R. W., Ray S. E., Pigott D. M., Shearer F. M., Johnson K., Earl L., Marczak L. B., Shirude S., Davis Weaver N., Gilbert M., Velayudhan R., Jones P., Jaenisch T., Scott T. W., Reiner R. C. Jr, Hay S. I., Nat Microbiol, 4, 1508–1515 (2019).

2. Troost B., Smit J. M., Curr. Opin. Virol., 43, 9–21 (2020).

3. Zandi K., Bassit L., Amblard F., Cox B. D., Hassandra Vish P., Moghaddam E., Yueh A., Libanio Rodrigues G. O., Passos I., Costa V. V., AbuBakar S., Zhou L., Kohler J., Teixeira M. M., Schinazi R. F., Antimicrob. Agents Chemother., 63, (2019).

4. Milisavljevic N., Konkolová E., Kozák J., Hodek J., Veselovská L., Sýkorová V., Čížek K., Pohl R., Eyer L., Svoboda P., Růžek D., Weber J., Nencka R., Bouřa E., Hocek M., ACS Infect Dis, 7, 471–478 (2021).

5. Matsuda A., Minakawa N., Sasaki T., Ueda T., Chem. Pharm. Bull., 36, 2730–2733 (1988).

6. Minakawa N., Takeda T., Sasaki T., Matsuda A., Ueda T., J. Med. Chem., 34, 778–786 (1991).

7. De Clercq E., Cools M., Balzarini J., Snoeck R., Andrei G., Hosoya M., Shigeta S., Ueda T., Minakawa N., Matsuda A., Antimicrob. Agents Chemother., 35, 679–684 (1991).

8. Okano Y., Saito-Tarashima N., Kurosawa M., Iwabu A., Ota M., Watanabe T., Kato F., Hishiki T., Fujimuro M., Minakawa N., Bioorg. Med. Chem., 27, 2181–2186 (2019).

9. Mehellou Y., Balzarini J., McGuigan C., ChemMedChem, 4, 1779–1791 (2009).

10. Warren T. K., Jordan R., Lo M. K., Ray A. S., Mackman R. L., Soloveva V., Siegel D., Perron M., Bannister R., Hui H. C., Larson N., Strickley R., Wells J., Stuthman K. S., Van Tongeren S.
A., Garza N. L., Donnelly G., Shurtleff A. C., Retterer C. J., Gharaibeh D., Zamani R., Kenny T., Eaton B. P., Grimes E., Welch L. S., Gomba L., Wilhelmsen C. L., Nichols D. K., Nuss J. E., Nagle E. R., Kugelman J. R., Palacios G., Doerffler E., Neville S., Carra E., Clarke M. O., Zhang L., Lew W., Ross B., Wang Q., Chun K., Wolfe L., Babusis D., Park Y., Stray K. M., Trancheva I., Feng J. Y., Barauskas O., Xu Y., Wong P., Braun M. R., Flint M., McMullan L. K., Chen S.-S., Fears R., Swaminathan S., Mayers D. L., Spiropoulou C. F., Lee W. A., Nichol S. T., Cihlar T., Bavari S., *Nature*, **531**, 381–385 (2016).

11. Eastman R. T., Roth J. S., Brimacombe K. R., Simeonov A., Shen M., Patnaik S., Hall M. D., *ACS Cent Sci.*, **6**, 672–683 (2020).

12. Pradere U., Garnier-Amblard E. C., Coats S. J., Amblard F., Schinazi R. F., *Chem. Rev.*, **114**, 9154–9218 (2014).

13. Puech F., Gosselin G., Lefebvre I., Pompon A., Aubertin A. M., Kirn A., Imbach J. L., *Antiviral Res.*, **22**, 155–174 (1993).

14. Tarashima N. S., Kumanomido Y., Nakashima K., Tanaka Y., Minakawa N., *J. Org. Chem.*, (2021).

15. Minakawa N., Matsuda A., Xia Z., Wiebe L. I., Knaus E. E., *J. Labelled Comp. Radiopharm.*, **38**, 809–824 (1996).

16. Ruda G. F., Alibu V. P., Mitsos C., Bidet O., Kaiser M., Brun R., Barrett M. P., Gilbert I. H., *ChemMedChem*, **2**, 1169–1180 (2007).

17. McGuigan C., Pathirana R. N., Mahmood N., Devine K. G., Hay A. J., *Antiviral Res.*, **17**, 311–321 (1992).
18. Ross B. S., Reddy P. G., Zhang H.-R., Rachakonda S., Sofia M. J., J. Org. Chem., 76, 8311–8319 (2011).

19. Pertusati F., McGuigan C., Chem. Commun., 51, 8070–8073 (2015).

20. Derudas M., Carta D., Brancale A., Vanpouille C., Lisco A., Margolis L., Balzarini J., McGuigan C., J. Med. Chem., 52, 5520–5530 (2009).

21. Sofia M. J., Bao D., Chang W., Du J., Nagarathnam D., Rachakonda S., Reddy P. G., Ross B. S., Wang P., Zhang H.-R., Bansal S., Espiritu C., Keilman M., Lam A. M., Steuer H. M. M., Niu C., Otto M. J., Furman P. A., J. Med. Chem., 53, 7202–7218 (2010).

22. Siegel D., Hui H. C., Doerfler E., Clarke M. O., Chun K., Zhang L., Neville S., Carra E., Lew W., Ross B., Wang Q., Wolfe L., Jordan R., Soloveva V., Knox J., Perry J., Perron M., Stray K. M., Barauskas O., Feng J. Y., Xu Y., Lee G., Rheingold A. L., Ray A. S., Bannister R., Strickley R., Swaminathan S., Lee W. A., Bavari S., Cihlar T., Lo M. K., Warren T. K., Mackman R. L., J. Med. Chem., 60, 1648–1661 (2017).

23. Dyson M. R., Coe P. L., Walker R. T., J. Med. Chem., 34, 2782–2786 (1991).

24. Van Draanen N. A., Freeman G. A., Short S. A., Harvey R., Jansen R., Szczek G., Koszalka G. W., J. Med. Chem., 39, 538–542 (1996).

25. Yoshimura Y., Kitano K., Yamada K., Satoh H., Watanabe M., Miura S., Sakata S., Sasaki T., Matsuda A., J. Org. Chem., 62, 3140–3152 (1997).

26. Bobek M., Whistler R. L., Bloch A., J. Med. Chem., 13, 411–413 (1970).

27. Minakawa N., Kaga D., Kato Y., Endo K., Tanaka M., Sasaki T., Matsuda A., J. Chem. Soc. Perkin 1, 2182–2189 (2002).
28. Wang W., Papov V. V., Minakawa N., Matsuda A., Biemann K., Hedstrom L., *Biochemistry*, **35**, 95–101 (1996).

29. Balzarini J., Stet L., Matsuda A., Wiebe L., Knauss E., De Clercq E., *Adv. Exp. Med. Biol.*, **431**, 723–728 (1998).

30. Nobori H., Toba S., Yoshida R., Hall W. W., Orba Y., Sawa H., Sato A., *Antiviral Res.*, **155**, 60–66 (2018).