Original Article

Pharmacologic action of oseltamivir on the nervous system

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ABSTRACT: Oseltamivir, an antiviral drug used for the treatment of influenza, contains the L-glutamic acid motif in its chemical structure. We focused on this structural characteristic of oseltamivir and examined the pharmacologic effects of the drug on the nervous system in invertebrate and vertebrate animal models. Injection of oseltamivir or L-glutamic acid into silkworm (*Bombyx mori*) larvae induced muscle relaxation. Oseltamivir and L-glutamic acid inhibited kainate-induced rapid muscle contraction, but neither drug affected insect cytokine paralytic peptide-induced slow muscle contraction. In the mammalian system, mice (*Mus musculus*) treated intracerebrally with oseltamivir developed convulsive seizures. Hydrolyzed oseltamivir, the active form containing a carboxylic acid, evoked epileptiform firing of hippocampal neurons in rat (*Rattus norvegicus*) organotypic hippocampal slice cultures. These results are the first to demonstrate that oseltamivir exerts pharmacologic effects on the nervous system in insects and mammals.

Keywords: Oseltamivir, Nervous system, L-Glutamic acid, Kainite, Pharmacologic effect

1. Introduction

Influenza is a highly transmissible viral disease. The recent emergence of fatal influenza virus types, especially H5N1-type, has raised concerns over a future global influenza pandemic (*1*). Oseltamivir phosphate (Tamiflu®; Figure 1, **1**) is the most widely used therapeutic agent for influenza (*2*). Oseltamivir exerts its activity through potent inhibition of neuraminidase localized on the surface of the influenza virus by mimicking the transition state of sialic acid hydrolysis (*3-5*). Because this process is essential for influenza transmission (*6-8*) oseltamivir is considered consistently effective against new types of influenza. To protect human beings from a future pandemic, the development of a novel synthetic route for **1** that enables efficient mass production has been studied intensively (*9-15*). Other than rare cases of asthma provocation in patients with hepatic dysfunction (*16*), oseltamivir is considered to be a safe drug without severe side effects (*17,18*). Recently in Japan, however, where 80% of the world supply of oseltamivir is consumed, it was reported that oseltamivir might cause abnormal behavior (such as hallucinations and impulsive behavior) in children under the age of 20. Comprehensive statistical investigations are now underway to clarify the relationship between the abnormal behavior and oseltamivir administration (*19*). To date, molecular-level or cellular-level studies that have addressed the pharmacologic effects of oseltamivir on the nervous system are limited. Izumi recently reported the pharmacologic effects of oseltamivir using juvenile rats and hippocampal slices of rats (*20*). In this paper, we describe the L-glutamic acid-like activities of oseltamivir in the nervous system of three animals (silkworms, mice, and rats).

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2. Materials and Methods

2.1. *Chemical synthesis of Tamiflu®*

Tamiflu® (Figure 1, **1**), Ro64-0802 (Figure 1, **2**), and its enantiomer (Figure 1, **4**) were synthesized by the previously reported method (*15*). For details, see Supplementary data.

2.2. *Silkworm larvae muscle contraction assay*

Silkworm eggs (*Bombyx mori*, Hu•Yo × Tukuba•Ne) were purchased from Ehime Sanshu (Ehime, Japan). Silkworm larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Kanagawa, Japan)

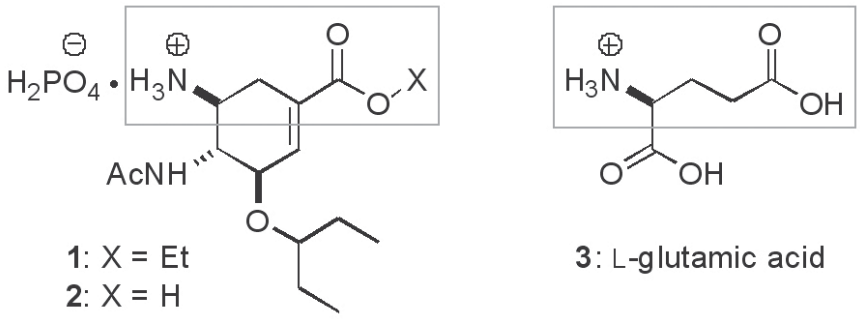

Figure 1. Structural analogy between oseltamivir and *L*-glutamic acid.

at 27°C. The measurement of muscle contraction activity using silkworms was described previously (*21*). Briefly, the heads of 5th instar silkworm larvae (3-4 g) were cut off and the peritrophic membranes removed. Each specimen was tied and attached to a transducer to measure isotonic contraction with a load of 27 g. Test samples were dissolved or suspended in phosphate-buffered saline, and injected into the body fluid of a specimen with a 1-mL syringe attached to a 27-gauge needle (Terumo, Tokyo, Japan). The intensity of the muscle contraction was expressed as the contraction value, calculated by measuring the maximum length of each specimen before (x cm) and after (y cm) the injection using the formula (x-y)/x. The muscle contraction stimulant kainate monohydrate was purchased from Wako (Osaka, Japan), and insect cytokine paralytic peptide (2.5 kDa) was synthesized using the solid-phase Fmock method (*22*). To test the effects of the inhibitors, 50 µL each of two samples (inhibitor and stimulant) were successively injected with an interval of 5 to 10 sec.

2.3. *Injection into cerebral ventricles of mice*

ICR mice (female, 6-week-old) were anesthetized with 300 µL of 1/10 diluted Nembutal (*i.p.*), and scalps were dissected to expose the cranial bones. After 3 to 5 h, 20 µL of the indicated samples were injected intracerebrally. Behaviors of mice were observed for at least 30 min after injection.

2.4. *Whole-cell patch-clamp recordings from rat hippocampal slices*

Organotypic cultures of hippocampal slices: Postnatal day 7 Sprague Dawley rats (SLC, Shizuoka, Japan) were deeply anesthetized by hypothermia, and their brains were aseptically removed (*23*), according to National Institutes of Health guidelines for laboratory animal care and safety. The posterior part of the brain was cut into 300-µm-thick transverse slices using a DTK-1500 vibratome (Dosaka, Japan) in aerated, ice-cold Gey's balanced salt solution supplemented with 25 mM glucose. The entorhino-hippocampi were dissected out under stereomicroscopic control and cultured using membrane interface techniques (*24,25*). Briefly, slices were placed on sterile 30-mm diameter membranes (Millicell-CM; Millipore, Bedford, MA, USA) and transferred into six-well tissue culture trays. Cultures were fed with 1 mL of 50% minimal essential medium (Invitrogen, Gaithersburg, MD, USA), 25% horse serum (Cell Culture Lab, Cleveland, OH, USA), and 25% Hank's Balanced Salt Solution and were maintained in a humidified incubator at 37°C in 5% CO_2. The medium was changed every 3.5 days.

Electrophysiologic recordings: Slices were placed in a recording chamber, perfused with artificial cerebrospinal fluid consisting of: 127 mM NaCl, 26 mM $NaHCO_3$, 1.6 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, and 10 mM glucose, bubbled with 95% O_2 and 5% CO_2 or Neurobasal (Gibco BRL, Gaithersberg, MD) at a rate of 2 to 3 mL/min. Whole-cell patch-clamp recordings were performed with glass pipettes (6-8 MΩ) filled with intracellular solution containing 120 mM K-gluconate, 20 mM KCl, 10 HEPES, 0.1 mM $CaCl_2$, 4 mM Mg-ATP, and 0.2 mM EGTA (pH 7.4, 280-300 mOsm). Recordings were performed with Axopatch 200B amplifiers (Molecular Devices, Union City, CA, USA). CA1 pyramidal cells were identified using an Olympus BX50WI microscope (Tokyo, Japan) and a 40Å~ objective under phase contrast control with a Cascade cooled CCD camera (Roper Scientific, Tucson, AZ, USA). Pipette seal resistances were typically >1 GΩ, and pipette capacitive transients were minimized prior to breakthrough. Postsynaptic currents were studied in voltage-clamp mode at -30 mV holding potential. Signals were low-pass filtered at 1 kHz, digitized at 10 kHz, and analyzed with pCLAMP 8.0 software (Molecular Devices). Drugs were applied locally via a puffer pipette placed near (20~50 µm) the patched neuron (50-70 hPa).

3. Results and Discussion

Oseltamivir (**1**) is a prodrug, and its biologically active form is the corresponding carboxylic acid **2** (Ro64-0802), which is produced through the hydrolysis of **1** by plasma and liver esterase (*26,27*).

The identification of an L-glutamic acid motif in the chemical structure of **2** (Figure 1) led us to hypothesize that oseltamivir has L-glutamic acid-like biologic activity. L-Glutamic acid is a major excitatory neurotransmitter in the vertebrate nervous system. Agonists of glutamic acid receptors induce hallucinative behaviors (*28*) and epileptic seizures (*29,30*) in experimental animals.

To examine our hypothesis experimentally, we first examined the pharmacologic effect of oseltamivir in an invertebrate model. Some of the authors of the present study previously proposed that silkworms (*Bombyx mori*) could serve as a useful invertebrate model for drug screening and evaluation, as the effects and pharmacologic kinetics in silkworms are generally comparable to those in mammalian models (*31-34*). In our previous work, we reported the biologic activity of L-glutamic acid in silkworms; kainate, a putative neurotransmitter receptor agonist in silkworm, induces muscle contraction in silkworm larvae, which is inhibited by L-glutamic acid, and L-glutamic acid itself causes muscle relaxation (*21*). Oseltamivir (2.5 mg) injected into the body fluid of silkworm larvae induces paralysis accompanied by muscle relaxation. This response resembles the effect of L-glutamic acid (ED_{50} = 15 μg). In addition, prior injection of 5 mg oseltamivir into silkworm larvae significantly inhibits kainate (2 μg)-induced muscle contraction (Supplementary data). On the other hand, another muscle contraction pathway independent of nervous system excitation (thus, not affected by L-glutamic acid), *i.e.*, the insect cytokine paralytic peptide pathway (*35*) is not affected by oseltamivir (Supplementary data). These results support our hypothesis that oseltamivir has a specific effect, similar to L-glutamic acid, on the nervous system.

Next we investigated the pharmacologic effect of oseltamivir on the mammalian central nervous system. Injection of 0.3 mg L-glutamic acid into the lateral ventricle of mice (*Mus musculus*) induced acute epileptiform convulsions, which lasted from several to 30 min (Figure 2). Intracerebral injection of more than 0.5 mg of oseltamivir caused intense convulsions, similar to those induced by L-glutamic acid (Figure 2). Phosphate-buffered saline, the solvent for both drugs, did not induce seizures in mice (Figure 2). Thus, oseltamivir acts as a convulsant agent in the central nervous system. We also performed whole-cell patch-clamp recordings from CA1 pyramidal cells in rat (*Rattus norvegicus*) cultured organotypic hippocampal slices to evaluate the electrophysiologic responses to the active form **2** (Figure 3). Results varied from neuron to neuron, and three types of responses were observed; i) generation of burst-like action potentials (n = 6), ii) subthreshold depolarization of membrane potentials (n = 4), and iii) no electrical response (n = 3), whereas L-glutamic acid (1 mM) induced burst-like action potentials in all neurons tested. The heterogeneous reactions induced by **2** are likely due to the diversity of neurons in the various parts of neural microcircuits. In tissue section cultures, the observed reactions could be induced not only by application of the drug to a single cell, but also by the complex activity of a large number of surrounding cells. Recently, the importance of experimental systems reflecting complex effects

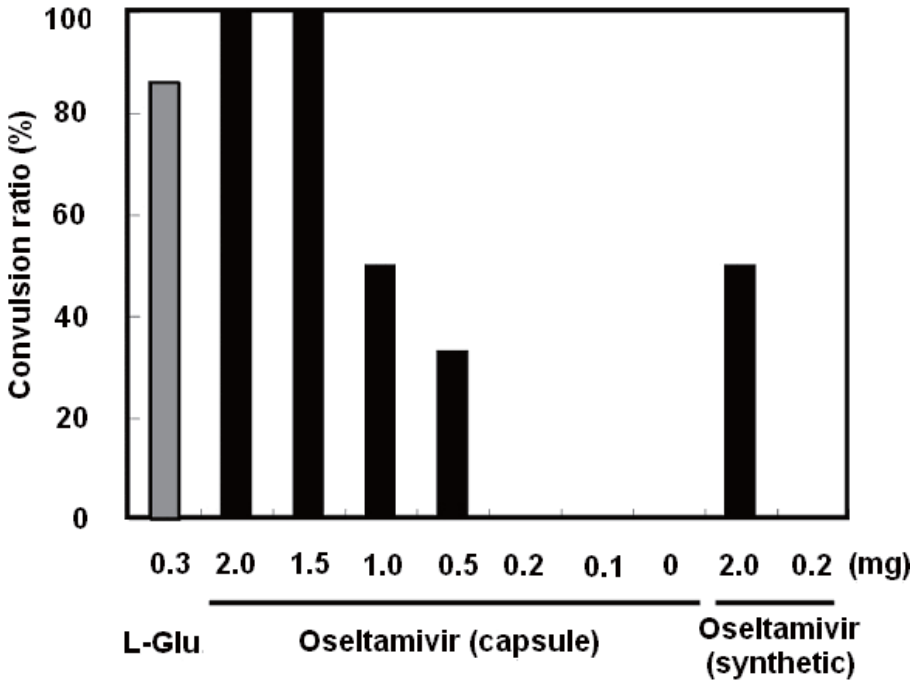

Figure 2. Convulsion induced by intracerebral injection of L-glutamic acid (L-Glu), oseltamivir (capsule), or synthetic oseltamivir in mice. Synthetic oseltamivir was administered at a dose of 0.2 mg (n = 5) and 2.0 mg (n = 6). Oseltamivir (capsule) was administered at a dose of 0.1 to 1.5 mg (n = 6), 2.0 mg (n = 8), and 0 mg (n = 7). Seven mice were injected with 0.3 mg L-glutamic acid.

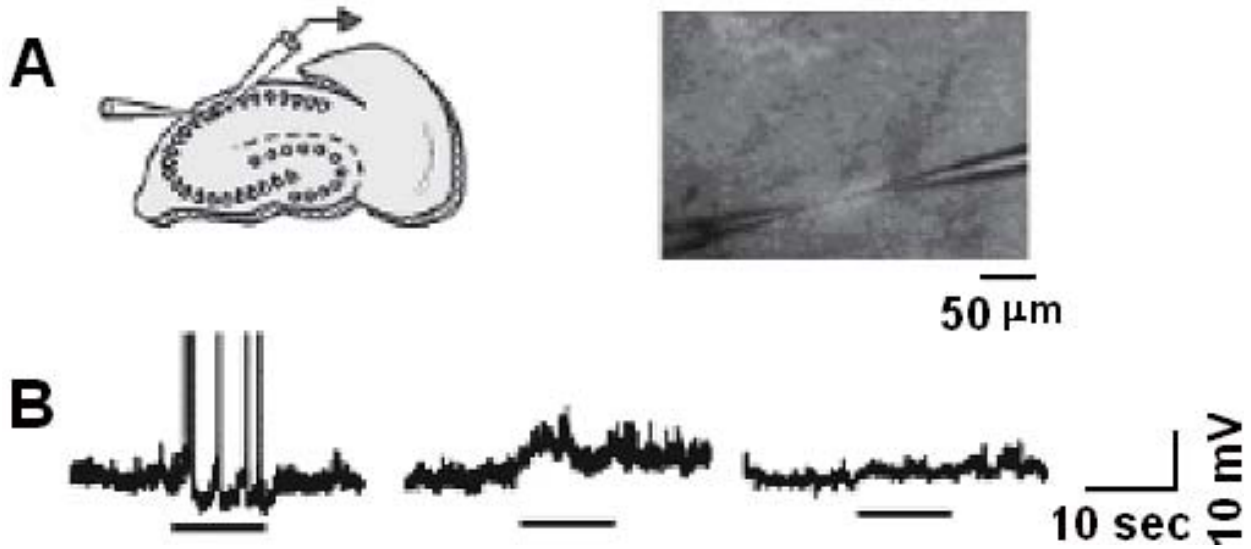

Figure 3. Excitatory effect of acid-form oseltamivir (**2**) on neurons in rat hippocampal slice cultures. A) Illustration and photograph of experimental procedures. Electrophysiologic responses were recorded from CA1 pyramidal cells through the right electrode in the current-clamp mode. Drugs were locally applied from the left electrode. B) Voltage responses to 1 mM acid form-oseltamivir (**2**). Underlines indicate periods of drug treatment. Representatives of three types of responses are shown.

in cell clusters for examination of the pharmacologic effects and toxicity of neuroactive substances has been emphasized (*36*). To identify the cells and receptors that interact with oseltamivir, studies using large-scale systems linked with the whole neuron networks are necessary. Importantly, the enantiomer of **2** (for the synthetic route, see Supplementary data) (1 mM) did not affect membrane potentials of hippocampal neurons (n = 5). This finding clearly indicated that the electrophysiologic effects of oseltamivir on neurons were stereospecific, and thus induced by specific interactions with biologic molecules.

The results of the above three types of experiments all demonstrated that oseltamivir induces specific responses in the nervous system identical to those induced by L-glutamic acid. Mammalian glutamate receptors are highly expressed in cortical and hippocampal neurons, and are classified into three subtypes; *N*-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)/kainate receptors, and metabotropic receptors. Agonists of these receptors excite neurons and induce seizures in mammals (*37*). Based on the results described here, oseltamivir may act on these receptors, thereby causing membrane depolarization in excitatory neurons and systemic seizures. In conclusion, these findings are the first to demonstrate that oseltamivir exerts L-glutamic acid-like effects in experimental systems reflecting higher biologic activities, *i.e.*, muscle contraction assay in silkworm larvae, seizure assay in mice, and electrophysiologic *ex vivo* membrane potential recordings. The C1 carboxylate, C4 acetamide, C5 amino moieties, and the position of the double bond of oseltamivir are important for the interaction between oseltamivir and viral neuraminidase (*3*). Modifying other parts of the molecule might eliminate the L-glutamic acid-like effects of oseltamivir without inducing a loss of its affinity to neuraminidase. Detailed studies toward mechanistic elucidation of oseltamivir's pharmacologic effects on the nervous system (especially, glutamate receptor subtype selectivity) and its chemical structure modification are ongoing in our group.

Acknowledgements

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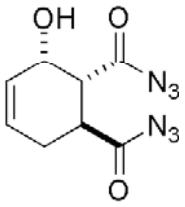
Supplementary Data

Synthesis of Tamiflu®

Overall Scheme

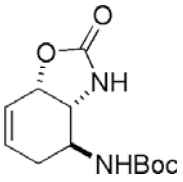
Tamiflu® (**1**), Ro64-0802 (**2**), and its enantiomer **4** were synthesized by the previously reported method (*1*).

(1*S,2*R**,3*S**)-3-Hydroxy-cyclohex-4-ene-1,2-dicarbonyl diazide (S-1):**

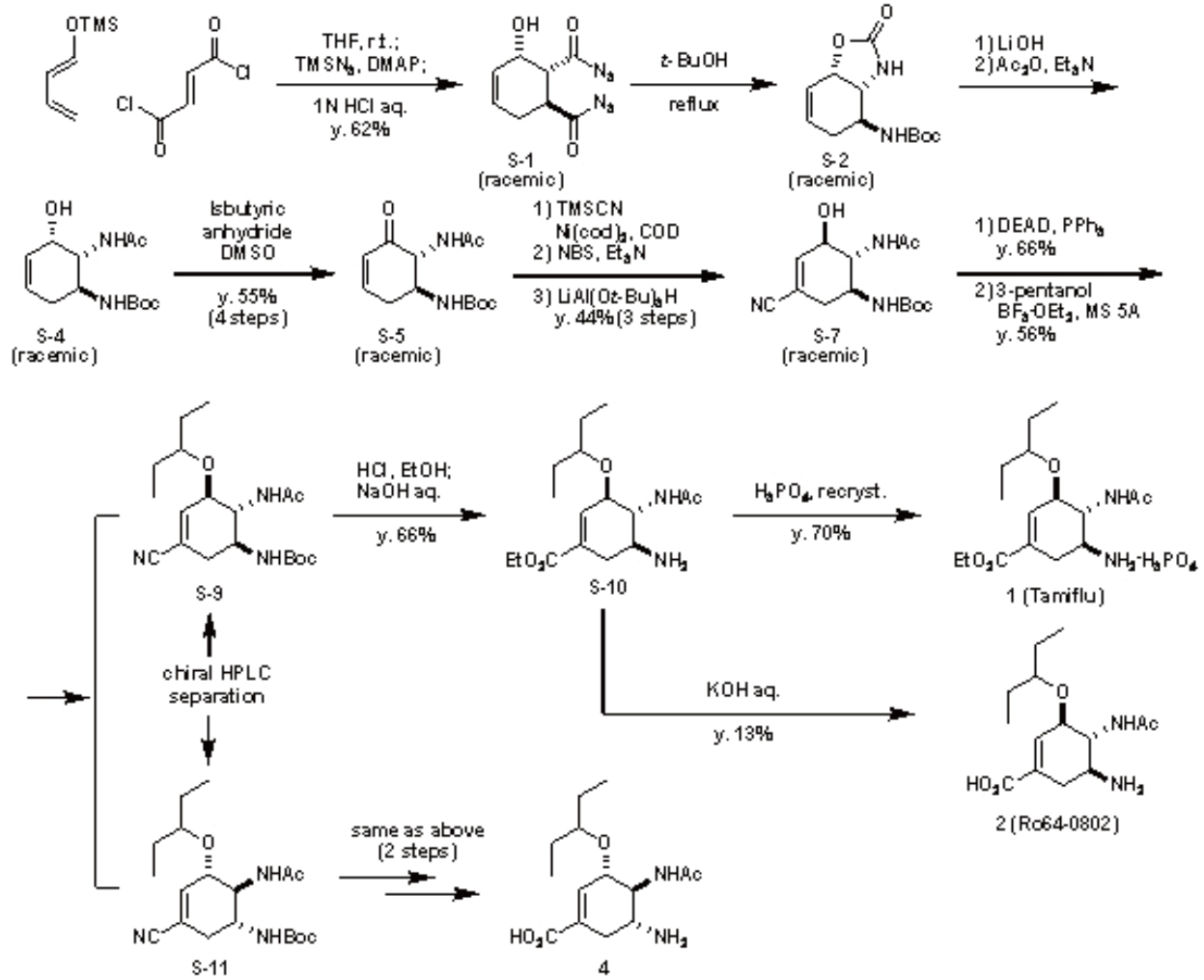


Fumaryl chloride (37.7 g, 247 mmol) was added slowly to a stirred solution of 1-(trimethylsilyloxy)-1,3-butadiene (40 g, 281 mmol) in THF (1,240 mL) at room temperature, and the mixture was stirred for 2 h. $TMSN_3$ (68.7 mL, 519 mmol) and DMAP (3 g, 24.7 mmol) were carefully added at room temperature, and the mixture was stirred for 2 h. After cooling to 4°C, 1 N HCl aq. (25.0 mL, 25.0 mmol) was carefully added, and the mixture was stirred at the same temperature for 10 min. The organic layer was separated and the aqueous layer was extracted twice with AcOEt (1,500 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution (500 mL) and brine (500 mL), dried over Na_2SO_4, and concentrated to give crude **S-1**, which was purified by silica gel column chromatography (neutral SiO_2 1,000 g, hexane /AcOEt = 4/1 to 2/1) to give **S-1** (36.0 g, 152.4 mmol; 62% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.92-5.86 (m, 2H), 4.48 (m, 1H), 2.96 (ddd, *J* = 5.3, 11.6, 12.0 Hz, 1H), 2.87 (dd, *J* = 4.0, 12.0 Hz, 1H), 2.49 (ddd, *J* = 5.2, 5.3, 17.7 Hz, 1H), 2.10-2.03 (m, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 181.9, 179.3, 128.7, 127.0, 63.8, 49.6, 37.9, 28.7; IR (neat, cm^{-1}) 3412, 2260, 2146, 1710; FAB-HRMS Calcd for $C_8H_9N_6O_3$ $[M+H]^+$: 237.0731, Found: 237.0726.

(2-Oxo-2,3,3aβ,4α,5,7aβ-hexahydro-benzoxazol-4-yl)-carbamic acid *tert*-butyl ester (S-2):



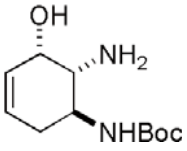
The solution of **S-1** (36.0 g, 152.4 mmol) in distilled *t*-BuOH (305 mL) was stirred at refluxing temperature for 15 h. Removal of the solvent under reduced pressure gave crude



Scheme 1. Overall synthetic scheme.

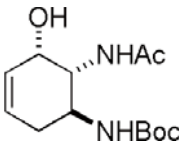
S-2 (38.8 g, 152.4 mmol) as a white solid, which was used for the next reaction without further purification. ^{1}H NMR (CD_3OD, 500 MHz) δ 6.10 (m, 1H), 5.88-5.86 (m, 1H), 5.04-5.03 (m, 1H), 3.77-3.73 (m, 1H), 3.55-3.50 (m, 1H), 2.37 (ddd, *J* = 5.2, 5.5, 17.2 Hz, 1H), 2.01-1.96 (m, 1H), 1.44 (s, 9H); ^{13}C NMR (CD_3OD, 125 MHz) δ 161.4, 158.1, 132.7, 123.7, 80.5, 75.3, 56.4, 51.0, 29.8, 28.7; IR (KBr, cm^{-1}) 3370, 3243, 1747, 1683; ESI-MS *m/z* 277 $[M + Na]^+$; FAB-HRMS Calcd for $C_{12}H_{19}N_2O_4$ $[M+H]^+$: 255.1339, Found: 255.1332.

(1*S,5*S**,6*R**)-(6-Amino-5-hydroxy-cyclohex-3-enyl)-carbamic acid *tert*-butyl ester (S-3):**



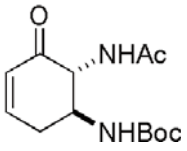
$LiOH•H_2O$ (32 g, 763 mmol) was added to a stirred solution of **S-2** (38.8 g, 152.4 mmol) in MeOH/H_2O (1/1, 1,530 mL) at room temperature, and the mixture was stirred at 65°C for 16 h. After evaporation of MeOH, $CHCl_3$/MeOH = 9/1 solution (500 mL) and brine (300 mL) were added. The aqueous layer was extracted with $CHCl_3$/MeOH = 9/1 solution (1,500 mL). The combined organic layers were dried over Na_2SO_4, and concentrated to give crude **S-3** (31.2 g, 136.7 mmol) as a pale brown solid, which was used for the next reaction without further purification. ^{1}H NMR (CD_3OD, 500 MHz) δ 5.77 (m, 2H), 4.11 (m, 1H), 3.66 (m, 1H), 2.61 (dd, *J* = 11.0, 11.0, 1H), 2.62-2.40 (m, 1H), 1.97-1.90 (m, 1H), 1.45 (s, 9H); ^{13}C NMR (CD_3OD, 125 MHz) δ 158.6, 129.6, 129.0, 80.1, 67.8, 67.8, 56.0, 33.7, 28.8; IR (KBr, cm^{-1}) 3301, 1675; ESI-MS *m/z* 229 $[M + H]^+$; FAB-HRMS Calcd for $C_{11}H_{21}N_2O_3$ $[M+H]^+$: 229.1547, Found: 2291550.

(1*S,5*S**,6*R**)-(6-Acetylamino-5-hydroxy-cyclohex-3-enyl)-carbamic acid *tert*-butyl ester (S-4):**



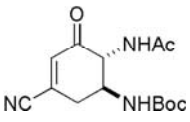
Et_3N (37.9 mL, 273.4 mmol) and Ac_2O (12.9 mL, 136.7 mmol) were slowly added to a stirred solution of **S-3** (31.2 g, 136.7 mmol) in THF (1,370 mL) at 4°C. The mixture was stirred at the same temperature for 15 min. The reaction was diluted with AcOEt (300 mL) and quenched with saturated NH_4Cl solution (150 mL). The organic layer was separated, and the aqueous layer was extracted with AcOEt (1,200 mL). The combined organic layers were washed with brine (300 mL), dried over Na_2SO_4, and concentrated to give crude **S-4** (37.0 g, 136.7 mmol) as a pale brown solid, which was used for the next reaction without further purification. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.89 (d, *J* = 8.3 Hz, 1H), 5.81-5.74 (m, 2H), 5.14 (d, *J* = 8.6 Hz, 1H), 4.08 (m, 1H), 4.08-3.90 (m, 2H), 2.48 (ddd, *J* = 4.7, 4.7, 17.7 Hz, 1H), 2.02-1.96 (m, 4H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.2, 156.8, 129.1, 127.4, 79.5, 66.6, 54.1, 46.2, 33.0, 28.3, 23.1; IR (KBr, cm^{-1}) 3364, 3290, 1686, 1663; ESI-MS m/z 293 $[M + Na]^+$; FAB-HRMS Calcd for $C_{13}H_{23}N_2O_4$ $[M+H]^+$: 271.1652, Found: 271.1657.

(1*S,6*R**)-6-Acetylamino-5-oxo-cyclohex-3-enyl)-carbamic acid *tert*-butyl ester (S-5):**



Dimethylsulfoxide (117 mL, 1640 mmol) was added to a stirred solution of **S-4** (37.0 g, 136.7 mmol) in AcOEt (1,370 mL) at room temperature. The reaction temperature was raised to refluxing temperature, and isobutyric anhydride (64 mL, 386.0 mmol) was added. The mixture was stirred at the same temperature for 25 h. The reaction was quenched with saturated $NaHCO_3$ solution (150 mL), the organic layer was separated, and the aqueous layer was extracted with AcOEt (1,000 mL). The combined organic layers were washed with brine (300 mL), dried over Na_2SO_4, and concentrated to give crude **S-5**, which was purified by recrystallization from hexane/2-propanol to afford **S-5** (17.0 g, 63.2 mmol). The filtrate was evaporated and purified by silica gel column chromatography (neutral SiO_2 450 g, hexane/AcOEt = 1/4 to AcOEt) to afford **S-5** (5.39 g, 20.1 mmol) as a white crystal. Both were combined and used for the next reaction (22.4 g, 83.3 mmol; 55% yield for 4 steps). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.96-6.93 (m, 1H), 6.37 (d, *J* = 6.6 Hz, 1H), 6.11 (dd, *J* = 2.9, 9.9 Hz, 1H), 5.70 (d, *J* = 7.6 Hz, 1H), 4.58 (dd, *J* = 6.6, 13.0 Hz, 1H), 3.94-3.86 (m, 1H), 2.92 (ddd, *J* = 5.5, 5.5, 18.9 Hz, 1H), 2.45-2.39 (m, 1H), 2.06 (s, 3H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 194.8, 172.3, 155,8, 148.6, 128.5, 79.7, 59.7, 53.5, 34.1, 28.3, 23.1; IR (KBr, cm^{-1}) 3329, 3283, 1689, 1664, 1626; ESI-MS *m/z* 291 $[M + Na]^+$; FAB-HRMS Calcd for $C_{13}H_{21}N_2O_4$ $[M+H]^+$: 269.1496, Found: 269.1493.

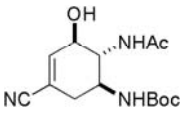
(1*S,6*R**)-6-Acetylamino-3-cyano-5-oxo-cyclohex-3-enyl)-carbamic acid *tert*-butyl ester (S-6):**



THF solution of **S-5** (0.17 M, 2.21 mL, 0.37 mmol), THF solution of 1,5-cyclooctadiene (0.40 M, 0.489 mL, 0.19 mmol), and distilled TMSCN (150 μL, 1.12 mmol) were added to a THF (1.98 mL) solution of $Ni(cod)_2$ (51.3 mg, 0.19 mmol) in a flame-dried flask at room temperature with stirring. The mixture was stirred at 65°C for 1 h. After the starting material was consumed, the mixture was filtered through a celite pad, and the filtrate was evaporated. The residue was dissolved in THF (3.73 mL), and cooled to 4°C. *N*-bromosuccinimide (69.7 mg, 0.39 mmol) was added to this solution, and the mixture was stirred at the same temperature for 1 h. The reaction temperature was raised to room temperature, and Et_3N (103 μL, 0.74 mmol) was added. The reaction

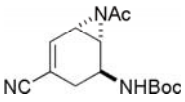
temperature was raised to refluxing temperature and stirred for 12 h. After cooling to room temperature, the mixture was diluted with AcOEt (5 mL), and quenched with 5% NaH_2PO_4 aqueous solution (2 mL). The organic layer was separated, and aqueous layer was extracted with AcOEt (15 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to give crude **S-6**, which was passed through a silica gel (neutral SiO_2 5 g, hexane/AcOEt = 1/1 to 1/2 to 1/6) to afford semi-purified **S-6** (73 mg). It was used for the next reaction without further purification.

(1*S,5*R**,6*R**)-(6-Acetylamino-3-cyano-5-hydroxy-cyclohex-3-enyl)-carbamic acid *tert*-butyl ester (S-7):**



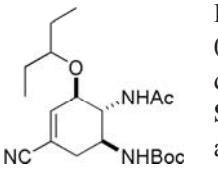
$LiAl(Ot\text{-}Bu)_3H$ (1.0 M in THF, 448 µL, 0.45 mmol) was added to a stirred solution of semi-purified **S-6** (66 mg) in THF (1.12 mL) at 4°C. The mixture was stirred at the same temperature for 45 min. The reaction was quenched with saturated NH_4Cl solution (1 mL), the organic layer was separated, and the aqueous layer was extracted with AcOEt (10 mL). The combined organic layers were washed with brine (5 mL), dried over Na_2SO_4, and concentrated to give crude **S-7**, which was purified by silica gel column chromatography (neutral SiO_2 3 g, AcOEt/MeOH = 100/1 to 50/1) to afford **S-7** (48 mg, 0.16 mmol; 44% yield for 3 steps) as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.12 (d, *J* = 5.2 Hz, 1H), 6.49 (s, 1H), 5.01 (d, *J* = 2.3 Hz, 1H), 4.82 (d, *J* = 8.0 Hz, 1H), 4.28-4.21 (m, 1H), 3.92-3.81 (m, 1H), 3.78-3.70 (m, 1H), 2.63 (dd, *J* = 16.9, 5.0 Hz, 1H), 2.32-2.22 (m, 1H), 2.00 (s, 3H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 173.6, 157.3, 145.3, 117.2, 109.8, 81.3, 73.1, 59.5, 47.3, 32.7, 28.2, 23.0; IR (KBr, cm^{-1}) 3322, 2979, 2225, 1683, 1625, 1571; ESI-MS: *m/z* 318 $[M+Na]^+$; FAB-HRMS: *m/z* calcd for $C_{14}H_{22}N_3O_4$ $[M+H]^+$: 296.1605, Found: 296.1602.

(1*R,2*S**,6*S**)-(7-Acetyl-4-cyano-7-aza-bicyclo[4.1.0]hept-4-en-2-yl)-carbamic acid *tert*-butyl ester (S-8):**



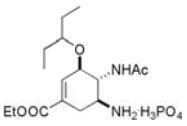
Diethyl azodicarboxylate (40% in toluene, 0.72 mL, 1.58 mmol) was added to a solution of triphenylphosphine (414 mg, 1.58 mmol) in THF (7.9 mL) at -20°C. Then **S-7** (234 mg, 0.79 mmol) in THF (2.5 mL) was added dropwise to the reaction mixture over 15 min at the same temperature. After stirring for 1 h, the reaction mixture was concentrated *in vacuo*. The residue was purified by flash column chromatography twice (first [removal of triphenylphosphine oxide]; hexane/AcOEt = 1/4 to AcOEt, and then AcOEt/MeOH = 20/1, second [removal of the residue derived from DEAD]; Et_2O/hexane = 3/1 to 5/1) to afford **S-8** (144 mg, 0.521 mmol; 66% yield) as an amorphous substance. ^{1}H NMR ($CDCl_3$) δ 6.88 (dd, *J* = 3.5, 3.7 Hz, 1H), 4.54 (brs, 2H), 3.12-3.04 (m, 2H), 2.53 (brd, *J* = 17.0 Hz, 1H), 2.35 (brd, *J* = 17.0, 1H), 2.13 (s, 3H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 180.8, 154.8, 140.0, 118.1, 112.3, 80.5, 41.3, 40.5, 31.4, 30.0, 28.3, 23.1; IR (neat, cm^{-1}) 3322, 2978, 2218, 1703, 1525; ESI-MS: *m/z* 300 $[M+Na]^+$; FAB-HRMS: *m/z* calcd for $C_{14}H_{20}N_3O_3$ $[M+H]^+$: 278.1499, Found: 278.1500.

(1*S*,5*R*,6*R*)-[6-Acetylamino-3-cyano-5-(1-ethyl-propoxy)-cyclohex-3-enyl]-carbamic acid *tert*-butyl ester (S-9):

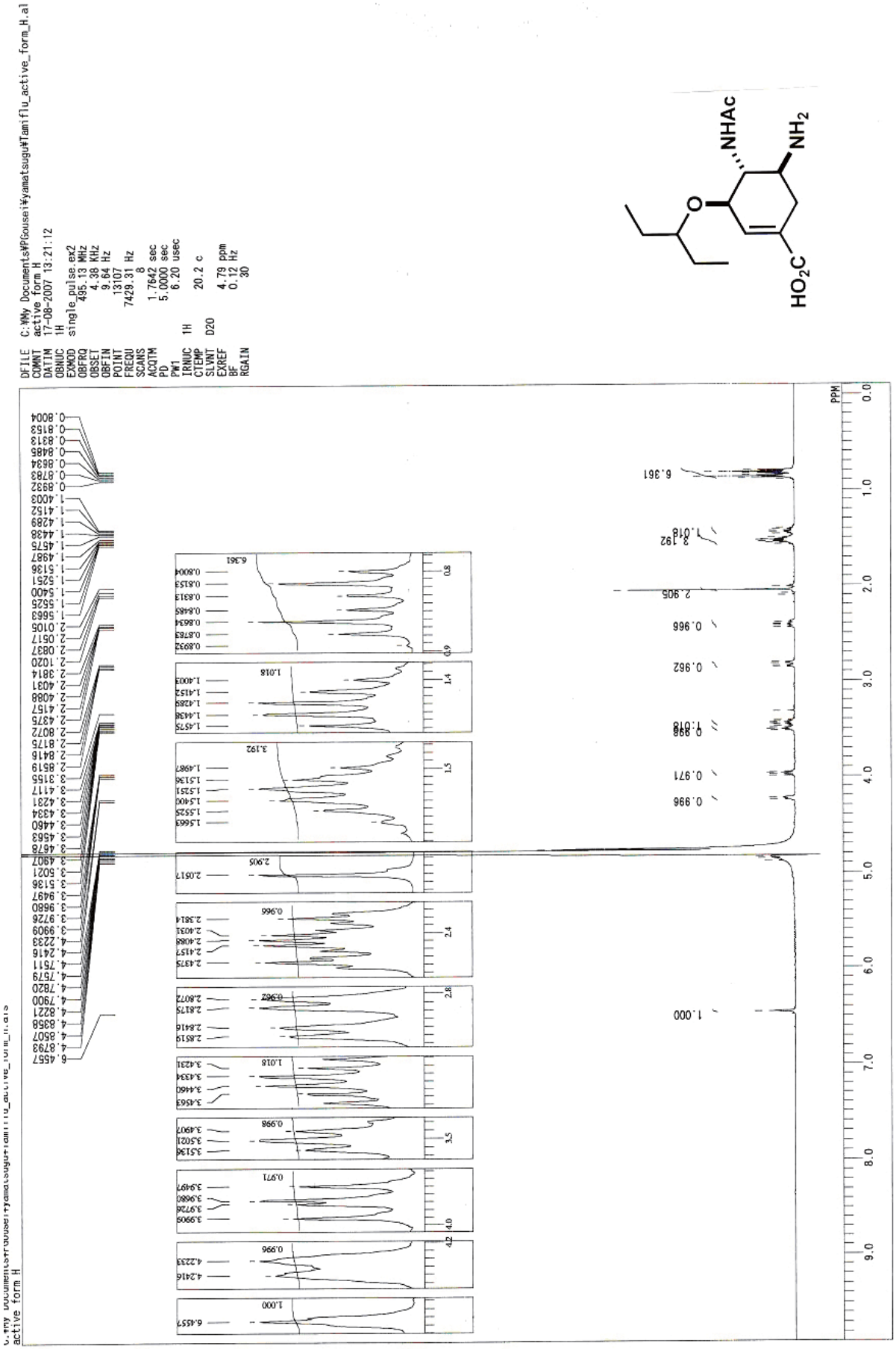


BF_3•OEt_2 (1 M in 3-pentanol, 0.65 mL, 0.645 mmol) was added dropwise over 15 min to a slurry of **S-8** (119.3 mg, 0.430 mmol) and activated molecular sieves 5A (430 mg) in 3-pentanol (4.3 mL) at -20°C. After stirring for 1 h, water (10 mL) was added at -20°C. The aqueous layer was extracted with AcOEt (15 mL) twice. The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4, and concentrated to give crude **S-9**, which was purified by flash column chromatography (hexane /AcOEt = 2/1 to 3/2 to 1/1) to afford **S-9** (88.1 mg, 0.241 mmol; 56% yield) as a white solid. Enantiomers were separated by chiral HPLC [Daicel Chiralpak AD-H, 2-propanol/hexane 1/20, flow 0.7 mL/min, detection at 220 nm; t_R 13.6 min (*ent*-**S-9**, $[\alpha]^{27}_D$ +84.2 (*c* 0.180, $CHCl_3$)) and 19.4 min (**S-9**, $[\alpha]^{27}_D$ -109.3 (*c* 0.140, $CHCl_3$))]. ^{1}H NMR ($CDCl_3$) δ 6.47 (s, 1H), 5.66 (brd, *J* = 8.6 Hz, 1H), 5.12, (brd, *J* = 8.6 Hz, 1H), 4.09-4.01 (m, 1H), 3.95-3.90 (m, 1H), 3.87-3.78 (m, 1H), 3.29 (quintet, *J* = 5.8 Hz, 1H), 2.60 (dd, *J* = 5.5, 18.1 Hz, 1H), 2.37-2.29 (m, 1H), 1.97 (s, 3H), 1.47 (quintet, *J* = 7.5 Hz, 4H), 1.40 (s, 9H), 0.87 (t, *J* = 7.5 Hz, 3H), 0.86 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 170.9, 156.1, 143.7, 117.5, 111.6, 82.6, 80.0, 75.0, 53.6, 48.3, 32.6, 28.3, 26.0, 25.6, 23.2, 9.4, 9.1; IR (KBr, cm^{-1}): 3335, 3287, 2968, 2220, 1686, 1654, 1541; ESI-MS: *m/z* 388 $[M+Na]^+$; FAB-HRMS: *m/z* calcd for $C_{19}H_{32}N_3O_4$ $[M+H]^+$: 366.2393, Found: 366.2401.

Ethyl (3*R*,4*R*,5*S*)-4-Acetamide-5-amino-3-(1-ethylpropoxy)cyclohexene-1-carboxylate phosphate (1) (Tamiflu®):



The solution of **S-9** (29.8 mg, 0.082 mmol) in 4.2 M HCl/EtOH (3.3 mL) was stirred at room temperature for 24 h. After cooling to 4°C, water (3.3 mL) was added to decompose the imino ester, and the mixture was stirred for 7 h. After addition of CH_2Cl_2 (6.6 mL), the mixture was stirred for a couple of minutes and the CH_2Cl_2 layer was removed. The aqueous layer was basified by 2 M NaOH aqueous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HO2C
O
PPM
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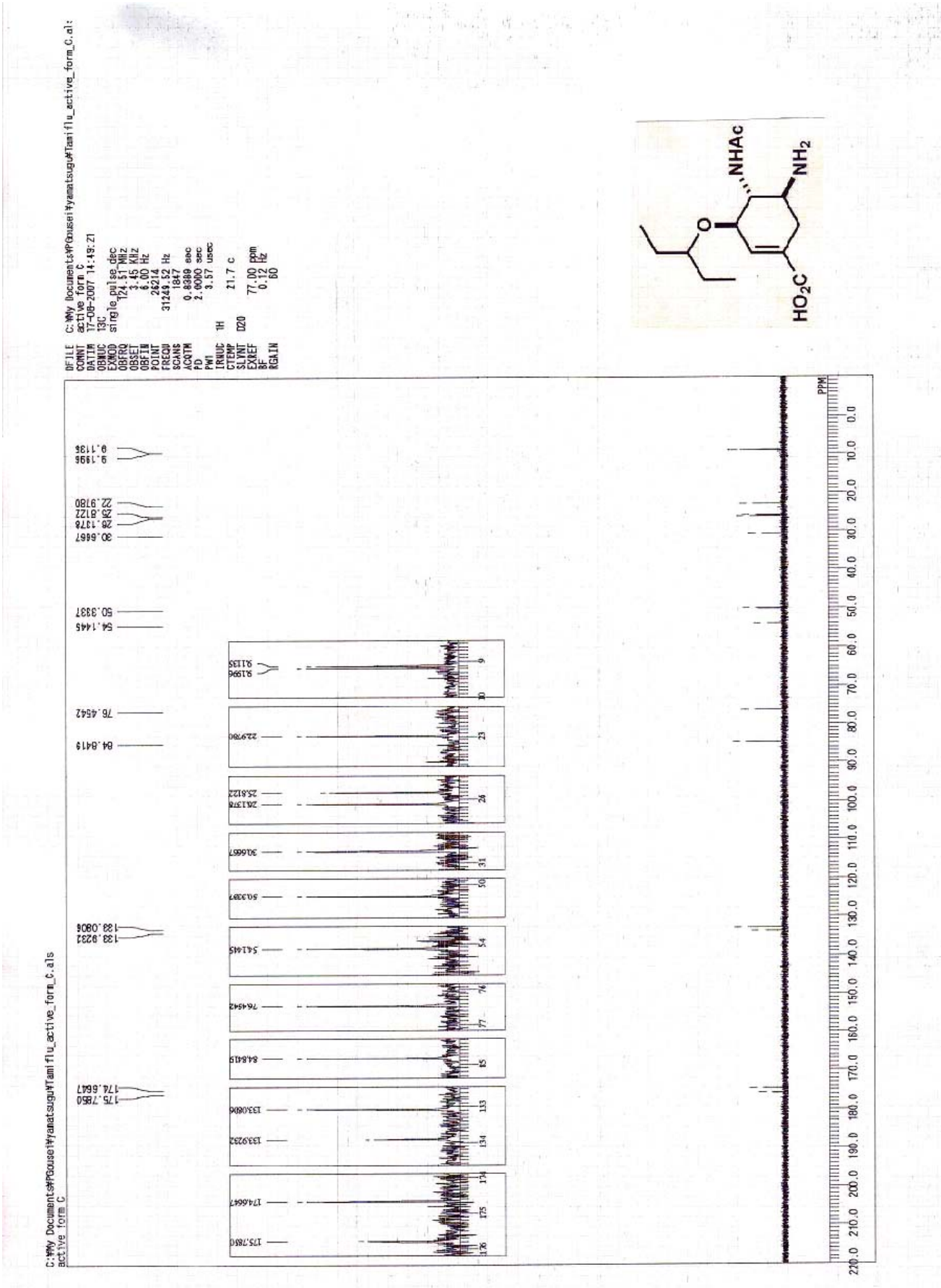
1.018
0.966
0.962
0.998
0.971
0.996
1.000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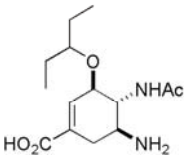
SLVNT D2O
EXREF 77.00 ppm
BF 0.12 Hz
RGAIN 60
NHAc
NH2
HO2C
PPM

solution to pH = 10, extracted with CH_2Cl_2, washed with brine, dried over Na_2SO_4, and concentrated to give the free base **S-10** (16.9 mg, 0.054 mmol; 66% yield). The free base (**S-10**, 74.6 mg, 0.239 mmol) in EtOH (1.44 mL) was slowly added to a solution of H_3PO_4 (85%, 33.1 mg, 0.287 mmol) in EtOH (2.41 mL) at 60°C. Crystallization commenced after a few minutes, and the suspension was cooled to 0°C. The crystal was collected and washed with acetone twice to afford Tamiflu® (**1**) (68.6 mg, 0.167 mmol; 70% yield) as a colorless crystal. ^{1}H NMR (D_2O) δ 6.91 (s, 1H), 4.39 (brd, *J* = 7.4 Hz, 1H), 4.34-4.26 (m, 2H), 4.11 (dd, *J* = 8.9, 11.6 Hz, 1H), 3.69-3.56 (m, 2H), 3.02 (dd, *J* = 5.1, 17.2 Hz, 1H), 2.62-2.53 (m, 1H), 2.14 (s, 3H), 1.64-1.46 (m, 4H), 1.35 (t, *J* = 7.2 Hz, 3H), 0.94 (t, *J* = 7.3 Hz, 3H), 0.90 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (D_2O) δ 178.1, 170.3, 140.7, 130.5, 87.2, 77.9, 65.3, 55.5, 52.0, 31.0, 28.3, 27.9, 25.2, 16.1, 11.4, 11.3; ^{31}P NMR (D_2O) δ 2.85; IR (KBr, cm^{-1}): 3195, 1718, 1661, 1551, 1246, 1127, 513; mp: 184-186°C; ESI-MS: *m/z* 313 $[M-H_3PO_4+H]^+$; FAB-HRMS: *m/z* calcd for $C_{16}H_{29}N_2O_4$ $[M-H_3PO_4+H]^+$: 313.2127. Found: 313.2124. $[\alpha]^{22}_D$ -30.5 (*c* 0.480, H_2O).

This analytical data completely matched with the reported one (*2*).

(3*R*,4*R*,5*S*)-4-Acetylamino-5-amino-3-(1-ethyl-propoxy)-cyclohex-1-enecarboxylic acid (2) (Ro64-0802):



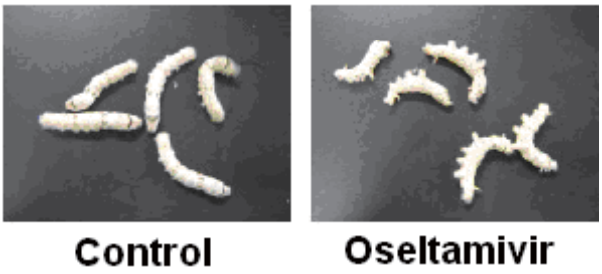
Aqueous KOH (1M; 0.320 mL, 0.320 mmol) was added to a THF (1.60 mL) solution of free-base **S-10** (50.0 mg, 0.160 mmol) at room temperature. The resulting solution was stirred at the same temperature for 1 h. The reaction mixture was acidified to pH = 4 with Amberlyst 15-DRY®, and filtered. The filtrate was evaporated, to afford the crude **2**, which was purified by reverse-phase PTLC (H_2O/CH_3CN = 20/1) to afford **2** (5.8 mg, 0.0204 mmol; 13% yield). ^{1}H NMR (D_2O) δ 6.46 (m, 1H), 4.24-4.22 (m, 1H), 3.97 (dd, *J* = 11.5, 9.2 Hz, 1 H), 3.51-3.41 (m, 2H), 2.83 (dd, *J* = 17.2, 5.2 Hz, 1H), 2.44-2.38 (m, 1H), 2.05 (s, 3H), 1.57-1.40 (m, 4H), 0.86 (t, *J* = 7.5 Hz, 3 H), 0.82 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (D_2O) δ 175.0, 174.7, 133.9, 133.1, 84.8, 76.5, 54.1, 50.3, 30.7, 26.1, 25.8, 23.0, 9.20, 9.11. IR (KBr, cm^{-1}): 3434, 1656, 1560; ESI-MS: *m/z* 307 $[M+Na]^+$.

This analytical data completely matched with the reported one (*3*).

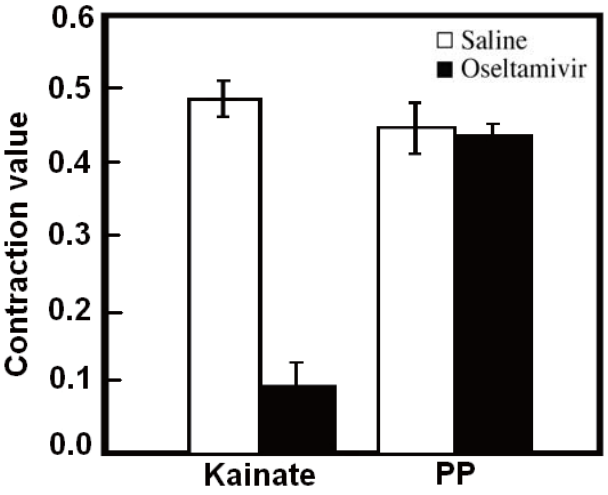
The enantiomer **4** [(3*S*,4*S*,5*R*)-4-Acetylamino-5-amino-3-(1-ethyl-propoxy)-cyclohex -1-enecarboxylic acid] was synthesized in the same way as **2**.

Paralytic effect of oseltamivir on silkworm larvae

The paralysis and muscle relaxation of 5th instar silkworm larvae induced immediately after injection of 0.9% NaCl or 2.5 mg oseltamivir (Tamiflu®, Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) are shown.



Inhibitory effect of oseltamivir on kainate-induced muscle contraction of silkworm larval specimen



Vehicle (50 μL, 0.9% NaCl) or oseltamivir (50 μL, 100 mg/mL capsule) was injected into a silkworm larval muscle specimen, and successively, 50 μL of kainate (40 μg/mL) or paralytic peptide (4 μg/mL) was injected. Contraction values were calculated as described above. Values represent the mean ± S.D., *n* = 3.

References

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2. Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver WG, Stevens RC. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J Am Chem Soc 1997; 119:681-690.
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