Tetrahedron 71 (2015) 266–270

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

A short synthetic pathway via three-component coupling reaction to tamiphosphor possessing anti-influenza activity

Yi-Wei Lo [a], Jim-Min Fang [a,b,*]

[a] Department of Chemistry, National Taiwan University, Taipei 106, Taiwan
[b] The Genomics Research Center, Academia Sinica, Taipei 115, Taiwan

ARTICLE INFO

Article history:
Received 8 October 2014
Received in revised form 17 November 2014
Accepted 23 November 2014
Available online 28 November 2014

Keywords:
Influenza
Neuraminidase inhibitor
Michael reaction
Wittig reaction
Organocatalyst

ABSTRACT

Three-component coupling reaction of (pent-3-oxy)acetaldehyde, (*Z*)-*N*-(2-nitrovinyl)acetamide, and tetraethyl 1,1-diylbis(phosphonate) is performed in a one-pot operation, followed by reduction of the nitro group and hydrolysis of the phosphonate ester, to afford 8.7% overall yield of tamiphosphor as a potent neuraminidase inhibitor with IC_{50} and EC_{50} values of 2.5 and 31.5 nM against wild-type H1N1 influenza virus. The tamiphosphor (5*R*)-epimer is a less active anti-influenza agent with IC_{50} and EC_{50} values of 39 and 117 nM.

1. Introduction

Seasonal influenza is a contagious respiratory illness, and new types of influenza viruses may emerge to cause global infections. The threat of influenza is even more serious due to the cross-species transmission of avian influenza viruses, such as H5N1 and H7N9, to humans in the recent years. Influenza neuraminidase (NA) is a viral surface glycoprotein that is responsible for releasing progeny influenza viruses by cleaving the linkage between the viral hemagglutinin and the sialo-receptor on the host cell.[1,2] Inhibition of NA has been utilized as a very effective strategy for development of anti-influenza drugs.[3] Tamiflu™, as the phosphate salt of oseltamivir (OS, **1a**),[4–7] is an orally available anti-influenza drug, which is hydrolyzed by endogenous esterases to generate the active ingredient oseltamivir carboxylic acid (OC, **1b**) as a potent NA inhibitor.[6,8–11] However, tamiflu-resistant influenza viruses such as the clinically relevant H275Y strain of H1N1 virus have emerged over past years.[12–16] Thus, many scientists have exerted great effort to develop new anti-influenza drugs that are also active to H275Y virus.[17–19]

We have previously explored tamiphosphor (TP, **2**),[17] a phosphonate congener of OC, as a potent NA inhibitor against various avian and human influenza viruses. Strecher's and our research teams have further demonstrated that the TP monoesters also possess remarkable anti-influenza activities based on the enzymatic, cell, and animal assays.[18,20–23] Moreover, the guanidine analogs of TP and its monoesters are potent NA inhibitors against H275Y virus with the IC_{50} values in nanomolar range.[17,18]

As delineated in Fig. 1, only a few methods have been utilized in the syntheses of TP and its derivatives. In the first synthesis of TP (path A),[17] D-xylose (**3**) is utilized as a starting material. After transformation of the 3β-OH group to 3α-NH_2 group, a diphosphorylmethyl substituent is implanted to the C-5 position for the subsequent intramolecular Wittig reaction (in the Horner–Wadsworth–Emmons (HWE) variant)[24,25] to establish the core structure of polysubstituted cyclohexene ring. The pent-3-oxy and amino substituents are then introduced to furnish ~10% yield of the final product of TP in overall 19 synthetic steps. The synthesis of TP was also achieved by using *N*-acetyl-D-glucosamine as another chiral-pool molecule with a preset acetamido group in the desired absolute configuration.[26] In path B,[20,27,28] the *N*-Boc-protected OC (**1c**) is subjected to photochemical Hunsdiecker–Barton halodecarboxylation.[29] A palladium-catalyzed phosphonylation of the halocyclohexene product **6** with diethyl phosphite is carried out to provide TP, after concomitant removal of the ethyl and Boc groups by treatment with Me_3SiBr. The pivotal intermediate **6** can also be prepared from (1*S*,2*S*)-3-bromocyclohex-3,5-diene-1,2-diol (**7**), a fermentation product of bromobenzene,[30,31] by regio- and stereoselective introduction of pent-3-oxy and acetamide groups along with proper manipulation of the desired functional groups (path C).[27] Though the synthesis via paths B and C can provide TP in fewer

* Corresponding author. Tel.: +886 2 33661663; fax: +886 2 23637812; e-mail address: jmfang@ntu.edu.tw (J.-M. Fang).

http://dx.doi.org/10.1016/j.tet.2014.11.062
0040-4020/

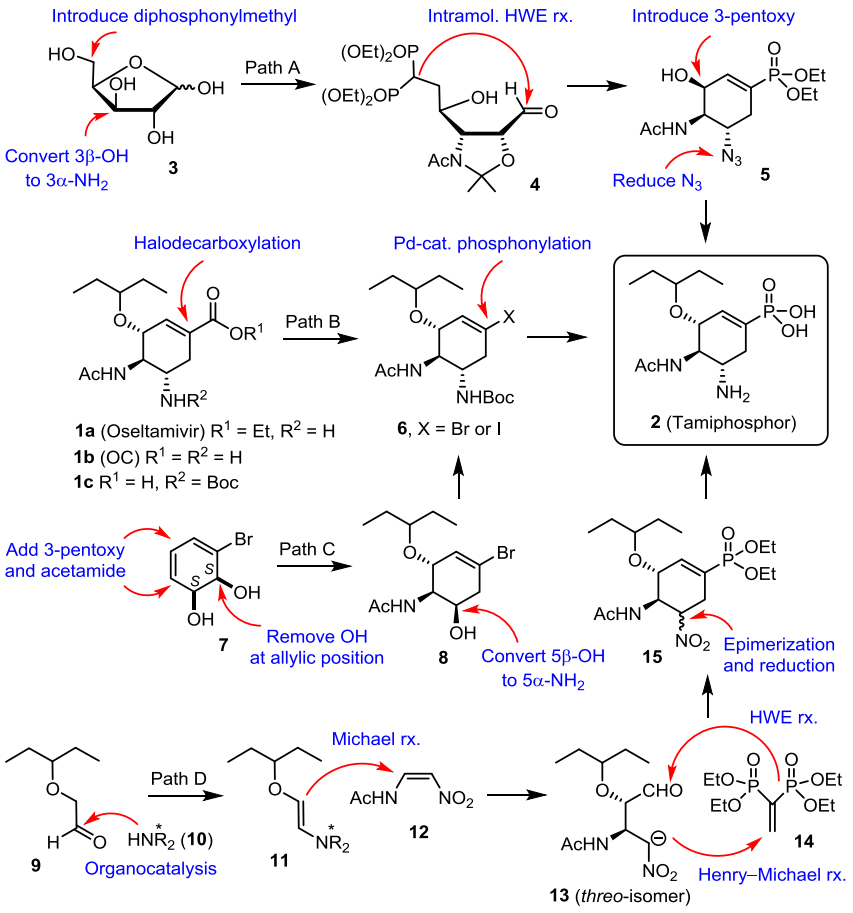


Fig. 1. Methods for the synthesis of tamiphosphor (**2**). (Path A) Carbohydrate molecule, e.g., D-xylose (**3**), is utilized as a chiral pool to construct the core structure of polysubstituted cyclohexene ring via an intramolecular Horner–Wadsworth–Emmons reaction. (Path B) The carboxylic acid in the oseltamivir derivative **1c** undergoes a photochemical Hunsdieker–Barton halodecarboxylation to give a halogen compound **6**, which is converted to the phosphonate ester via a palladium-catalyzed coupling reaction. (Path C) The fermentation product **7** of bromobenzene is sequentially processed to the common intermediate **6**. (Path D) In this study, a one-pot three-component coupling reaction of 2-(pent-3-oxy)acetaldehyde (**9**), (*Z*)-*N*-(2-nitrovinyl)acetamide (**12**), and 1,1-diphosphonylethene (**14**) is devised to construct the polysubstituted cyclohexene-1-phosphonate (**15**) for elaboration to tamiphosphor.

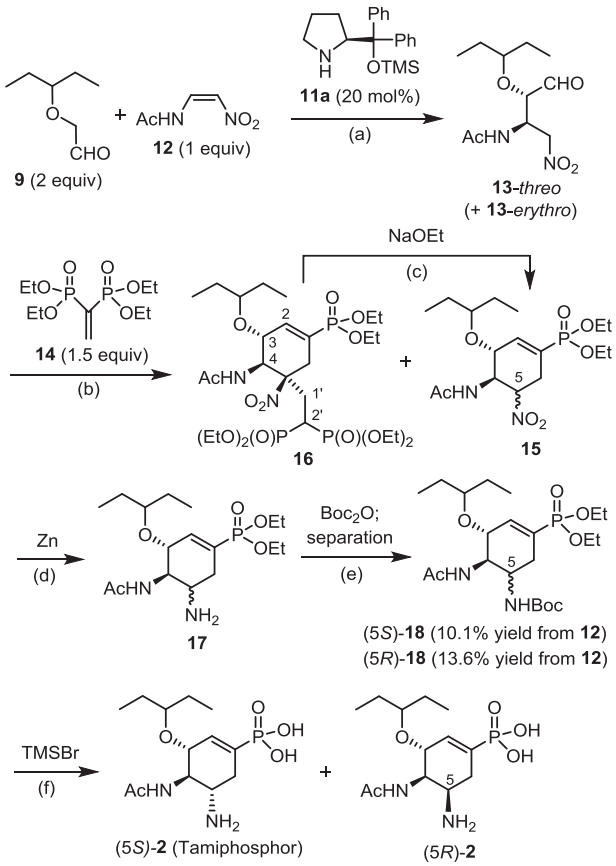
steps, some problems such as the photochemical of **1c** and microbial preparation of chiral diol **7** in large scales remain to be solved.

To develop TP and its derivatives for the therapeutic applications, it is needed to devise practical and large-scale synthetic methods. Inspired by the expedient synthesis of oseltamivir that uses one-pot multiple-component coupling reactions to construct the key intermediate of polysubstituted cyclohexene-1-carboxylate,[32–36] we aimed to investigate whether such straightforward methodology could be applied to synthesize TP? Path D shows our synthetic design to prepare polysubstituted cyclohexene-1-phosphonate **15** in one-pot operation by a sequence of reactions comprising an organocatalytic condensation of 2-(pent-3-oxy)acetaldehyde (**9**) with (*Z*)-*N*-(2-nitrovinyl)acetamide (**12**), a Henry–Michael reaction to trap the anion of the nitro intermediate **13** with tetraethyl ethene-1,1-diylbis(phosphonate) (**14**), and an intramolecular HWE reaction. In comparison with ethyl 2-(diethoxyphosphoryl)acrylate, $H_2C{=}C(CO_2Et)PO(OEt)_2$, used in oseltamivir synthesis,[32–36] 1,1-diphosphorylethene **14** would be a less reactive electrophile in Michael reaction because the sp^2-hybridized C–C double bond is not fully conjugated with the phosphoryl groups in the tetrahedral configuration.

2. Results and discussion

We first validated the asymmetric Michael reaction of aldehyde **9** with nitroalkene **12** using (*S*)-diphenylprolinol trimethylsilyl ether (**11a**) as the chiral organocatalyst to furnish the addition product **13**, predominantly in the *threo*-isomer (Scheme 1).[35] The *threo*-isomer showed the CHO group at δ_H 9.61, whereas the *erythro* isomer displayed the aldehyde proton at δ_H 9.57. We then carried on the reaction of **13** (as a mixture of *threo* and *erythro* isomers) with bisphosphorylethene **14** in the presence of Cs_2CO_3. The ^1H NMR spectrum of the crude product showed a vinyl proton (H-2) at δ_H 6.60 with a large coupling constant (J_{H-P}=21.6 Hz),[17] indicating that cyclohexene-1-phosphonate **15** was formed by tandem Henry–Michael reaction and intramolecular HWE reaction. The elimination species of phosphoric acid diethyl ester, $(OEt)_2P(O)(OH)$ generated from HWE reaction, exhibited the phosphorus(V) signal at δ_P=0 in the ^{31}P NMR spectrum. However, an appreciable amount of the side product **16**, showing a sodiated molecular ion $[M+Na]^+$ at *m*/*z* 729.2616, was also observed. Compound **16** was presumably derived from a second Henry–Michael reaction of **14** with **15**.[33] The NOESY correlation between H-4 (at δ_H 4.58) and the methylene protons (H-1′ at δ_H 2.69) of C5 substituent was consistent with the attack of **14** at the **15**-anion from the less hindered face. There are three phosphorus signals occurring at δ_P 17.2, 23.0, and 23.4 in compound **16**. The characteristic vinyl proton (H-2) appeared at δ_H 6.60 (J_{H-P}=22 Hz), and the methine proton (H-2′) adjacent to the phosphoryl groups exhibited at δ_H 2.57 with a large H–P coupling constant of 25.4 Hz. The NOESY correlation between H-3 (at δ_H 3.84) and NH (at δ_H 6.31) of C4 acetamide group revealed the trans-relationship at C3 and C4.

Our attempts to reduce the formation of **16**, e.g., by conducting the reaction at low temperature (−30 °C) or using less amounts of



Scheme 1. Synthesis of tamiphosphor (**2**) and its (5*R*)-epimer via multiple-component reactions. Reagents and reaction conditions: (a) $ClCH_2CO_2H$ (40 mol %), CH_2Cl_2, 0 °C, 24 h; (b) Cs_2CO_3, 0 °C, 24 h; (c) NaOEt, EtOH, 0 °C, 7.5 h; (d) Zn, 2 M $HCl_{(aq)}$, EtOH, 25 °C, 6 h; (e) Boc_2O, Et_3N, CH_2Cl_2, 25 °C, 18 h; separation of (5*S*)-**18** and (5*R*)-**18** by flash chromatography; (f) Me_3SiBr, CH_2Cl_2, 25 °C, 18 h; aqueous NH_4HCO_3; 86% yield. The steps (a)–(c) were conducted in one-pot operation.

bisphosphonate **14**, failed. However, we found that **16** underwent a retro-Michael reaction on treatment with a strong base (e.g., NaOEt) to give the desired product **15** as a less polar compound according to the TLC analysis. Compound **15** existed as an inseparable mixture of two 5-epimers. The desired epimer, (5*S*)-**15**, showed the H-5 and the methine proton on the pent-3-oxy group at δ_H 3.80 and 3.31, respectively, whereas the corresponding protons in (5*R*)-**15** occurred at δ_H 4.02 and 3.45. The released side product **14** was readily scavenged by NaOEt, via an oxa-Michael reaction, to avoid re-addition to **15**.

In the one-pot process, the amine-promoted reaction of **9** (2 equiv) with **12** (1 equiv) gave an intermediate **13**, as a mixture of *threo*- and *erythro*-isomers, which was subsequently treated with bisphosphonate **14** (1.5 equiv) and Cs_2CO_3 at 0 °C for 24 h, followed by addition of NaOEt (5 equiv) in EtOH solution for reversion of **16**, to accomplish the synthesis of **15** without contamination of **16**. The product **15** existed as a 1:1.2 mixture of two diastereomers (5*S*)-**15** and (5*R*)-**15** as shown by the ^{1}H NMR spectrum. According to the previous reports,[32–36] we also attempted to perform the thia-Michael reaction of cyclohexene-1-phosphonate **15** with *p*-toluenethiol, so that the cyclohexene ring would be converted to a -cyclohexane framework for better epimerization of (5*R*)-**15** (in 4,5-cis configuration) to the desired (5*S*)-**15** (in 4,5-trans configuration). However, the anticipated thia-Michael reaction of **15** could not be realized by using *p*-toluenethiol and other thiols under thermal or radical conditions, presumably because the cyclohexene-1-phosphonate was a relatively weak Michael acceptor.

The nitro group in **15** (as a mixture of 5*S*/5*R* epimers, 1:1.2) was selectively reduced by zinc powder in acidic conditions. The crude amine product **17** was obtained by acid–base extraction, and isolated as the Boc-protected compound **18** by subsequent treatment with Boc_2O. The two epimers were then successfully separated by flash chromatography to give (5*S*)-**18** and (5*R*)-**18**, respectively, in 10.1% and 13.6% isolated yields (calculated from nitroalkene **12**). According to our previously reported procedure,[27] (5*S*)-**18** was treated with bromotrimethylsilane to afford tamiphosphor **2** having the (5*S*)-configuration in 86% yield by concomitant hydrolysis of the phosphonate group and removal of Boc group. The epimer (5*R*)-**2** was obtained from (5*R*)-**18** by a similar procedure.

The NA inhibitory activities were measured by a fluorescence assay using 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) as the substrate, while the anti-influenza activities were evaluated by the cytopathic effect of H1N1 virus (A/WSN/1933) on Madin–Darby canine kidney (MDCK) cells. Tamiphosphor was a potent anti-influenza agent (IC_{50}=2.5 nM and EC_{50}=31.5 nM), whereas its epimer (5*R*)-**2** showed inferior activities with the IC_{50} and EC_{50} values of 38.7 and 116.6 nM, respectively. This result was in agreement with the molecular model of NA–tamiphosphor complex,[17] in which the 5–NH_3^+ substituent of tamiphosphor exhibited considerable electrostatic interactions with the acidic residues of Glu119, Asp151, and Glu227 in the S2 site of NA. This study thus provides direct evidence that the C-5 aminium group in (*S*)-chirality is appropriate to bind with influenza NA. In comparison, a recent study revealed that the (4*S*)-epimer of OC completely lost anti-influenza activity.[37] The influence of wrong stereodisposition in the C4-acetamido group appeared to be more drastic than that in the C5-amino group for anti-influenza activity.

3. Conclusion

Starting from the chiral amine-promoted Michael reaction of aldehyde **9** with nitroenamide **12**, the sequential reactions comprising a second Michael addition with 1,1-diphosphorylethene **14**, an HWE reaction and an reversion of the side product **16** were carried out by one-pot operation to give the (3,4,5-trisubstituted) cyclohexene-1-phosphonate **15**. After nitro reduction and protection of the resulting amine, the two diastereomers of **18** were separated by flash chromatography, and then individually treated with bromotrimethylsilane to afford tamiphosphor and its (5*R*)-epimer. This synthetic protocol could be performed in open system without rigorous exclusion of oxygen or moisture. The whole process involved only one chromatographic separation of (5*S*)-**18** and (5*R*)-**18**. Thus, 8.7% overall yield (based on nitroenamide **12**) of tamiphosphor was obtained by a straightforward manner, and upgraded to gram-scale synthesis. The starting materials **9**, **12** and **14** were readily prepared from inexpensive reagents by known methods. The chiral prolinol derivative was employed as an eco-friendly organocatalyst to establish the desired absolute configurations at the C-3 and C-4 positions. The side product **16** was effectively reverted by sodium ethoxide to the key intermediate **15**. Taken together, this method appears to be more appealing to tamiphosphor synthesis than the previously reported methods.[17,20,26–28] In this study, we also compare the IC_{50} and EC_{50} values of tamiphosphor with its epimer (5*R*)-**2** to evaluate the influence of the disposition of 5-amino group in anti-influenza activity in a quantitative sense.

4. Experimental section

4.1. General

All the reagents and solvents were reagent grade and used without further purification unless otherwise specified. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel using aqueous *p*-anisaldehyde as visualizing

agent. Silica gel (0.040–0.063 mm particle sizes) was used for column chromatography. Flash chromatography was performed on silica gel of 60–200 μm particle size. Molecular sieves were activated under high vacuum at 220 °C over 6 h.

Melting points were recorded on a Yanaco or Electrothermal MEL-TEMP 1101D apparatus in open capillaries and are not corrected. Optical rotations were measured on digital polarimeter of Japan JASCO Co. DIP-1000. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Infrared (IR) spectra were recorded on Nicolet Magna 550-II or Thermo Nicolet 380 FT-IR spectrometers. Nuclear magnetic resonance (NMR) spectra were obtained on Varian Unity Plus-400 (400 MHz) or Bruker Avance-400 (400 MHz) spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to δ_H 7.24/δ_C 77.0 (central line of t) for $CHCl_3/CDCl_3$, δ_H 4.80 for H_2O/D_2O, or δ_H 3.31/δ_C 48.2 for MeOH-d_4. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (double of doublets), and br (broad). Coupling constants (J) are given in hertz (Hz). The ESI-MS experiments were conducted on a Bruker Daltonics BioTOF III high-resolution mass spectrometer.

Compound **11a** was commercially available. Compounds **9**,[32] **12**,[34] and **14**[38,39] were prepared according to the previously reported methods.

4.2. Diethyl (3*R*,4*R*,5*S*)-4-acetamido-5-*tert*-butoxycarbonylamino-3-(1-ethylpropoxy)cyclohex-1-ene-1-phosphonate (18)[27]

Freshly prepared aldehyde **9** (1.0 g, 7.7 mmol) was added to a suspension of (*S*)-diphenylprolinol trimethylsilyl ether (**11a**, 250 mg, 0.76 mmol), nitroenamide **12** (0.50 g, 3.8 mmol), and chloroacetic acid (152 mg, 1.52 mmol) in CH_2Cl_2 at 0 °C. The mixture was stirred for 24 h, then bisphosphonate **14** (1.36 g, 5.76 mmol) and Cs_2CO_3 (11.5 mmol, 3.76 g) were added at 0 °C. After stirring for 24 h at 0 °C, the mixture was concentrated under reduced pressure at 0 °C. The residue containing **15** and **16** was dissolved in absolute alcohol (EtOH, 10 mL) as purchased without prior removal of water. NaOEt (1.3 g, 19 mmol) was added to the solution at 0 °C. The mixture was stirred at the same temperature for 7.5 h, and then quenched with cold 2 M HCl (10 mL). The aqueous layer was extracted with CH_2Cl_2 (20 mL) for three times. The combined organic extracts were washed with saturated $NaHCO_3$ solution (25 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give a crude product **15**.

The above-prepared crude product was dissolved in EtOH (10 mL) and added 2 M aqueous HCl (10 mL). Activated zinc powder (1754 mg, 25.8 mmol) was added to the resulting solution and stirred for 6 h at 25 °C before filtration. Aqueous ammonia (5 mL, 28%) was added to the filtrate. The aqueous layer was extracted three times with 10% MeOH/CH_2Cl_2. The combined organic phase was extracted three times with 2 M HCl. The combined aqueous extracts were adjusted to pH 10 with 28% aqueous ammonia and extracted three times with 10% MeOH/CH_2Cl_2. The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude amine product **17**. The crude amine was dissolved in CH_2Cl_2. To the solution was added triethylamine (313 mg, 3.1 mmol, 0.4 mL), followed by addition of di-*tert*-butyl dicarbonate (338 mg, 1.55 mmol). The mixture was stirred for 18 h at 25 °C until the reaction was completed as shown by TLC analysis. The mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/2-propanol=15:1) to afford (5*S*)-**18** (183 mg, 10.1% yield form **12**) and (5*R*)-**18** (247 mg, 13.5% yield form **12**).

*4.2.1. Compound (5S)-**18**.* $C_{22}H_{41}N_2O_7P$; white solid; mp 173–175 °C (lit.[27] mp 167–169 °C); $[\alpha]_D^{20}$ −65.5 (*c* 1.0, $CHCl_3$) (lit.[27] $[\alpha]_D^{20}$ −88.8 (*c* 1.14, $CHCl_3$)); IR (film) 3389, 2933, 1695, 1565, 1172, 1025 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.57 (1H, d, *J*=21.6 Hz), 5.90 (1H, d, *J*=8.8 Hz), 5.10 (1H, d, *J*=9.2 Hz), 4.09–3.98 (5H, m), 3.89–3.87 (1H, br s), 3.80–3.75 (1H, m), 3.30 (1H, m), 2.62–2.55 (1H, m), 2.22–2.15 (1H, m), 1.95 (3H, s), 1.50–1.44 (4H, m), 1.39 (9H, s), 1.30–1.27 (6H, m), 0.87–0.79 (6H, m); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.9, 156.3, 141.5, 127.4 (C-1, d, J_{C-P}=181 Hz), 82.1, 79.8, 76.0, 62.1, 62.0, 54.5, 49.1, 31.2, 28.3 (3×), 26.0, 25.5, 23.3, 16.3, 16.4, 9.5, 9.1; ^{31}P NMR (162 MHz, $CDCl_3$) δ 17.99; HRMS (ESI): calcd for $C_{22}H_{42}N_2O_7P$: 477.2730, found: *m*/*z* 477.2545 $[M+H]^+$.

*4.2.2. Compound (5R)-**18**.* $C_{22}H_{41}N_2O_7P$; pale yellow oil; $[\alpha]_D^{20}$ −68.8 (*c* 1.0, $CHCl_3$); IR (film) 3297, 2978, 1687, 1536, 1168, 1033 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 6.60 (1H, d, *J*=22 Hz), 4.2 (1H, br s), 4.20–4.06 (5H, m), 3.97–3.95 (1H, m), 3.48 (1H, m), 2.55–2.49 (1H, m), 2.26–2.19 (1H, m), 1.98 (3H, s), 1.60–1.46 (4H, m), 1.44 (9H, s), 1.40–1.28 (6H, m), 0.95–0.87 (6H, m); ^{13}C NMR (100 MHz, CD_3OD) δ 174.0, 159.3, 141.2, 129.4 (C-1, d, J_{C-P}=180 Hz), 83.5, 80.48, 74.2, 63.9, 63.7, 52.7, 47.7, 29.3, 29.2, 28.9 (3×), 27.6, 27.59, 16.9, 16.8, 10.4, 10.0; ^{31}P NMR (162 MHz, $CDCl_3$) δ 18.93; HRMS (ESI): calcd for $C_{22}H_{42}N_2O_7P$: 477.2730, found: *m*/*z* 477.2545 $[M+H]^+$.

4.3. (3*R*,4*R*,5*S*)-4-Acetamido-5-amino-3-(1-ethylpropoxy)cyclohex-1-ene-1-phosphonic acid (2, tamiphosphor)[27]

Diethyl phosphonate (5*S*)-**17** (50 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (1 mL) and treated with bromotrimethylsilane (160 mg, 1.05 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 18 h. After which, the mixture was concentrated under reduced pressure. The residue was taken up in water (0.5 mL), stirred for 2 h at room temperature, and subject to lyophilization. The residual pale yellow solids were washed with Et_2O (3×20 mL) to give white solids, which were dissolved in aqueous NH_4HCO_3 (0.1 M solution, 2 mL), stirred for 1 h at room temperature, and then lyophilization to afford tamiphosphor **2** as ammonium salts (32 mg, 86% yield).

$C_{13}H_{25}N_2O_5P$; white solid; mp 246–250 °C (lit.[27] 238–240 °C); $[\alpha]_D^{20}$ −48.51 (*c* 1.0, H_2O) (lit.[27] $[\alpha]_D^{20}$ −56.7 (*c* 1.2, H_2O)); IR (film) 3125, 3032, 2960, 1653, 1558, 1019 cm^{-1}; ^{1}H NMR (600 MHz, D_2O) δ 6.33 (1H, d, $J_{P\text{-}1}$=19.2 Hz), 4.26 (1H, d, *J*=9.0 Hz), 3.94 (1H, dd, *J*=12.0, 8.7 Hz), 3.58–3.55 (2H, m), 2.86–2.81 (1H, m), 2.54–2.48 (1H, m), 2.11 (3H, s), 1.62–1.56 (3H, m), 1.51–1.46 (1H, m), 0.92 (3H, t, *J*=7.8 Hz), 0.87 (3H, t, *J*=7.8 Hz); ^{13}C NMR (150 MHz, D_2O) δ 177.9, 136.9, 134.9 (C-1, d, $J_{P\text{-}1}$=172 Hz), 87.1, 78.8, 55.7, 52.2, 32.0, 28.2, 27.9, 25.1, 11.3, 11.2; ^{31}P NMR (162 MHz, D_2O) δ 10.16; HRMS calcd for $C_{13}H_{26}N_2O_5P$ $[M+H]^+$: 321.1579, found: *m*/*z* 321.1569.

4.4. (3*R*,4*R*,5*R*)-4-Acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene phosphonic acid ((5*R*)-2)

Diethyl phosphonate (5*R*)-**17** (50 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (1 mL) and treated with bromotrimethylsilane (160 mg, 1.05 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 18 h. After which, the mixture was concentrated under reduced pressure. The residue was taken up in water (0.5 mL), stirred for 2 h at room temperature, and subject to lyophilization. The residual pale yellow solid residue was washed with Et_2O (3×20 mL) to give white solids, which was dissolved in aqueous NH_4HCO_3 (0.1 M solution, 2 mL), stirred for 1 h at room temperature, and then lyophilization to afford (5*R*)-**2** as ammonium salts (33 mg, 89% yield).

$C_{13}H_{25}N_2O_5P$; white solid; mp 218–224 °C (dec); $[\alpha]_D^{20}$ −58.83 (*c* 1.0, H_2O); IR (film) 3037, 2967, 2875, 1647, 1540, 1070 cm^{-1}; ^{1}H NMR (600 MHz, D_2O) δ 6.15 (1H, d, $J_{P\text{-}2}$=18.8 Hz), 4.12 (1H, d, *J*=8.1 Hz), 3.94 (1H, dd, *J*=7.2, 4.3 Hz), 3.77–3.74 (1H, m), 3.59–3.56 (1H, m), 2.79–2.73 (1H, m), 2.40–2.36 (1H, m), 2.04 (3H, s), 1.65–1.56 (3H, m), 1.50–1.44 (1H, m), 0.95 (3H, t, *J*=7.4 Hz), 0.89 (3H, t, *J*=7.4 Hz);

^{13}C NMR (150 MHz, D_2O) δ 178.2, 139.1 (C-1, d, $J_{P\text{-}1}$=170 Hz), 132.2, 85.8, 75.5, 50.4, 49.4, 28.6, 28.0, 27.9, 24.6, 12.3, 10.9; ^{31}P NMR (162 MHz, D_2O) δ 10.05; HRMS calcd for $C_{13}H_{26}N_2O_5P$ $[M+H]^+$: 321.1579, found: *m*/*z* 321.1578.

4.5. Determination of influenza virus $TCID_{50}$

Influenza A/WSN/1933 (H1N1) (from Dr. Shin-Ru Shih, Chang Gung University, Taiwan) was cultured in the allantoic cavities of 10-day-old embryonated chicken eggs for 72 h, and purified by sucrose gradient centrifugation. Madin–Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (Manassas, Va), and were grown in DMEM (Dulbecco's modified Eagle medium, GibcoBRL) containing 10% fetal bovine serum (GibcoBRL) and penicillin–streptomycin (GibcoBRL) at 37 °C under 5% CO_2.

The $TCID_{50}$ (50% tissue culture infectious dose) was determined by serial dilution of the influenza virus stock onto 100 μL MDCK cells at 1×10^5 cells/mL in 96-well microplates. The infected cells were incubated at 37 °C under 5.0% CO_2 for 48 h and added to each wells with 100 μL per well of CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega). After incubation at 37 °C for 15 min, absorbance at 490 nm was read on a plate reader. Influenza virus $TCID_{50}$ was determined using Reed–Muench method.[40,41]

4.6. Determination of NA activity by fluorescent assay

The neuraminidase activity was measured using diluted allantoic fluid harvest from influenza A/WSN/1933 (H1N1) infected embryonated eggs. A fluorometric assay was used to determine the NA activity with the fluorogenic substrate 2-(4-methylum belliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma). The fluorescence of the released 4-methylumbelliferone was measured in Envision plate reader (Perkin–Elmer, Wellesley, MA) using excitation and emission wavelengths of 365 and 460 nm, respectively. Neuraminidase activity was determined at 200 μM of MUNANA. Enzyme activity was expressed as the fluorescence increase during 15 min incubation at room temperature.

4.7. Determination of IC_{50} of NA inhibitor

NA inhibition was determined by mixing inhibitor and neuraminidase for 10 min at room temperature followed by the addition of 200 μM of substrate. Inhibitor IC_{50} value was determined from the dose–response curves by plotting the percent inhibition of NA activity versus inhibitor concentrations using Graph Pad Prism 4.

4.8. Determination of EC_{50} of NA inhibitor

The anti-flu activities of neuraminidase inhibitors were measured by the EC_{50} values that were the concentrations of NA inhibitor for 50% protection of the H1N1 cytopathic activities. Fifty microliters diluted H1N1 at 100 $TCID_{50}$ were mixed with equal volumes of NA inhibitors at varied concentrations. The mixtures were used to infect 100 μL of previously seeded MDCK cells at 1×10^5 cells/mL in 96-wells. After 48 h incubation at 37 °C under 5% CO_2, the cytopathic effects (CPE) were determined with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay reagent as described above. Inhibitor EC_{50} values were determined by fitting the curves of percent CPE versus the concentrations of NA inhibitor using Graph Pad Prism 4.

Acknowledgements

We thank Dr. Yih-Shyun E. Cheng (the Genomics Research Center, Academia Sinica) for bioassays, Dr. Jiun-Jie Shie (the Genomics Research Center, Academia Sinica) for helpful discussion, as well as the Ministry of Science and Technology for financial support (NSC 101-2325-B-002-024).

Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2014.11.062.

References and notes

1. von Itzstein, M. *Nat. Rev. Drug Discov.* **2007**, *6*, 967–974.
2. Lowen, A. C.; Mubareka, S.; Steel, J.; Palese, P. *PLoS Pathog.* **2007**, *3*, 14770–14776.
3. Moscona, A. *N. Engl. J. Med.* **2005**, *353*, 1363–1373.
4. Li, W. X.; Escarpe, P. A.; Eisenberg, E. J.; Cundy, K. C.; Sweet, C.; Jakeman, K. J.; Merson, J.; Lew, W.; Williams, M.; Zhang, L. J.; Kim, C. U.; Bischofberger, N.; Chen, M. S.; Mendel, D. B. *Antimicrob. Agents Chemother.* **1998**, *42*, 647–653.
5. Sidwell, R. W.; Huffman, J. H.; Barnard, D. L.; Bailey, K. W.; Wong, M. H.; Morrison, A.; Syndergaard, T.; Kim, C. U. *Antiviral Res.* **1998**, *37*, 107–120.
6. Kim, C. U.; Lew, W.; Williams, M. A.; Wu, H. W.; Zhang, L. J.; Chen, X. W.; Escarpe, P. A.; Mendel, D. B.; Laver, W. G.; Stevens, R. C. *J. Med. Chem.* **1998**, *41*, 2451–2460.
7. Widmer, N.; Meylan, P.; Ivanyuk, A.; Aouri, M.; Decosterd, L. A.; Buclin, T. *Clin. Pharmacokinet.* **2010**, *49*, 741–765.
8. Varghese, J. N.; Epa, V. C.; Colman, P. M. *Protein Sci.* **1995**, *4*, 1081–1087.
9. Kim, C. U.; Lew, W.; Williams, M. A.; Liu, H. T.; Zhang, L. J.; Swaminathan, S.; Bischofberger, N.; Chen, M. S.; Mendel, D. B.; Tai, C. Y.; Laver, W. G.; Stevens, R. C. *J. Am. Chem. Soc.* **1997**, *119*, 681–690.
10. Sweeny, D. J.; Lynch, G.; Bidgood, A. M.; Lew, W.; Wang, K. Y.; Cundy, K. C. *Drug Metab. Dispos.* **2000**, *28*, 737–741.
11. McClellan, K.; Perry, C. M. *Drugs* **2001**, *61*, 263–283.
12. Gubareva, L. V.; Webster, R. G.; Hayden, F. G. *Antimicrob. Agents. Chemother.* **2001**, *45*, 3403–3408.
13. de Jong, M. D.; Thanh, T. T.; Khanh, T. H.; Hien, V. M.; Smith, G. J. D.; Chau, N. V.; Cam, B. V.; Qui, P. T.; Ha, D. Q.; Guan, Y.; Peiris, J. S. M.; Hien, T. T.; Farrar, J. *N. Engl. J. Med.* **2005**, *353*, 2667–2672.
14. Dharan, N. J.; Gubareva, L. V.; Meyer, J. J.; Okomo-Adhiambo, M.; McClinton, R. C.; Marshall, S. A.; George, K. S.; Epperson, S.; Brammer, L.; Klimov, A. I.; Bresee, J. S.; Fry, A. M.; Grp, O.-R. W. *J. Am. Med. Assoc.* **2009**, *301*, 1034–1041.
15. Weinstock, D. M.; Zuccotti, G. *J. Am. Med. Assoc.* **2009**, *301*, 1066–1069.
16. Moscona, A. *N. Engl. J. Med.* **2009**, *360*, 953–956.
17. Shie, J.-J.; Fang, J.-M.; Wang, S.-Y.; Tsai, K.-C.; Cheng, Y.-S. E.; Yang, A.-S.; Hsiao, S.-C.; Su, C.-Y.; Wong, C.-H. *J. Am. Chem. Soc.* **2007**, *129*, 11892–11896.
18. Cheng, T.-J. R.; Weinheimer, S.; Tarbet, E.-B.; Jan, J.-T.; Cheng, Y.-S. E.; Shie, J.-J.; Chen, C.-L.; Chen, C.-A.; Hsieh, W.-C.; Huang, P.-W.; Lin, W.-H.; Wang, S.-Y.; Fang, J.-M.; Hu, O.-Y. P.; Wong, C.-H. *J. Med. Chem.* **2012**, *55*, 8657–8670.
19. Abed, Y.; Pizzorno, A.; Boivin, G. *Antimicrob. Agents. Chemother.* **2012**, *56*, 4375–4380.
20. Carbain, B.; Collins, P. J.; Callum, L.; Martin, S. R.; Hay, A. J.; McCauley, J.; Streicher, H. *ChemMedChem* **2009**, *4*, 335–337.
21. Carbain, B.; Martin, S. R.; Collins, P. J.; Hitchcock, P. B.; Streicher, H. *Org. Biomol. Chem.* **2009**, *7*, 2570–2575.
22. Stanley, M.; Martin, S. R.; Birge, M.; Carbain, B.; Streicher, H. *Org. Biomol. Chem.* **2011**, *9*, 5625–5629.
23. Stanley, M.; Cattle, N.; McCauley, J.; Martin, S. R.; Rashid, A.; Field, R. A.; Carbain, B.; Streicher, H. *MedChemComm* **2012**, *3*, 1373–1376.
24. Fleet, G. W. J.; Shing, T. K. M. *J. Chem. Soc., Chem. Commun.* **1983**, 849–850.
25. Streicher, H.; Meisch, D.; Bohner, C. *Tetrahedron* **2001**, *57*, 8851–8859.
26. Chen, C.-A.; Fang, J.-M. *Org. Biomol. Chem.* **2013**, *11*, 7687–7699.
27. Shie, J.-J.; Fang, J.-M.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2008**, *47*, 5788–5791.
28. Gunasekera, D. S. *Synlett* **2012**, 573–576.
29. Barton, D. H. R.; Crich, D.; Motherwell, W. B. *J. Chem. Soc., Chem. Commun.* **1983**, 939–941.
30. Boyd, D. R.; Sheldrake, G. N. *Nat. Prod. Rep.* **1998**, *15*, 309–324.
31. Hudlicky, T.; Gonzalez, D.; Gibson, D. T. *Aldrichimica Acta* **1999**, *32*, 35–62.
32. Ishikawa, H.; Suzuki, T.; Hayashi, Y. *Angew. Chem., Int.Ed.* **2009**, *48*, 1304–1307.
33. Ishikawa, H.; Suzuki, T.; Orita, H.; Uchimaru, T.; Hayashi, Y. *Chem.—Eur. J.* **2010**, *16*, 12616–12626.
34. Zhu, S. L.; Yu, S. Y.; Wang, Y.; Ma, D. W. *Angew. Chem., Int. Ed.* **2010**, *49*, 4656–4660.
35. Weng, J.; Li, Y.-B.; Wang, R.-B.; Lu, G. *ChemCatChem* **2012**, *4*, 1007–1012.
36. Rehák, J.; Huťka, M.; Latika, A.; Brath, H.; Almássy, A.; Hajzer, V.; Durmis, J.; Toma, Š.; Šebesta, R. *Synthesis* **2012**, *44*, 2424–2430.
37. Sartori, A.; Dell'Amico, L.; Battistini, L.; Curti, C.; Rivara, S.; Pala, D.; Kerry, P. S.; Pelosi, G.; Casiraghi, G.; Rassu, G.; Zanardi, F. *Org. Biomol. Chem.* **2014**, *12*, 1561–1569.
38. Degenhardt, C. R.; Burdsall, D. C. *J. Org.Chem.* **1986**, *51*, 3488–3490.
39. Chaleix, V.; Lecouvey, M. *Tetrahedron Lett.* **2007**, *48*, 703–706.
40. Reed, L. J.; Müench, H. *Am. J. Hyg.* **1938**, *27*, 493–497.
41. Burleson, F. G.; Chambers, T. M.; Wiedbrauk, D. L. *Virology, A Laboratory Manual*; Academic: San Diego, CA, 1992.