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FULL PAPERS

OSELTAMIVIR ANALOG WITH BORON CLUSTER MODULATOR

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Abstract: Synthesis of novel neuraminidase inhibitor – carborane ester of oseltamivir carboxylic acid is described, and its physicochemical and spectral characteristics is provided. Surprisingly, carborane analog of oseltamivir is of an order of magnitude less active than its precursor, the corresponding ethyl ester, which is the active principle of pharmaceutical preparations used in influenza prophylactics and therapy.

Keywords: antiviral activity, neuraminidase inhibitor, oseltamivir analog, carborane cluster

Abbreviations: API – active pharmaceutical ingredient, DCC – dicyclohexylcarbodiimide, DDD – drug discovery and development, DTGS – deuterated triglycine sulfate, DMAP – 4-dimethylaminopyridine, DMEM – Dulbecco's modified Eagle's medium, DMSO – dimethyl sulfoxide, ESI MS – electrospray ionization mass spectroscopy, FBS – fetal bovine serum, HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, IAV – influenza A virus, IR – infrared spectroscopy, MDCK – Madin-Darby canine kidney cells, MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, PMS – phenazine methosulfate, SiC – silicon carbide, TLC – thin layer chromatography, TPCK – L-1-tosylamide-2-phenylethyl chloromethyl ketone

The threat of pandemic influenza poses very serious challenge for public health services throughout the world (1, 2) although situation of individual countries vary greatly, reflecting local status of pharmaceutical industry. Antiviral drug discovery is less serendipitous than drug discovery and development (DDD) in other therapeutic areas and majority of marketed drugs are the products of true rational design (3–5). Neuraminidase inhibitors represent good example of such approach, in which structural analogs of enzymatic process transition state are employed for blocking the release of newly formed virus particles, thus preventing further proliferation of the pathogen. Our involvement in process development for oseltamivir generics, combined with continuous interest in pharmaceutical innovation prompted us to combine expertise for design of novel analogs of the leading drug (Tamiflu®), exploiting carborane clusters as innovative replacement of the standard ester residue of the API. Exemplary synthesis of the carborane analog (6) of oseltamivir is described in detail, and evaluation of its biological activity in antiviral test, in comparison with the reference pro-drug and its active form (oseltamivir carboxylic acid) is presented.

Oseltamivir phosphate – the prodrug of oseltamivir carboxylate (Ro 64-0802; GS4701) and the active ingredient of anti-influenza drugs – is a carbocyclic analog of sialic acid transition state, when transformed by neuraminidase enzyme. The active pharmaceutical ingredient (API) launched by Roche in 1999 is manufactured by chemical synthesis from natural products such as shikimic or quinic acids (7), although effective approaches to the total synthesis have also been disclosed (8, 9) (Scheme 1).

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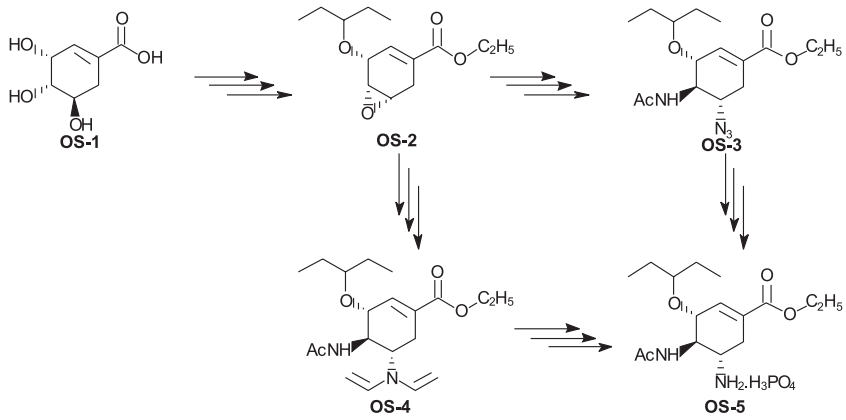
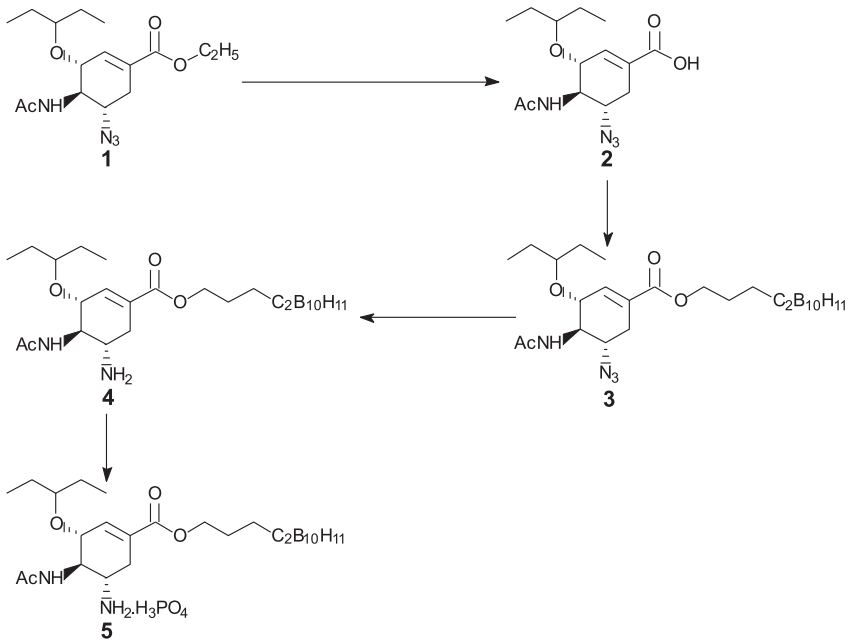


Scheme 1. Possible synthetic ways of oseltamivir phosphate (**OS-5**) from shikimic acid (**OS-1**)



Scheme 2. Synthesis of carborane analog of oseltamivir **5** from commercially available azido-ester **1**

It is well known, that oseltamivir is a pro-drug, which has to undergo hydrolytic biotransformation to the corresponding carboxylic acid in order to become active as neuraminidase inhibitor. Therefore, we have decided to obtain and test for antiviral activity some other, unreported esters. Since boron clusters are already installed in medicinal chemistry as proven moderators of pharmacophoric properties (6), we have chosen to replace the ester residue in oseltamivir by carborane. Direct transesterification of the API seemed unattainable and stepwise procedures involving carboxylic acid were considered first. Although standard basic ester hydrolysis of oseltamivir base is relatively simple operation, the resulting amino acid would require protection and deprotection in order to obtain desired ester. Therefore, oseltamivir precursor, with azido group in place of the amino function, was selected for introduction of carborane residue. Synthesis of the new analog of oseltamivir is depicted in Scheme 2.

In contrast to oseltamivir carboxylate and its ethyl ester, which both exhibit high inhibitory activity in the plaque reduction assay, the new carborane

ester shows only 1/10 of the expected activity. At present, we can offer no rational explanation for the reason for this unexpected, dramatic difference in activity.

EXPERIMENTAL

Materials and Methods

4-Dimethylaminopyridine (DMAP) and *N,N`*-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents were purchased in the highest available quality. Column chromatography was performed on 230-400 mesh silica gel obtained from Sigma-Aldrich (Steinheim, Germany). TLC analysis was performed on F_{254} silica gel plates purchased from Sigma-Aldrich (Steinheim, Germany). Compounds were visualized by use of UV light (254 nm). If not stated otherwise, ^{1}H, ^{11}B and ^{13}C NMR spectra were recorded with a Bruker Avance III 600 MHz spectrometer. The spectra were recorded at 600.3, 192.6 and 150.9 MHz, respectively. ^{1}H NMR chemical shifts (δ) are reported in parts per million (ppm) relative to a residual proton peak of the dichloromethane-d_2 (δ = 5.3 for CD_2Cl_2). ^{13}C NMR chemical shifts (δ) are reported in ppm relative to the solvent peak (δ = 53.8 for CD_2Cl_2). $BF_3/(C_2H_5)_2O$ was used as standard for ^{11}B and $^{11}B\{^{1}H\}$ NMR. The following abbreviations were used to explain the multiplicities: s – singlet, d – doublet, t –- triplet, m – multiplet, dd – doublet of doublets, td – triplet of doublets, br – broad, brd – broadened. Coupling constants (*J*) are reported in hertz (Hz). The electrospray mass spectra (ESI MS) were recorded with a Varian 500-MS LC Ion Trap mass spectrometer. The masses are those of the positive ions. Infrared absorption spectra (IR) were recorded using a Nexus Fourier-transform infrared spectrometer (Thermo-Nicolet 6700) equipped with a silicon carbide (SiC) air-cooled source, a caesium iodide beam splitter, and DTGS (deuterated triglycine sulfate) detectors. Samples were prepared by diluting compounds with potassium bromide (70–140 mg of KBr per sample) and then pressing in the stainless-steel die to form disc of 0.8 cm diameter.

Cells, virus, drugs, reagents

Madin-Darby canine kidney cells (MDCK) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.2% bovine serum albumin, 25 mM HEPES buffer, 100 U/mL of penicillin, 100 µg/mL of streptomycin, at 37°C under 5% CO_2.

Influenza A virus H5N2 strain was originally obtained from National Veterinary Institute in Puławy, Poland.

Stock solutions of all tested compounds were prepared in dimethyl sulfoxide (DMSO) and stored at –20°C until future use.

Mouse anti-influenza A virus M1 monoclonal antibody was purchased from Abcam (Cambridge, UK). Anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

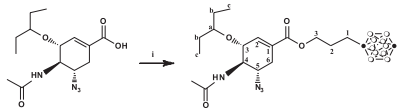
Synthetic procedures

Synthesis of (3R,4R,5S)-4-acetamido-5-azido-3-(1-ethylopropoxy)-cyclohex-1-ene-1-carboxylic acid (2)

A solution of ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate (**1**, 29.55 mmol, 10.00 g) in methanol (67 mL) was treated with a solution of NaOH (59.18 mmol, 2.367 g, 2 eq.) in water (21 mL). The reaction mixture was stirred at room temperature for 24 h and then neutralized with 5% hydrochloric acid. The excess of methanol was evaporated *in vacuo*. The residue was acidified to pH 2 with 5% hydrochloric acid and extracted with dichloromethane (3 × 24 mL). The combined extracts were dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The crude product was crystallized from a mixture of acetone-hexane (10/40 mL). Yield: 74.5% (6.83 g).

TLC (MeOH-AcOEt-NH_3aq (50:30:1, v/v) Rf = 0.6; ^{1}H NMR (200 MHz, DMSO-d_6, δ, ppm): 0.78 (3H, t, *J* = 7.3 Hz, CH_3(c)), 0.83 (3H, t, *J* = 7.3 Hz, CH_3(c)), 1.40 (4H, br m, 2 × CH_2(b)), 1.83 (3H, s, CH_3 of acetyl group), 2.06 (1H, br m, H-6), 2.69 (1H, brd dd, *J* = 16.9 and 5.1 Hz, H-6), 3.34 (1H, pentet, *J* = 5.6 Hz, CH(a)), 3.63 (1H, dd, *J* = 11.0 and 8.4 Hz, H-4), 3.79 (1H, td, *J* = 11.0 and 4.95 Hz, H-5), 4.12 (1H, br d, *J* = 8.4 Hz, H-3), 6.61 (1H, br s, H-2), 8.11 (1H, d, *J* = 8.4 Hz, NH); ^{1}H NMR (200 MHz, $CDCl_3$, 213,705 , ppm): 0.90 (3H, t, *J* = 7.3 Hz, CH_3(c)), 0.91 (3H, t, *J* = 7.5 Hz, CH_3(c)), 1.51 (4H, br m, 2 × CH_2(b)), 2.05 (3H, s, CH_3 of acetyl group), 2.24 (1H, br m, H-6), 2.87 (1H, brd dd, *J* = 17.6 and 5.5 Hz, H-6), 3.25 (1H, dd, *J* = 11.0 and 8.4 Hz, H-4), 3.34 (1H, pentet, *J* = 5.95 Hz, CH(a)), 4.38 (1H, td, *J* = 10.8 and 5.4 Hz, H-5), 4.65 (1H, br d, *J* = 9.2 Hz, H-3), 5.84 (1H, d, *J* = 7.3 Hz, NH), 6.91 (1H, t, *J* = 2.1 Hz, H-2); ^{13}C NMR (50.32 MHz, DMSO-d_6, δ ppm) 8.8, 9.5, 22.8, 25.1, 25.6, 29.8, 54.5, 58.3, 74.5, 81.1, 128.1 (C-1), 137.7 (C-2), 166.9 (CO of carboxyl group), 169.6 (CO of acetyl group).

Synthesis of 1,12-dicarba-closo-dodecaborane(12)-1-prop-3-yl (3R,4R,5S)-4-acetamido-5-azido-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate (3)

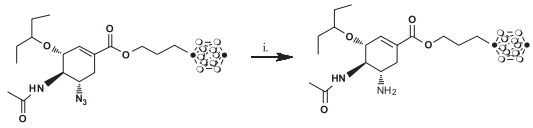


i) 1-(3-hydroxypropyl)-1,12-dicarba-closo-dodecacarborane(12), DMAP, DCC, CH_2Cl_2

To a solution of the (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylic acid (**2**, 0.109 mmol, 33.74 mg) and 1-(3-hydroxypropyl)-1,12-dicarba-closo-dodecaborane (12) (0.435 mmol, 87.85 mg) in dry dichloromethane (675.3 mL), was added DMAP (0.016 mmol, 1.99 mg, 0.15 eq.). The solution was cooled to 0–4°C in an ice bath and DCC (0.1413 mmol, 36.79 mg, 1.3 eq.) was added. The reaction mixture was stirred under an argon atmosphere at room temperature until TLC analysis (hexane:AcOEt, 1:1, v/v) showed completion of the reaction (ca.1.5 h). The solvent was evaporated to dryness under vacuum and the residue was purified by silica gel column chromatography eluting with hexane/diethyl ether (2:1, v:v). Yield 97.4% (52.4 mg).

TLC (hexane:AcOEt, 1:1, v/v): Rf = 0.5; ^{1}H NMR (600 MHz, CD_2Cl_2, δ, ppm): 0.89–0.95 (6H, m, 2 × CH_3(c)), 1.44–1.61 (6H, m, CH_2-2 from linker and 2 × CH_2(b)), 1.20–2.90 (10H, m, carborane), 1.74–1.77 (2H, m, CH_2-1 from linker), 2.03 (3H, s, CH_3 of acetyl group), 2.18–2.24 (1H, m, H-6), 2.76 (1H, br s, CH of carborane), 2.79–2.82 (1H, dd, H-6, *J* = 17.4 and 5.4 Hz), 3.33–3.37 (1H, m, CH(a)), 3.45–3.52 (1H, m, H-4), 3.95–4.02 (2H, m, CH_2O from linker), 4.08–4.12 (1H, m, H-5), 4.42 (1H, m, H-3), 5.97 (1H, d, NH, *J* = 7.8 Hz), 6.74 (1H, m, H-2); ^{13}C NMR (150.95 MHz, CD_2Cl_2, δ, ppm): 9.81 and 10.32 (CH_3(c)), 24.11 (CH_3 of acetyl group), 26.49 and 27.09 (CH_2(b)), 29.20 (CH_2-2 from linker), 31.25 (C-6), 36.21 (CH_2-1 from linker), 57.96 (C-4), 58.66 (C-5), 59.29 (CH of carborane), 64.54 (CH_2O from linker), 74.89 (C-3), 82.82 (CH(a)), 84.60 (C of carborane), 128.65 (C-1), 138.94 (C-2), 166.25 (CO of carboxyl group), 171.55 (CO of acetyl group); ^{11}B NMR (192.59 MHz, CD_2Cl_2, decoupled: δ, ppm): –12.61, –15.04 (2s, 10B, carborane); (coupled: δ, ppm): –12.18, –13.03, –14.61, –15.47 (2d, 10B, carborane); IR (KBr, cm^{-1}): 3069, 2607, 2100, 1720, 1656; MS (ESI, +Ve): m/z = 496.93 $[M + 1]^+$, 518.20 $[M + Na^+]$, 534.56 $[M + K^+]$ ($C_{19}H_{38}B_{10}N_4O_4$, calculated: 495.39 (100 %)).

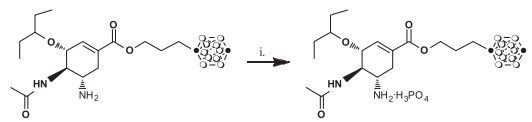
Synthesis of 1,12-dicarba-closo-dodecaborane(12)-1-prop-3-yl (3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate (4)



i) PPh_3, H_2O, THF, 65°C

To a solution of 1,12-dicarba-closo-dodecaborane(12)-1-prop-3-yl (3*R*,4*R*,5*S*)-4-acetamido-5-azido-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate (**3**, 251 mg, 0.51 mmol) in THF(6 mL) were added triphenylphosphine (202.5 mg, 0.75 mmol) and water (0.75 mL). The solution was vigorously stirred and heated at 65°C (ca. 5 h) until TLC analysis (CH_3OH:$CHCl_3$, 17:3, v/v) showed complete conversion of an intermediate product (Rf = 0.05) to the expected amine (Rf = 0.2). The solvent was evaporated to dryness under vacuum and a crude product was purified by silica gel column chromatography using a linear gradient of methanol in dichloromethane as an eluting solvent system. Yield 88.3% (210 mg). Amine **4** was characterized as the phosphate salt **5**.

Synthesis of 1,12-dicarba-closo-dodecaborane(12)-1-prop-3-yl (3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate phosphate [1:1] (5)



i) 85% H_3PO_4, EtOH-AcOEt

To a solution of 1,12-dicarba-closo-dodecaborane(12)-1-prop-3-yl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate (**4**, 210 mg, 0.45 mmol) in ethyl acetate (2.5 mL), 0.87 mL of a solution of 85% phosphoric acid in ethanol (prepared from 298 mg of 85% phosphoric acid in 5 mL of ethanol) was added. The resulting solution was vigorously stirred and heated at 70°C for 10 min, after which a precipitate began to appear. The suspension was stirred at room temperature for an additional 30 min. The crystals were filtered, washed with cold ethyl acetate and finally dried under vacuum at room temperature. Yield 61.0% (155 mg).

TLC (CH_2Cl_2:CH_3OH, 4:1, v/v): Rf = 0.5; ^{1}H NMR (600 MHz, CD_2Cl_2, δ, ppm): 0.80 (3H, t, CH_3(c)), 0.86 (3H, t, CH_3(c)), 1.20–2.90 (10H, m, carborane), 1.37–1.48 (6H, m, CH_2-2 from linker and 2 × CH_2(b)), 1.72–1.75 (2H, m, CH_2-1 from linker), 1.88 (3H, s, CH_3 of acetyl group), 2.14–2.20

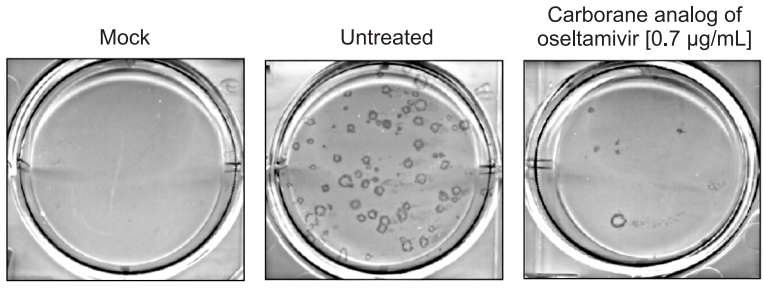


Figure 1. Effect of the carborane analog of oseltamivir on IAV plaque formation in MDCK cells. MDCK cells were mock infected or infected with influenza A virus. At 1 h post infection, cells were treated with the carborane analog of oseltamivir or left untreated (positive control). Three days post infection, cells were fixed and immunoperoxidase monolayer assay using monoclonal antibody specific for M1 protein was performed to detect IAV plaques

(1H, dd, H-6), 2.66–2.70 (1H, dd, H-6, J = 17.4 and 5.4 Hz), 3.12–3.17 (1H, m, H-4), 3.33–3.37 (1H, m, CH(a)), 3.63–3.68 (2H, m, H-5 and CH of carborane), 3.89–3.98 (2H, m, CH_2O from linker), 4.11–4.12 (1H, m, H-3), 6.16 (2H, br s, NH_2), 6.59 (1H, s, H-2), 8.20 (1H, d, NH, J = 9.0 Hz); ^{13}C NMR (150.95 MHz, CD_2Cl_2, δ, ppm): 8.94 and 9.50 (2 × CH_3(c)), 23.24 (CH_3 of acetyl group), 25.28 and 25.74 (2 × CH_2(b)), 28.14 (CH_2-2 from linker), 30.12 (C-6), 34.70 (CH_2-1 from linker), 48.79 (C-4), 54.04 (C-5), 59.21 (CH of carborane), 63.32 (CH_2O from linker), 74.85 (C-3), 81.26 (CH(a)), 83.82 (C of carborane), 127.63 (C-1), 138.65 (C-2), 165.21 (CO of carboxyl group), 170.44 (CO of acetyl group); ^{11}B NMR {^{1}H BB} (192.59 MHz, CD_2Cl_2, δ, ppm): –12.60 (s, 5B, carborane); –15.01 (s, 5B, carborane); ^{11}B NMR (192.59 MHz, CD_2Cl_2, δ, ppm): = –12.59 (d, 5B, carborane); –15.01 (d, 5B, carborane); IR (KBr, cm^{-1}): 1648, 1711, 2603, 2875, 2932, 2967, 3067, 3176; MS (ESI, +Ve): m/z = 469.90 $[M]^+$ (52%) ($C_{19}H_{40}B_{10}N_2O_4$, calculated: 469.40 (100 %)).

Biological activity tests

Cytotoxicity assay

To determine MDCK cell viability CellTiter 96 AQueous non-radioactive cell proliferation assay (MTS) (Promega) was performed. MDCK cells were grown and incubated on 96-well plates at 37°C in the presence of different concentrations of compounds (in triplicate) for 2 days. Cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) for 3 h at 37°C. The absorbance at 490 nm was read using a microplate reader. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

Plaque reduction assay

For determination of antiviral activity, the plaque reduction assay was performed, as previously described [10]. In brief, MDCK cell monolayers in 12-well plates were infected with IAV for 1 h at 37°C. Unbound virus was removed by washing with serum-free medium, and the cell monolayers were then overlaid with fresh serum-free medium containing 1.2% Avicel, 2 µg/mL TPCK-trypsin and increasing amounts of inhibitors. After 3 days, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and virus-infected cells were immunostained by incubating for 1 h with a monoclonal antibody specific for M1 protein diluted 1:1000 in PBS containing 1% Tween 20 and 5% FBS. Anti-mouse horseradish peroxidase (HRP)-conjugated antibody was used as secondary antibody (diluted 1:1000 in PBS containing 1% Tween 20 and 5% FBS). Plaques were detected using H_2O_2/AEC (3-amino-9-ethylcarbazole) and counted. IC_{50} was calculated as the concentration at which the number of plaques was reduced by 50% compared to untreated infected control cells.

A carborane analog of oseltamivir was evaluated at different concentrations for its anti-IAV activity *in vitro* (Fig. 1) and monitored by the MTS assay in the MDCK cells. Oseltamivir phosphate and osetamivir carboxylic acid were used as the reference drugs. Oseltamivir phosphate and oseltamivir carboxylic acid appeared to have similar IC_{50} values against H5N2 virus. Unexpectedly, newly obtained carborane ester showed at least a 10 times less potent inhibitory activity against influenza virus with IC_{50} of 0.5 µg/mL determined in plaque reduction assay. The difference in the observed inhibitory property of synthesized compounds indicated that unlike in other cases, this particular combination of proven pharmacophore with boronate cluster resulted in deleterious change in inhibitory activity.

CONCLUSIONS

Novel derivative of the oseltamivir acid carboxylic acid was obtained by exploiting intermediates from technical synthesis of the antiviral drug active pharmaceutical ingredient. The new chemical entity was characterized with appropriate analytical and spectral data. The exchange of ethyl ester group for carborane cluster in oseltamivir carboxylate offers no advantage in terms of antiviral activity.

Acknowledgment

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REFERENCES

1. Combating the threat of pandemic influenza; drug discovery approaches, Torrence P.F., Ed., J Wiley & Sons, Hoboken, NJ 2007.
2. McCellan K., Perry C.M.: Drugs 61, 263 (2001).
3. Moscona A.: N. Engl. J. Med. 353, 1363 (2005).
4. Von Itzstein M., Wu W.-Y., Kok G.B., Pegg M.S., Dyason J.C., Jin B., Phan T.V. et al.: Nature 363, 418 (1993).
5. Runrotmongkol T., Frecer V., De-Eknamkul W., Hannonguba S., Miertus S.: Antivir. Res. 82, 51 (2009).
6 Lesnikowski Z.J.: New opportunities in boron chemistry for medical applications. in Boron Sciences. New technologies and applications, Hosmane N.S. Ed., pp. 3–19, CRC Press, Boca Raton, FL 2011.
7. Shibasaki M., Kanai M.: Eur. J. Org. Chem. 2008, 1839.
8. Trost B.M., Zhang T.: Angew. Chem. Int. Ed. Engl. 47, 3759 (2008).
9. Satoh N., Akiba T., Yokoshima S., Fukuyama T.: Tetrahedron 65, 3239 (2009).
10. Matrosovich M., Matrosovich T., Garten W., Klenk H.D.: Virol. J. *3*, 63 (2006).