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Dihydrodibenzothiepine: Promising hydrophobic pharmacophore in the influenza cap-dependent endonuclease inhibitor

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ABSTRACT

This work describes a set of discovery research studies of an influenza cap-dependent endonuclease (CEN) inhibitor with a carbamoyl pyridone bicycle (CAB) scaffold. Using influenza CEN inhibitory activity, antiviral activity and pharmacokinetic (PK) parameters as indices, structure activity relationships (SAR) studies were performed at the N-1 and N-3 positions on the CAB scaffold, which is a unique template to bind two metals. The hydrophobic substituent at the N-1 position is extremely important for CEN inhibitory activity and antiviral activity, and dihydrodibenzothiepine is the most promising pharmacophore. The compound **(*S*)-13i** showed potent virus titer reduction over oseltamivir phosphate in an *in vivo* mouse model. The CAB compound described herein served as the lead compound of baloxavir marboxil with a tricyclic scaffold, which was approved in Japan and the USA in 2018.

Influenza is a viral respiratory infection caused by influenza virus, with high fever, general malaise, cough, and sore throat appearing as symptoms. Seasonal influenza is repeatedly epidemic around the world, with 3–5 million people becoming infected every year.[1] In 2009, the strain A(H1N1)pdm09 became prevalent and spread rapidly because it was significantly different in antigenicity from the seasonal influenza circulating until then and humans had not developed immunity to it. Types/subtypes including another subtype of new pandemic influenza might affect the severity profile and lead to a large number of deaths.[2,3] Moreover, a pandemic might occur again in the future.

At present, neuraminidase inhibitors such as oseltamivir, zanamivir, peramivir, and laninamivir are used to treat influenza.[4] However, problems are arising such as the emergence of viruses resistant to these therapeutic agents and new viruses against which they are ineffective.[5] Thus, there is a growing need for new medication which has a different mechanism for its antiviral effect. We started research on an influenza cap-dependent endonuclease (CEN) inhibitor in 2006 and discovered baloxavir marboxil, which was approved in Japan and the US in 2018.

CEN has two metal ions (Mg^{2+} or Mn^{2+}) in its active site and is an enzyme nesessary for virus replication.[6] It has been reported that compounds **1**–**3**, the scaffold of which can chelate to these two metal ions, inhibit the CEN activity (Fig. 1).[7–9] However, they had insufficient antiviral activity and were not developed as clinical candidates. We previously reported that compound **4a**, with a carbamoyl pyridone bicycle (CAB) scaffold found by structure activity relationships (SAR) studies of our compound library, inhibited influenza CEN activity and thereby interfered with influenza virus replication.[10] **4a** is largely composed of two parts. The bicyclic carbamoyl pyridone moiety coordinates with the metal cofactor present in the active site of CEN. The lipophilic benzhydryl group at the N-1 position of the CAB core is considered to play an important role in binding to the enzyme by hydrophobic interaction. In this paper, we report on optimization of the lipophilic side chain at the N-1 position and the substituent at the N-3 position of the CAB scaffold. As a result, we discovered that the compound having dihydrodibenzothiepine at the N-1 position exerted remarkably potent CEN inhibitory activity, antiviral activity and reduction of virus titer in an *in vivo* mouse model.

The synthetic route is shown in Scheme 1. Amination of the commercially available pyrone **5** using ammonia gave pyridone **6**. After condensation of carboxylic acid with the corresponding amine, *N*-amination using *O*-(2,4-dinitrophenyl)hydroxylamine was performed to give intermediate **8**. Bicyclic pyridone derivatives **9** were synthesized by cyclization with paraformaldehyde. Subsequently, compound **4** and **10**–**14** were obtained by *N*-alkylation with alkylhalide and debenzylation with TFA, or addition of alcohol accompanied by debenzylation under acidic condition.

The dihydrodibenzothiepines having a substituent on the benzene ring were synthesized according to the literature.[11] First, ethyl

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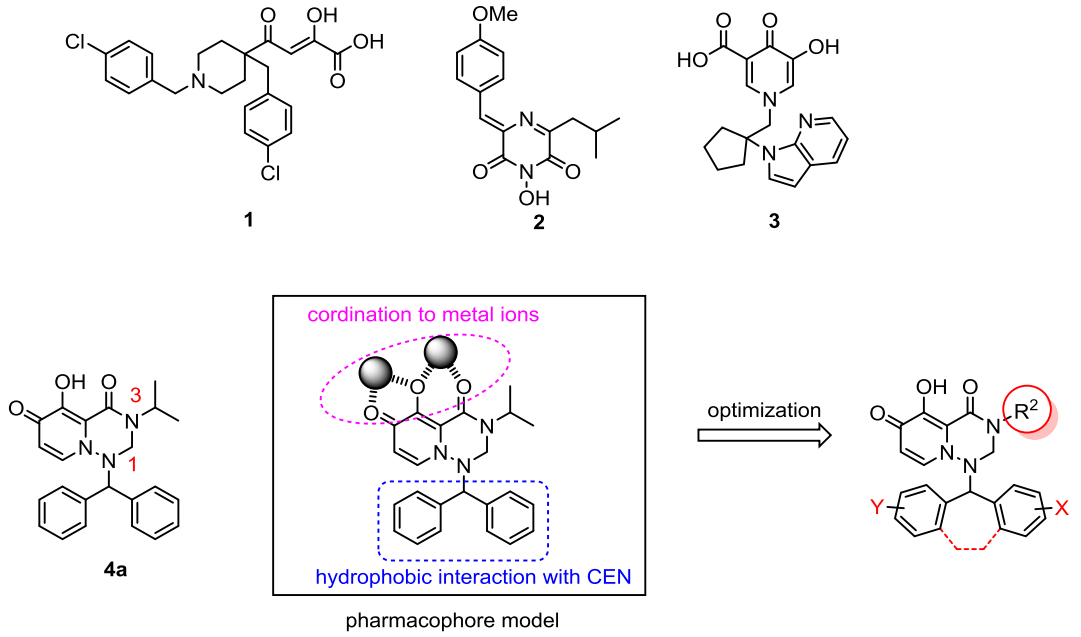
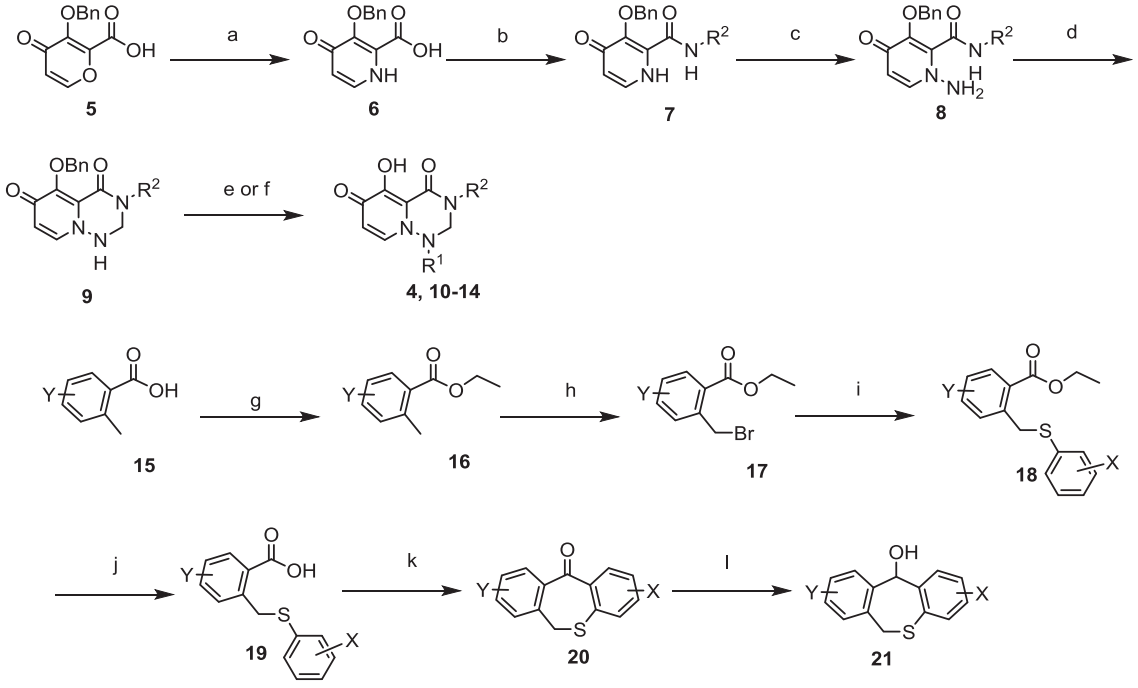


Fig. 1. Reported influenza CEN inhibitors and our design of new inhibitors.

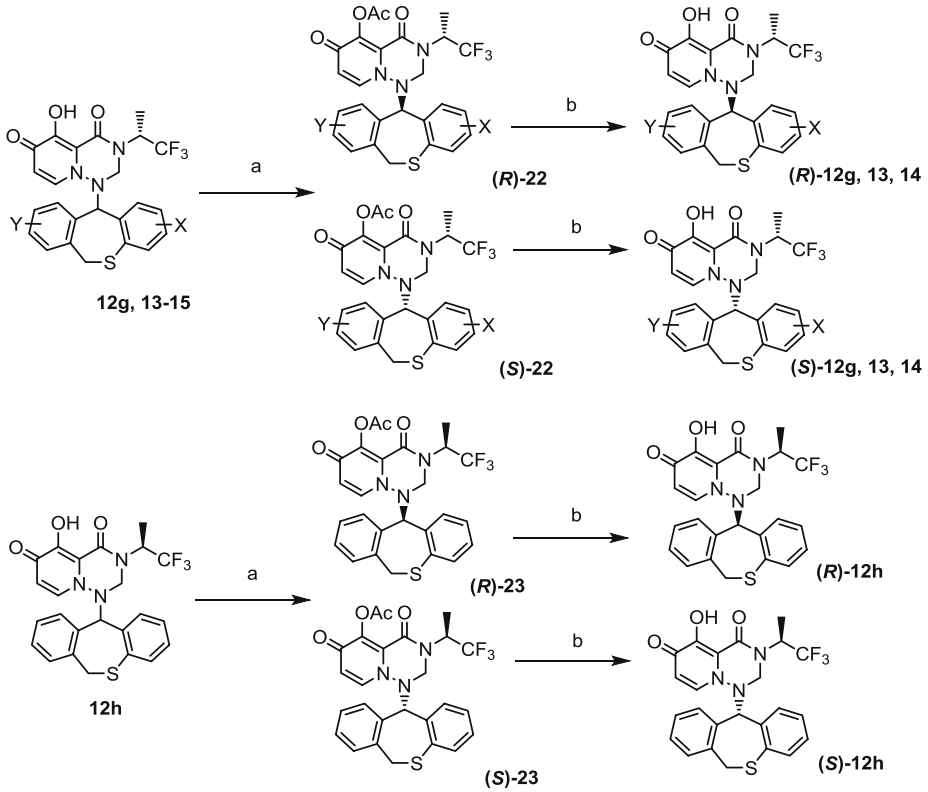


Scheme 1. Synthesis of CAB compounds. Reagents and conditions: (a) 28% NH_3 aq., r.t.; (b) (1) WSC-HCl, HOBt, DMF, r.t., (2) amine, r.t.; (c) *O*-(2,4-dinitrophenyl) hydroxylamine, K_2CO_3, DMF, r.t.; (d) paraformaldehyde, AcOH, toluene, 100 °C; (e) (1) R^1-X, Cs_2CO_3, DMF, r.t., (2) TFA, r.t.; (f) R^1-OH, H_2SO_4, AcOH, r.t.; (g) EtI, K_2CO_3, DMF, r.t.; (h) NBS, CCl_4, reflux; (i) benzenethiol, K_2CO_3, acetone, reflux; (j) 2 N NaOH aq., EtOH, r.t.; (k) PPA, 120 °C; (l) $NaBH_4$, MeOH, 0 °C ~ r.t.

esterification of 2-methylbenzoic acid **15** having a substituent and bromination of methyl group afforded **17**. After reaction with a benzenethiol, the benzoic acid **19** was obtained by hydrolysis of the ethyl ester. The dihydrodibenzothiepine ring was constructed by treatment with polyphosphoric acid (PPA) and reduction of the ketone gave alcohol **21**.

In the case of compounds having a 1,1,1-trifluoroisopropyl group at the N-3 position, acetylation of the hydroxyl group of the CAB scaffold made it possible to separate the two diastereomers by usual silica gel column chromatography. By hydrolyzing the acetyl group, we succeeded in obtaining derivatives with a dihydrodibenzothiepine of *R* or *S* configuration, respectively (Scheme 2). The relative and absolute stereochemistry of **(*R*)-23** was confirmed by single crystal X-ray and that of other compounds was determined based on this result (Fig. 2).

First, we studied the effect of substituents on the benzhydryl group at the N-1 position (Table 1). In the case (**4b**-**4f**) of single substitution of Me or Cl, no great improvement was observed in either CEN inhibitory activity or antiviral activity. Second, when two substituents were symmetrically introduced, some improvement in antiviral activity was observed in **4h** having two Cl atoms, but in other cases, the activities decreased (**4g**, **4j**, **4k**). We thought that this was due to the reduction of cell membrane permeability by the introduction of a polar substituent



Scheme 2. Separation of stereoisomers of CAB compounds. Reagents and conditions: (a) Ac_2O, Et_3N, DMAP, CH_2Cl_2, r.t.; (b) 2 N NaOH aq., THF-MeOH, r.t.

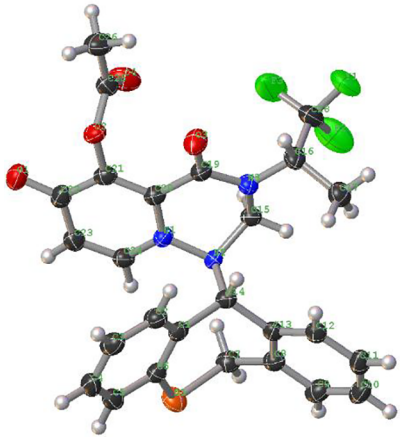


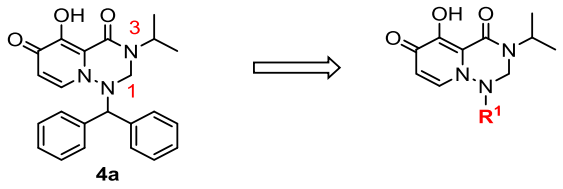
Fig. 2. Single crystal X-ray structure of **(*R*)-23**: Thermal ellipsoids are drawn at the 30% probability level.

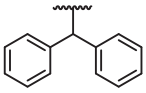
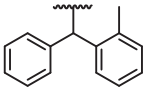
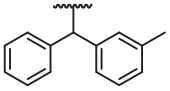
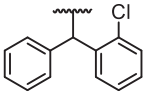
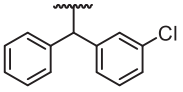
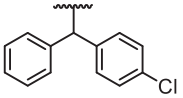
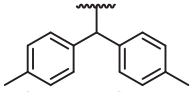
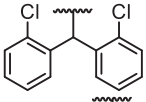
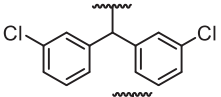
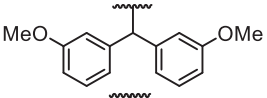
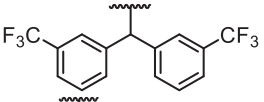
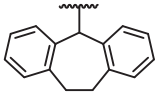
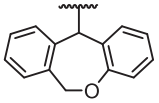
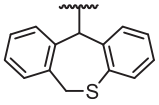
or an increase in molecular size. To improve membrane permeability, we designed dihydrodibenzoannulene **10**, dihydrodibenzooxepin **11** and dihydrodibenzothiepine **12** derivatives by linking two benzene rings of a benzhydryl group to constrain the free rotation. Although no significant improvement in activity was observed in **11**, both the CEN inhibitory activity and the antiviral activity were improved about fourfold in **10**. Moreover, despite its racemic form, **12a** demonstrated almost equal activity to that of **10**. The N-1 substituent of compound **11** is less lipophilic than those of **10** and **12**. Therefore, the lower activities of **11** seem to be due to the reduction of affinity for the enzyme and/or cell membrane permeability. Based on this result, we selected dihydrodibenzothiepine as a substituent at the N-1 position for further optimization.

Table 2 shows the effect of the substituent at the N-3 position of the CAB scaffold with dihydrodibenzothiepine at the N-1 position. As we demonstrated in the previous paper, the region around the N-3 position is exposed to the solvent and does not have much effect on the potency of the compounds.[10] Therefore, we aimed to use the N-3 subtituent to adjust the physicochemical properties of the molecule. Since compound **12a** had a large rat iv clearance value (32.7 mL/min/kg), we investigated whether it would be improved by modification of the isopropyl group at the N-3 position. Conversion to a cyclopropyl group or substitution with a polar group (OMe, NMe_2) and deuterium instead worsened clearance and did not significantly alter antiviral activity (**12b-e**). However, in the case of substitution with a F atom, the clearance of **(*S*)-12g** with (*R*)-1,1,1-trifluoro-2-propyl group was improved (15.8 mL/min/kg). This can be attributed to improved stabilisation in rat microsomes and a decreased fu value in plasma. In addition, **(*S*)-12g** was the most potent of the four isomers (**12g**, **12h**) and demonstrated more than 3 times higher antiviral activity than **12a**, indicating a good balanced profile in both activity and PK profile.

Finally, **(*S*)-12g** was used as a lead compound to optimize the substituent on the dihydrodibenzothiepine at the N-1 position (Table 3). Since introduction of the Cl atom on the benzhydryl group (**4h** and **4i**) in Table 1 improved the antiviral activity, we decided to thoroughly investigate the effect of a halogen atom (F or Cl) on the dihydrodibenzothiepine ring. The F atom led to about 4-fold more CEN inhibitory activity than **(*S*)-12g** when introduced at the positions 7 or 8 (**(*S*)-13e**, **(*S*)-13f**), but there was no significant change at other positions. In addition, when the F atom was introduced at both positions 7 and 8, further improvement of CEN inhibitory activity was observed (**(*S*)-13i**). **(*S*)-14f** and **(*S*)-14h** having a Cl atom at position 8 or 10 showed CEN inhibitory activity below 10 nM. The enhanced enzyme inhibitory activity of these compounds suggests the presence of small lipophilic pockets in the vicinity of the dihydrodibenzothiepine ring that can accommodate F and Cl atoms. Although many compounds exhibit antiviral activity of EC50 values of low nM, there is a discrepancy between the values of IC50 and EC50 in some cases. The reason for this is unknown, but one possibility is that the cellular assay system reached the limit of detection. As described above, **(*S*)-13i** and

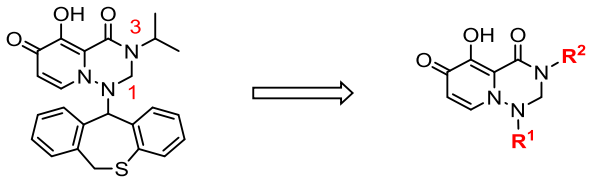
Table 1
SAR of N-1 position of CAB scaffold.



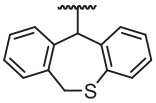
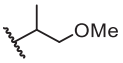
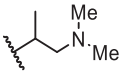
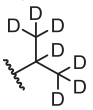
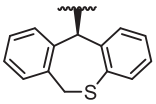
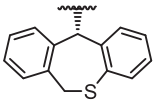
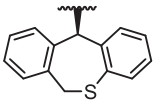
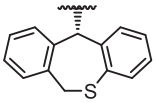
compound	R^1	CEN IC50[a] (nM)	CPE EC50[b] (nM)
4a		286	81.6
4b		142	73.4
4c		276	219
4d		210	77.8
4e		101	75.9
4f		291	94.0
4g		394	440
4h		37.0	48.2
4i		230	65.8
4j		1310	589
4k		456	386
10		60.1	20.1
11		264	71.0
12a		61.9	16.9

Reported values were the median of two or more experiments. [a] CEN inhibitory activity was measured by enzymatic assay as reported previously.[9] [b] Madin-Darby bovine kidney (MDBK) cells were infected with influenza A virus (A/WSN/33 dilutions at 50 $TCID_{50}$/well in the 96 well plate), and incubated with test compounds at 37 °C in a CO_2 incubator for 72 hr, and the concentration of test compound resulting in 50% cell protection was reported as the EC_{50}.

Table 2
SAR of N-3 position of CAB scaffold.



12a

compound	R^1	R^2	CEN IC50[a] (nM)	CPE EC50[b] (nM)	Met. Stab. Microsome[c] (%)	Rat iv Cl[d] (mL/min/kg)	Fu[e] (%)
12a			61.9	16.9	72.6	32.7	7.68
12b			35.0	13.7	73.7	46.0	7.83
12c			57.9	20.7	58.6	49.1	10.6
12d			151	84.1	76.4	98.7	NC
12e			43.4	11.7	74.4	39.1	6.01
12f			113	27.5	84.9	33.5	NC
(*R*)-12 g			1890	131	NT	NT	NT
(*S*)-12 g			49.7	4.69	98.1	15.8	1.03
(*R*)-12 h			285	21.7	NT	NT	NT
(*S*)-12 h			112	18.6	79.8	NT	NT

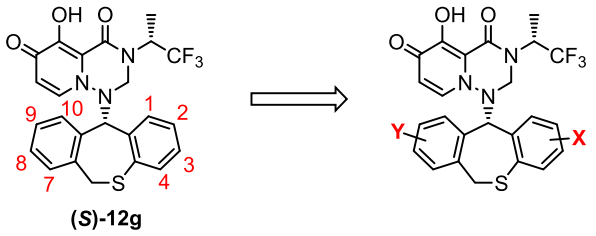
Reported values were the median of two or more experiments. Values of PK parameters are the means of duplicate experiments. [a] CEN inhibitory activity was measured by enzymatic assay as reported previously.[9] [b] Madin-Darby bovine kidney (MDBK) cells were infected with influenza A virus (A/WSN/33 dilutions at 50 $TCID_{50}$/well in the 96 well plate), and incubated with test compounds at 37 °C in a CO_2 incubator for 72 hr, and the concentration of test compound resulting in 50% cell protection was reported as the EC_{50}. [c] Metabolic stability in the presence of rat liver microsomes was represented as the % compound remaining at a concentration of 2 μM after 30 min, incubated at 37 °C. [d] Rat clearance was measured by LC/MS/MS after a single intravenous administration. [e] Free fraction ratio in the presence of rat serum. NT means not tested. NC means not calculated.

(*S*)-14h were discovered as compounds having not only strong CEN inhibitory activity but antiviral activity. Furthermore, when the PK profile was examined, **(*S*)-13i** showed lower rat iv clearance value than **(*S*)-14h** (**(*S*)-13i**: 11.0 mL/min/kg vs. **(*S*)-14h**: 19.6 mL/min/kg). Therefore compound **(*S*)-13i** was selected for further evaluation.

In the mouse model inoculated with A/WSN/33 influenza virus (100 $TCID_{50}$/mouse), **(*S*)-13i** or oseltamivir phosphate was orally administered twice a day (BID) at 5 days after infection (Fig. 3). At 24 h after the first dosage, the amount of virus titer (log $TCID_{50}$/mL) in the lung was measured using Madin-Darby canine kidney (MDCK) cells. When **(*S*)-13i** was administered at 0.4 mg/kg BID, the viral load was reduced by one-tenth compared to the administration of a clinical dose equivalent to oseltamivir phosphate (5 mg/kg BID). Furthermore, by increasing the dose of **(*S*)-13i** to 10 mg/kg BID, the lung viral load was reduced to about one-thirtieth of that after administration of oseltamivir phosphate (5 mg/kg BID). Thus, **(*S*)-13i** exhibited a superior virus reduction effect to that of oseltamivir phosphate *in vivo*. This result suggests that a CEN inhibitor is potentially useful for the treatment of influenza.

In conclusion, we optimized the N-1 and N-3 positions of the CAB scaffold of **4a** as a lead compound which was reported in the previous paper. As a result of SAR studies using CEN inhibitory activity and antiviral activity as an index, we found that the lipophilic substituent at the N-1 position was extremely important for the activity, with dihydrodibenzothiepine being the most promising. Furthermore, by considering PK parameters, we discovered that **(*S*)-13i** having (*S*)-7,8-difluoro-6,11-dihydrodibenzothiepine at the N-1 position and (*R*)-1,1,1-trifluoroisopropyl group at the N-3 position was an outstanding

Table 3
Optimization of the substituent on the dihydrodibenzothiepine ring.



(*S*)-compound	X or Y	CEN IC50[a] (nM)	CPE EC50[b] (nM)	Met. Stab. Microsome[c] (%)	Rat iv Cl[d] (mL/min/kg)	Fu[e] (%)
12g	H	49.7	4.69	98.1	15.8	1.03
13a	1-F	37.1	15.1	97.4	9.09	1.19
13b	2-F	39.2	8.16	103	15.4	0.975
13c	3-F	48.2	14.3	93.9	13.4	0.807
13d	4-F	66.4	3.41	95.6	10.3	0.574
13e	7-F	12.5	3.85	91.8	8.37	0.978
13f	8-F	12.3	3.54	95.3	11.6	0.383
13g	9-F	44.3	4.68	98.9	21.3	1.29
13h	10-F	31.8	3.75	102	18.0	1.2
13i	7,8-F,F	5.57	4.28	81.1	11.0	0.411
14a	1-Cl	76.2	18.8	85.4	10.2	0.589
14b	2-Cl	24.9	70.8	NT	NT	NT
14c	3-Cl	62.1	21.2	101	12.2	0.303
14d	4-Cl	23.2	13.5	92.3	6.78	0.329
14e	7-Cl	30.0	3.04	102	7.36	0.293
14f	8-Cl	6.99	10.5	96.1	5.70	0.117
14g	9-Cl	16.6	4.21	94.1	12.4	0.514
14h	10-Cl	9.95	3.52	93.3	19.6	1.79

Reported values were the median of two or more experiments. Values of PK parameters are the means of duplicate experiments. [a] CEN inhibitory activity was measured by enzymatic assay as reported previously.[9] [b] Madin-Darby bovine kidney (MDBK) cells were infected with influenza A virus (A/WSN/33 dilutions at 50 $TCID_{50}$/well in the 96 well plate), and incubated with test compounds at 37 °C in a CO_2 incubator for 72 hr, and the concentration of test compound resulting in 50% cell protection was reported as the EC_{50}. [c] Metabolic stability in the presence of rat liver microsomes was represented as the % compound remaining at a concentration of 2 μM after 30 min, incubated at 37 °C. [d] Rat clearance was measured by LC/MS/MS after a single intravenous administration. [e] Free fraction ratio in the presence of rat serum. NT means not tested. NC means not calculated.

compound. In a mouse model inoculated with A/WSN/33 influenza virus, the virus reduction effect of **(*S*)-13i** exceeded oseltamivir phosphate. We continued optimizing the compound, which led to the discovery of baloxavir marboxil (Fig. 4). Details of this will be reported in the near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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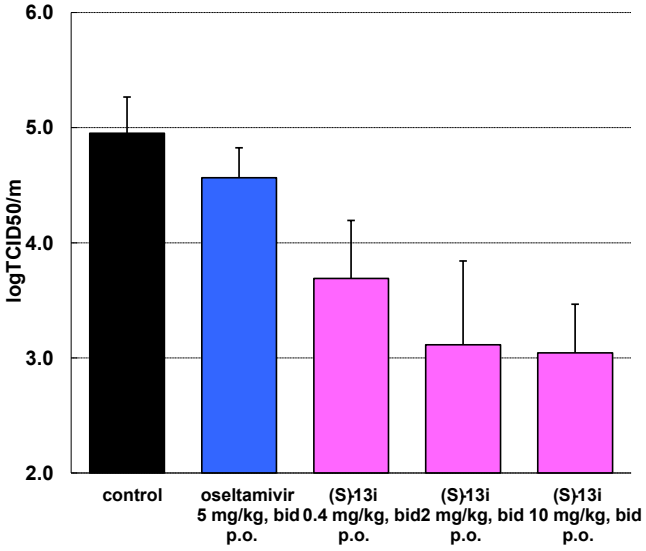

Fig. 3. *In vivo* efficacy of **(*S*)-13i** in the mouse model inoculated with A/WSN/33 influenza virus. **(*S*)-13i** or oseltamivir phosphate was orally administered twice a day (BID) at 5 days after infection. Next, the amount of virus titer (log $TCID_{50}$/mL) in the lung at 24 h after the first dosage was measured.

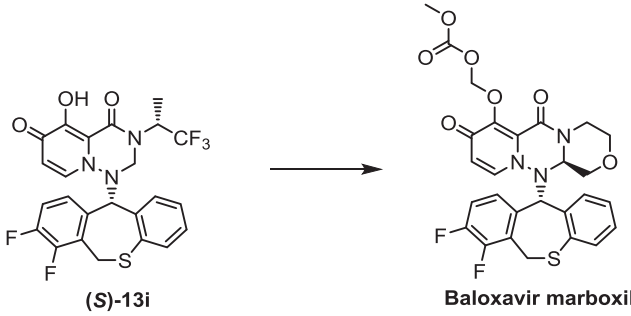

Fig. 4. Road to baloxavir marboxil from **(*S*)-13i.**

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127547.

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