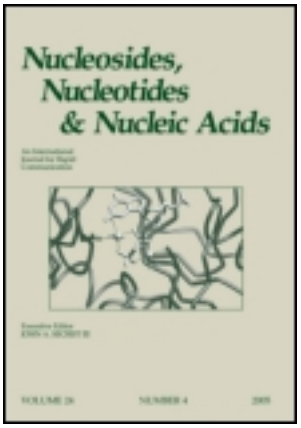

Nucleosides, Nucleotides and Nucleic Acids

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Synthesis and Biological Activity of Bimorpholine and its Carbanucleoside

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF BIMORPHOLINE AND ITS CARBANUCLEOSIDE

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□ *A new enantiomerically pure carbacyclic nucleoside analogue with bimorpholine as a nonaromatic nucleobase was synthesized. The nucleoside analogue and bimorpholine were tested for cytotoxicity using an MTT assay and* the *xCELLigence System. Both assays revealed that compound* ***3*** *was highly cytotoxic at a 50 μM concentration while the cytotoxic effect of compound* ***1*** *was much less prominent. No antiretroviral activity was detected for this compound. In contrast, it acted as a potent inhibitor of hepatitis C virus (HCV) replication. Most likely this effect originates largely from the cytotoxicity of the compound; however, it is possible that a specific mechanism of HCV inhibition also exists.*

Keywords Bimorpholines; carbacyclic nucleoside analogue; hepatitis C virus

INTRODUCTION

Substituted morpholines are widely occurring structural motifs in various bioactive natural products, as well as in several pharmaceutically important compounds.[1] The spectrum of the biological properties of morpholine-containing compounds is very wide and includes antiviral activity.[2–4] On the other hand, nucleosides are a very important group of antiviral compounds. In recent decades, carbocyclic nucleosides have received a great deal of attention because they are recognized by enzymes and receptors that

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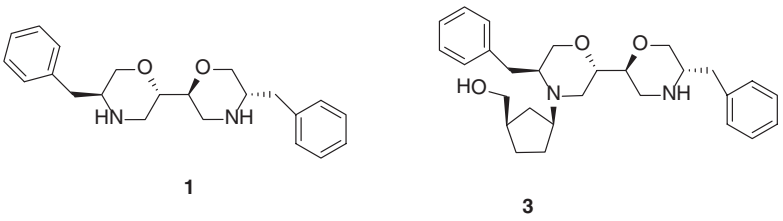


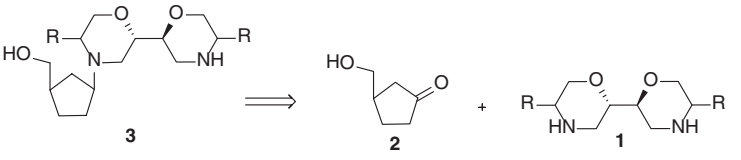
FIGURE 1 Tested compounds.

also respond to standard nucleosides.[5,6] Carbocyclic nucleosides are inert against the action of enzymes that cleave glycosidic bonds, making them metabolically more stable. In connection with our ongoing project on the synthesis[7,8] and investigation of antiviral compounds,[9] we present here the results of our studies of the nonnucleoside antiviral compound **1**. Our preliminary screening of 5,5′-disubstituted-2,2′-bimorpholine derivatives revealed no cytotoxicity (at a 50 μM concentration) using a standard MTT assay; no clear activity against retroviruses (human immunodeficiency virus type 1) was detected in a biological assay (data not shown). Surprisingly, one of the compounds had potent anti-HCV (hepatitis C virus) activity at 50 μM and that prompted us to derivatize it with sugar analogues. Thus, we used this potential antiviral compound as a nucleobase and designed and synthesized the new carbocyclic nucleoside analogue **3** (Figure 1). The compounds were tested for the ability to suppress replication of HCV, a positive single-strand RNA virus that has infected an estimated 3% of the world's population and is one of the main causes of liver cirrhosis and cancer.[10,11]

RESULTS AND DISCUSSION

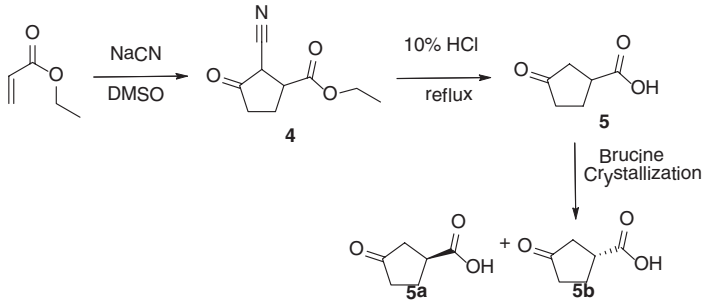
Synthesis of the Compounds

The synthesis of bimorpholine **1** was described by us previously.[12,13] Although glycosyl derivatives with secondary amines are known,[14] the reaction of bimorpholine with ribose (acylated or nonacylated) resulted in an unstable hemiaminal. Carbocyclic nucleosides, on the other hand, give a stable C–N bond. The synthetic scheme to 2,3-dideoxy carbocyclic nucleosides with bimorpholine as a base is very straightforward (Scheme 1).



SCHEME 1 Retrosynthetic route to nonaromatic nucleoside carba-analogue **3**.

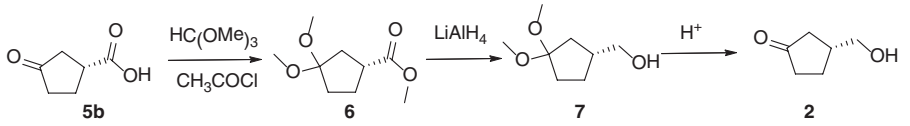
The target **3** can be obtained by the reductive amination of cyclopentanone **2** with bimorpholine **1**. Our approach started with 3-oxocyclopentanecarboxylic acid **5** that was prepared using the literature procedure reported by Stetter and Kuhlmann[15] (Scheme 2). The skeleton of the carbocycle was formed via a cascade of reactions that involved a conjugate addition of sodium cyanide to ethyl acrylate, an attack of the formed ester enolate on the second molecule of ethyl acrylate and cyclization. This one-pot procedure gave a cyclopentanone derivative **4** in a 42% yield. Simultaneous acidic hydrolysis and decarboxylation afforded racemic acid **5** in a 64% yield.



SCHEME 2 Synthesis and resolution of enantiomers of keto acid **5**.

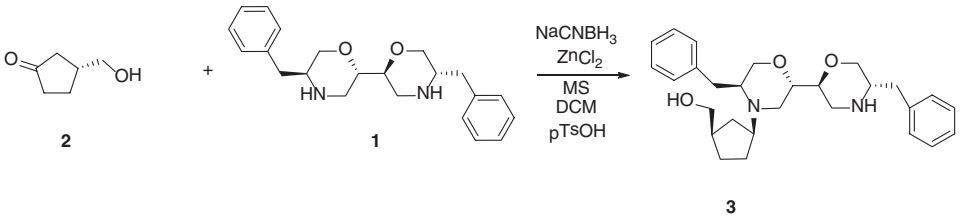
The treatment of racemic acid **5** with (−)-brucine and the crystallization of diastereoisomeric salts from water[16] enabled us to resolve enantiomers. *R*-enantiomer **5b** was obtained after four sequential crystallizations in 98% of ee. The enantiomeric purity of acid **5b** was determined after derivatization with 2-bromo-2′-acetonaphthone by chiral HPLC on column Chiralcel ODH.

Next, the selective reduction of a carboxylic group in the presence of a carbonyl group was performed (Scheme 3). The esterification and acetalization of keto acid **5b** with trimethyl orthoformate, followed by a reduction of the ester in compound **6,** and the deprotection of the carbonyl group in compound **7** afforded the hydroxy ketone **2** in 85% overall yield.[17]



SCHEME 3 Synthesis of hydroxy ketone **2**.

The final step of the synthesis was reductive amination (Scheme 4). There was no reaction between bimorpholine **1** and ketone **2** with $NaBH_4$ in the presence of acetic acid. However, the reaction with zinc-modified cyanoborohydride[18] led to the mixture of isomers of the target carbocyclic nucleoside analogue **3** with 40% of the unreacted starting bimorpholine. Column chromatography on silica gel afforded pure single more stable *cis*



SCHEME 4 Reductive amination of hydroxy ketone **2**.

isomer of **3** in a 15% yield. Configuration of this isomer was determined by a detailed ^{1}H and ^{13}C NMR analysis with the help of 2D FT experiments and with a comparison of present results with NMR data from *cis* and *trans* isomers of dideoxynucleoside analogues.[7]

BIOLOGICAL ACTIVITIES OF SYNTHESIZED COMPOUNDS

Analysis of the Cytotoxicity of Compounds

The observed inhibition of the replication of HCV in a cell culture can result from the direct antiviral effect of the compound. Alternatively, it may be related to its ability to specifically inhibit the activity of the essential cellular co-factor of HCV (an example of such an inhibitor is the cyclophilin inhibitor Alisporivir),[19] or may originate from a mild general cytotoxic effect, as the replication of HCV in a cell culture is very sensitive to toxic compounds, even in concentrations that are not toxic to the cells. As compounds **1** and **3** lack obvious similarities with known HCV inhibitors, either indirect mechanism of action was suggested.

An analysis performed by use of the MTT assay demonstrated that compound **3** is toxic for Huh7-Lunet and Huh-Luc/neo-ET (human hepatoma cells, which carry an autonomously replicating subgenomic HCV replicon, expressing Firefly luciferase as a reporter protein)[20] at a 50 μM concentration. The analysis of compound **1** was compromised by a large experiment-to-experiment variation in results; overall, no cytotoxicity (which would be statistically significant) was observed at 50 μM (data not shown). Such behavior, however, suggested that the compound had some effect on the cells, and hence, more sensitive methods were required. Therefore, the effects of both compounds on the cells were analyzed using the xCELLigence System (Roche), which measures electrical impedance across interdigitated microelectrodes integrated on the bottom of tissue culture E-Plates. The impedance measurement provides quantitative information about the biological status of cells, including cell number and viability. Most importantly, this system makes it possible to measure the same sample over the entire experiment, greatly reducing the variation in the results.

TABLE 1 Results of the analysis of cytotoxic properties of the compounds

Compound concentration	6 hours	24 hours	48 hours	72 hours
1, 50 μM	1.01	0.79	0.75	0.78
1, 10 μM	1.04	0.91	0.83	0.76
1, 1 μM	1.00	0.97	0.97	0.95
3, 50 μM	0.50	0.14	0.04	< 0.01
3, 10 μM	0.95	0.77	0.71	0.59
3, 1 μM	0.95	0.90	0.83	0.74

Note: Normalized cell indexes (values are normalized to the time point of the compound administration) are shown. Cell viability is presented as a ratio of the Normalized cell index of cells treated with the tested compounds to the Normalized cell index of the cells treated with relevant concentrations of DMSO (taken as 1).

First, we compared the action of compounds **1**, **3** and DMSO as a diluent control to Huh-Luc/neo-ET cells. Three concentrations of the substances, 50, 10, and 1 μM, and relevant DMSO concentrations (0.5%, 0.1%, and 0.01%) were used. The normalized results from one of the three reproducible experiments are shown in Table 1.

As is evident from the presented data, compound **1** was moderately toxic at high concentrations (50 and 10 μM) and had no or almost no toxic effects at 1 μM. Consistent with the results from the MTT assays, compound **3** was found to be extremely toxic at high concentrations (50 μM), and moderately toxic at lower concentrations (10 and 1 μM).

To confirm or rule out the possibility that toxic effects of compound **1** were related to the presence of the HCV replicon in cells, we compared the effects of compound **1** to Huh-Luc/neo-ET cells and Huh7-Lunet cells (the parental line for Huh-Luc/neo-ET, which did not contain the HCV replicon) using a similar assay. The results of one reproducible experiment are shown in Table 2.

Though some slight experiment-to-experiment differences (probably related to slightly different cell densities and growth conditions) were observed, the tendency always remained the same (the differences between

TABLE 2 Results of the analysis of cytotoxic action of compound **1** to the Huh-7 Lunet cells, with or without HCV replicon.

Compound concentration	Huh7-Lunet 24 hours	Huh-Luc/neo-ET 24 hours	Huh7-Lunet 48 hours	Huh-Luc/neo-ET 48 hours
1, 50 μM	0.78	0.79	0.62	0.61
1, 10 μM	0.83	0.94	0.62	0.87
1, 1 μM	1.04	1.01	0.86	1.01

Note: Cell viability is presented as a ratio of the normalized cell index of cells treated with the tested compound to the normalized cell index of the cells treated with relevant concentrations of DMSO (taken as 1 for each concentration).

TABLE 3 Effect of compound **1** on the HCV replication.

Concentration	6 hours	24 hours	48 hours	72 hours
50 μM	1.03	0.51	0.25	0.12
10 μM	1.23	0.75	0.88	0.85
1 μM	0.88	0.83	1.19	1.68

Note: Luciferase activity, which is proportional to the HCV RNA copy number, was measured at the indicated time points and normalized to the total protein content. At each time point, the luciferase activity in control cells treated with relevant amounts of DMSO is taken as 1. The data from one reproducible experiment is shown.

the HCV replicon containing cells and Huh7-Lunet cells were statistically nonsignificant). Thus, it is evident from the data that the presence of the HCV replicon does not make Huh-7 Lunet cells more susceptible to it nor does the cytotoxic effects protect cells against the cytotoxic effects of the compound.

Anti-HCV Activity of Compound 1

Based on the cytotoxicity analysis, we excluded compound **3** from subsequent analysis and tested next the action of compound **1** on HCV replication. In this experiment, Huh-Luc/neo-ET cells were incubated with the indicated concentrations of compound **1** or relevant concentrations of the DMSO used as a control. At indicated time points, Luc activity was measured and normalized to the total protein in the cell lysate; the results are presented in Table 3.

As is evident from the presented data, compound **1** drastically inhibited HCV replication at a 50 μM concentration. At 10 μM, the inhibition was almost completely lost and even some enhancement of the HCV replication was observed at a 1 μM concentration.

CONCLUSIONS

Nonaromatic nucleoside carba-analogue **3** is extremely cytotoxic at high concentrations. Compound **1** is a potent inhibitor of HCV replication at a 50 μM concentration; in contrast, at 10 μM, its ability to inhibit HCV is greatly reduced. As at the same concentration, some cytotoxic effects were observed (Table 1), it is likely that the action of compound **1** against HCV is not direct but is mediated by its general cytotoxicity. However, it should be noted that while the cytotoxic effects of compound **1** on the cells were nearly identical at both the 50 and 10 μM concentrations (Table 1), the HCV replication was inhibited only at the higher (50 μM) concentrations; similarly, the cytotoxic effects were relatively mild (nearly undetectable using an MTT assay) and the effect against HCV was more prominent. Therefore, it is possible that the cytotoxicity of compound **1** is not its only mechanism of

action against HCV and that some specific mechanism of inhibition exists. Additional research is needed to verify this possibility, and if the specific inhibition exists, to unravel its mechanism.

EXPERIMENTAL

Full assignment of ^{1}H and ^{13}C chemical shifts is based on the 1D and 2D FT NMR spectra on a Bruker AMX500, AvanceIII 400. Deuterosolvent peaks ($CHCl_3$ $\delta = 7.27$, $CDCl_3$ $\delta = 77.00$) or the TMS peak was used as chemical shift references. High-resolution mass spectra were recorded on an LTQ Orbitrap (Thermo Electron). Optical rotations were obtained using a Anton Paar GWB Polarimeter MCP 500. Chiral HPLC was performed using the Chiralcel OD-H (250 × 4.6 mm).

Reactions sensitive to oxygen or moisture were conducted under an argon atmosphere in flame-dried glassware. Anhydrous dichloromethane was freshly distilled over CaH_2 and anhydrous diethyl ether, over $LiAlH_4$. Commercial reagents were generally used as received. The petroleum ether used had bp 40°C–60°C.

Ethyl 2-Cyano-3-oxocyclopentanecarboxylate 4

NaCN (18.4 g, 0.380 mol) is suspended in DMSO (74.5 mL) at 80°C under Ar. Ethyl acrylate (81.5 mL, 0.750 mol) is added to the reaction mixture slowly over 2.5 hours and the mixture is heated for an additional 2 hours at 80°C. After stirring at room temperature for 17 hours, the reaction is finished with the addition of glacial acetic acid (23.6 mL) and water and stirred for 1 hour at room temperature. The reaction mixture is extracted with CH_2Cl_2, back-extracted with water and sat. NaCl solution, and dried over Na_2SO_4. The solvent is removed and the mixture is purified by Kugelrohr distillation to afford the desired product as a colorless oil (28.5 g, 42% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.27 (m, 2H), 3.64 (d, $J = 10.9$ Hz, 1H), 3.35 (td, $J = 10.6, 6.8$ Hz, 1H), 2.60–2.32 (m, 3H), 2.11–1.98 (m, 1H), 1.33 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 203.84, 171.03, 115.21, 62.17, 45.73, 42.25, 36.52, 25.02, 14.12.

3-Oxocyclopentanecarboxylic Acid 5

Ethyl 2-cyano-3-oxocyclopentanecarboxylate **4** (28.5 g, 0.157 mol) is dissolved in an 18% HCl solution (660 mL) and heated to reflux for 1 hour. The mixture is concentrated and extracted with CH_2Cl_2. After removal of the solvent, the desired carboxylic acid **5** is obtained (20.1 g, 64% yield).

Racemic carboxylic acid (6 g, 0.05 mol) and (−)-brucine (22.2 g, 0.056 mol) are dissolved in water (107 mL) and heated to reflux until a clear solution is obtained. The mixture is allowed to cool to room temperature.

The formed crystals are filtered and washed with water and dried. After four sequential crystallizations, 98% ee is obtained.

Enantiomerically pure (*R*)-3-oxocyclopentanecarboxylic acid (−)-brucine salt is dissolved in water and heated to reflux until a clear solution is obtained. NH_4OH is added and filtered, the crystals are washed with water, and the filtrate is concentrated, acidified with 1M HCl, and extracted with Et_2O. (*R*)-3-oxocyclopentanecarboxylic acid **5b** is obtained as soft yellow crystals (1.1 g, 18% yield, mp = 48°C).

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.36 (s, 1H), 3.25–3.13 (m, 1H), 2.55–2.14 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.76, 180.25, 40.92, 40.70, 37.35, 26.46. **5b** $[\alpha]_D^{25} = +20$ (c 1.9, MeOH), (lit. $[\alpha]_D^{25} = +22$ (c 1.9, MeOH).[16] Chiral HPLC (Chiralcel OD-H, *n*-Hexane:iPrOH 8:2, 1 mL/minutes, UV 254 nm, t_R = 25.07 minutes (*R*-enantiomer) and t_R = 26.8 minutes (*S*-enantiomer).

(*R*)-Methyl 3,3-dimethoxycyclopentanecarboxylate (6)

(*R*)-3-oxocyclopentanecarboxylic acid **5b** (978 mg, 6.0 mmol) is dissolved in methanol (12 mL); orthoformate (6.0 mL) and acetylchloride (0.4 mL, 6.0 mmol) are added and the mixture is stired for 5 hours. Na_2CO_3 (3 g) and petroleum ether (15 mL) are added and the mixture is stired for an additional hour. After filtration and solvent removal, the crude product is obtained as a light yellow oil (1.1 g, quantitative yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.69 (s, 3H), 3.22 (s, 3H), 3.20 (s, 3H), 2.94–2.83 (m, 1H), 2.12 (dd, J = 23.4, 8.6 Hz, 1H), 2.06 (dd, J = 13.4, 8.8 Hz, 1H), 2.02–1.77 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.84, 110.86, 51.80, 49.68, 49.07, 41.07, 37.21, 33.52, 26.60.

(*R*)-(3,3-Dimethoxycyclopentyl)methanol 7

Anhydrous Et_2O (10 mL) is added to a flame-dried, round bottom flask under Ar, $LiAlH_4$ (294 mg, 7.8 mmol) is added, and the mixture is cooled to 0°C. (*R*)-methyl 3,3-dimethoxycyclopentanecarboxylate **6** (1.1 g, 6 mmol) is dissolved in anhydrous Et_2O (10 mL), slowly added to the reaction mixture, and heated to reflux for 3 hours. The reaction mixture is cooled to 0°C and quenched with H_2O and 4N NaOH and dried over Na_2SO_4. After filtration and solvent removal, the crude product is obtained as a light yellow oil (911 mg, 95% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.59–3.48 (m, 2H), 3.21 (s, 6H), 2.41–2.17 (m, 2H), 2.00 (dd, J = 13.3, 8.8 Hz, 1H), 1.95–1.70 (m, 3H), 1.56 (ddd, J = 13.3, 7.5, 1.7 Hz, 1H), 1.50–1.37 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 111.42, 66.73, 49.36, 49.25, 38.95, 37.39, 33.47, 25.76.

(*R*)-3-(Hydroxymethyl)cyclopentanone 2

(*R*)-(3,3-dimethoxycyclopentyl)methanol **7** (911 mg, 5.7 mmol) is dissolved in an Et_2O/H_2O (1:1, 20 mL) mixture, a few drops of 1M HCl is added and the mixture is stired overnight. The solvent is removed, and the mixture is purified by silica gel column chromatography (petroleum ether:acetone 16%) to give product **2** as a colorless oil (610 mg, 94% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.73–3.64 (m, 2H), 2.52–2.42 (m, 1H), 2.42–2.35 (m, 1H), 2.35–2.27 (m, 1H), 2.27–2.18 (m, 1H), 2.18–2.10 (m, 2H), 2.03 (ddd, J = 10.0, 8.4, 0.9 Hz, 1H), 1.75 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 219.63, 65.75, 41.67, 38.99, 38.02, 25.63. $[\alpha]_D^{25}$ = +84 (c 0.85, acetone).

(1*R*,3*S*)-((2*S*,2′*S*,5*S*,5′*S*)-5,5′-Dibenzyl-2,2′-bimorpholino)cyclopentyl)methanol 3

Molecular sieves 4Å are added to a round bottom flask and sealed, dried, and filled with Ar. (2*S*,2′*S*,5*S*,5′*S*)-5,5′-dibenzyl-2,2′-bimorpholine (120 mg, 0.340 mmol) and (*R*)-3-(hydroxymethyl)cyclopentanone (38.8 mg, 0.340 mmol) are dissolved in dry CH_2Cl_2 (5 mL) and added to the reaction flask via a syringe. In a separate flask, $NaCNBH_3$ (32.1 mg, 0.51 mmol) and $ZnCl_2$ (34.8 mg, 0.26 mmol) are dissolved in CH_2Cl_2 (5 mL), stired, and then transferred to the reaction mixture. After 14 hours, a crystal of pTsOH is added and the mixture is refluxed for 2 days. The reaction is quenched with the addition of 1N NaOH, extracted with CH_2Cl_2, and dried over Na_2SO_4. The crude product is purified by silica gel column chromatography using CH_2Cl_2:NH_3/MeOH 0.5% to give product **3** as a colorless, sticky oil (23 mg, yield 15%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32–7.20 (m, 5H), 7.20–7.10 (m, 5H), 3.90 (dd, J = 11.0, 2.9 Hz, 1H), 3.67–3.58 (m, 2H), 3.58–3.53 (m, 2H), 3.53–3.45 (m, 2H), 3.33–3.19 (m, 2H), 3.10 (dt, J = 15.8, 8.0 Hz, 1H), 3.02–2.92 (m, 1H), 2.81–2.69 (m, 4H), 2.62 (dt, J = 16.2, 8.1 Hz, 1H), 2.49–2.37 (m, 3H), 2.21–2.05 (m, 2H), 1.89 (m, 1H), 1.77–1.62 (m, 2H), 1.59–1.45 (m, 1H), 1.45–1.33 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.59, 137.70, 129.11, 128.99, 128.60, 128.38, 126.55, 126.25, 76.38, 76.26, 72.66, 70.31, 66.79, 60.20, 59.72, 55.58, 47.57, 45.87, 39.36, 38.57, 34.81, 33.14, 26.49, 22.17. IR: ν = 3413, 2949, 2860, 1603, 1453, 1107. HRMS (EI+) calculated 451.2955 [M+H], found 451.2953 [M+]. $[\alpha]_D^{25}$ = +76 (c 0.66, $CHCl_3$).

Analysis of Cytotoxic Activity of Compounds by xCELLigence System

Cytotoxicity of the compounds was determined using Huh-Luc/neo-ET or Huh7-Lunet cells and the xCELLigence system (Roche). First, we put 50 μl of cell growth media (Dulbecco Modified Eagle Media, 1x Pen/Strep, 10% Foetal Calf Serum) into the well and one sweep to determine if the

basic electrical impedance was made. Then, the cells were seeded on a plate in the amount of 1500 cells per well. For the first 2 hours, sweeps were made every minute (a total of 120 sweeps), and after that, every 30 minutes (a total of 44 sweeps). Twenty-four hours later, substances in the indicated amounts were added and the cells were incubated for 72 hours. For the first 2 hours, sweeps were made every minute (a total of 120 sweeps), and after that, every 30 minutes (a total of 140 sweeps).

Procedure for Testing Anti-HCV Activity

About 45 000 Huh-luc/neo-ET cells were seeded on 60 mm diameter dishes. Twenty-four hours later, compound **1** or DMSO at indicated concentrations was added. Cells were incubated with the compounds for the indicated time, and after that, they were lysed, and luciferase activity in the lysates was measured according to the protocol of Luciferase Assay systems (Promega). The total protein concentration in the lysates was determined using a Bradford assay (Bio-Rad) and the luminescence was normalized to the total protein concentration.

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