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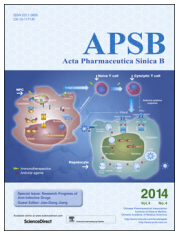

ORIGINAL ARTICLE

Synthesis and biological evaluation of sophocarpinic acid derivatives as anti-HCV agents

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KEY WORDS

Sophocarpinic acid;
Matrine;
Anti-HCV;
Antiviral activity;
Structure–activity relationship

Abstract Chronic hepatitis C virus (HCV) infection has become a major public health burden worldwide. Twenty-two sophocarpinic acid or matrine derivatives were synthesized and their anti-HCV activities were evaluated *in vitro*. The structure-activity analysis revealed that (i) sophocarpinic acids with a D-seco 3-ring structure scaffold were more favorable than matrines with a 4-ring scaffold; (ii) the introduction of an electron-withdrawing group on the phenyl ring in 12-*N*-benzenesulfonyl $\Delta^{\beta\gamma}$ sophocarpinic acids was beneficial for the antiviral activity against HCV. Among them, compounds **9h** and **9j** exhibited the most potent inhibitory activities on HCV replication with selectivity indies of 70.3 and 30.9, respectively. Therefore, both were selected as antiviral candidates for further investigation.

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1. Introduction

Chronic hepatitis C virus (HCV) infection has become a major public health burden worldwide. The World Health Organization (WHO) reported that over 3% of the global population with approximately 180 million individuals is estimated to be infected with HCV, with a 3–4 million new cases appearing every year globally[1,2]. Among all countries, China accommodates the largest HCV-infected population, with more than 41 million people infected, and the incidence rate is rising year by year[2]. In nearly 85% of the cases, the disease progresses into chronicity, and 30 percent of the cases may progress to liver cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC)[3].

There is no vaccine to prevent HCV infection. The standard therapy for HCV in the clinic is the combination of pegylated-interferon with ribavirin[4,5]. The regimen is only effective in approximately 40% of patients infected with HCV genotype 1, the prevalent HCV genotype in the United States, Europe, and China, and is associated with significant side effects[3]. The introduction of Telaprevir and Boceprevir was temporarily effective with HCV patients, but drug-resistant mutations soon appeared[6–9]. Sofosbuvir, a new HCV NS5B RNA polymerase inhibitor approved by FDA in 2013 showed promise in dealing with drug-resistant HCV[10]. However, a S282T mutation in NS5B was found in genotype 1a, 1b and 2a replicons, and caused a reduced susceptibility to sofosbuvir, again raising the need for new drugs with novel modes of action[11].

Matrine (**1**, Fig. 1), a quinolizidine natural product extracted from *Sophora flavescens*, has been used clinically for HBV treatment for decades and has a novel mechanism of action[12,13]. Clinical reports showed that compound **1** was also effective for HCV patients in China[14]. Bearing a unique 4-ring core scaffold, compound **1** strongly provoked our interest to explore its anti-HCV structure-activity relationship (SAR) in an effort to discover a new chemical entity (NCE) against HCV with a novel mechanism. SAR studies have been carried out in our lab, revealing that the 5*S* configuration in the core scaffold is beneficial for anti-HCV activity[15,16]. In the present study, the 5*S* configuration was maintained and further SAR analysis was directed toward the carbon-carbon double bond in sophocarpine (**2**, Fig. 1), another natural product extracted from *Sophora flavescens*. Based on this strategy, a series of (*E*)-$\Delta^{\beta\gamma}/\Delta^{\alpha\beta}$-sophocarpinic acid (**3/4**, Fig. 1) derivatives bearing a D-seco 3-ring scaffold were synthesized and evaluated for their anti-HCV activities.

2. Results and discussion

2.1. Chemistry

As showed in Scheme 1, compound **2**, as the starting material, was hydrolyzed in strong base and formed an isomeric mixture of **3** and **4**. The target compounds (*E*)-12-*N*-substituted-$\Delta^{\beta\gamma}/\Delta^{\alpha\beta}$-sophocarpinic acids (**9/10**) were acquired by using a three-step sequence including carboxyl protection with diphenyldiazomethane as the protective agent, 12-*N*-alkylation or acylation in the presence of potassium carbonate, and deprotection in *m*-cresol[15–19], with overall yields of 5%–12%. The regio-isomers were separated before alkylation or acylation.

To gain sophocarpinic acid derivatives without the double bond, **2** was refluxed in aqueous base as illustrated in Scheme 2 to gain the key intermediate **11**, which was hydrolyzed in strong base to form a 3-ring scaffold product **12**, followed by a three-step sequence including carboxyl protection *via* diphenyldiazomethane, 12-*N*-alkylation in the presence of potassium carbonate and deprotection in *m*-cresol to gain the desired product **15** in an overall yield of 16.1%[15–19]. Matrine derivatives **16a-d** (Table 1) as a class of anti-HBV agents were prepared as previously reported[17].

2.2. SAR analysis for anti-HCV activity in vitro

All the synthesized compounds were tested for their anti-HCV activity and cytotoxicity in Huh7.5 cells using specific real-time RT-PCR assay, as described in our previous publication[20]. Anti-HCV activity was evaluated by measuring both EC_{50} (for anti-HCV activity) and TC_{50} (for cytotoxicity) values. As a key indication, the selectivity index (SI) was calculated as a ratio of TC_{50} to EC_{50}. Anti-HCV activity of a given compound was estimated by combining its EC_{50} value with its SI. Twenty-two sophoridinic acid or sophoridine analogs and their anti-HCV effectiveness are shown in Tables 1 and 2.

The SAR study for anti-HCV activity was initially focused on the influence of the substitutions on ring D while the 4-ring scaffold was unchanged. As indicated in Table 1, matrine compounds **16a-c** afforded similar or lower SI values compared to compound **1**, while compound **16d** bearing a methylamino showed a higher SI value, which hinted that suitable substitutions on ring D could enhance the activity against HCV.

Table 2 disclosed the SAR analysis on the D-meso 3-ring scaffold derivatives. The compounds bearing a carbon-carbon

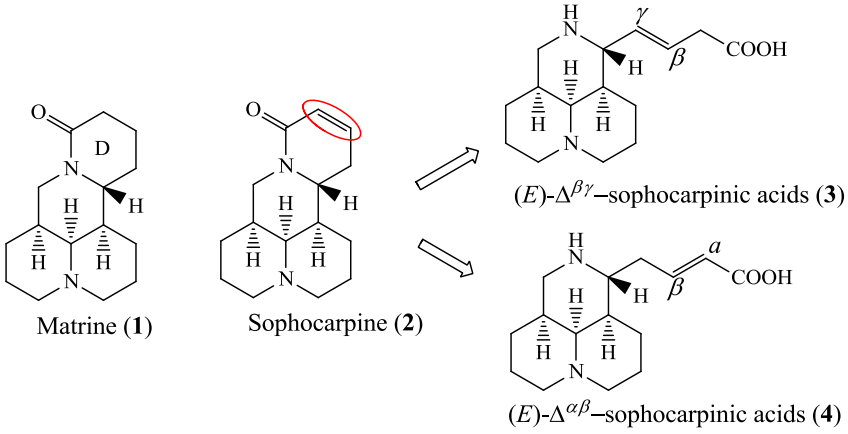
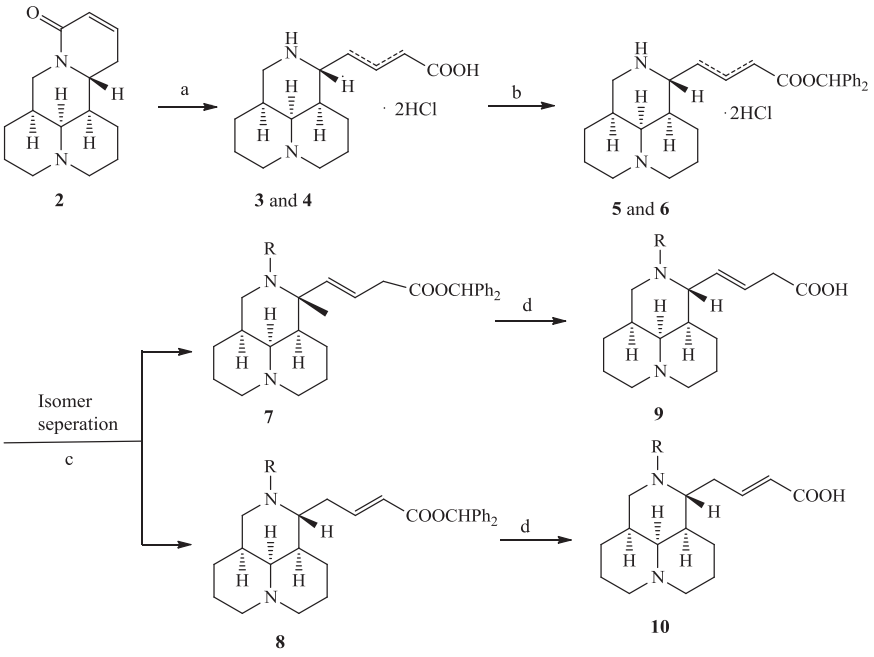
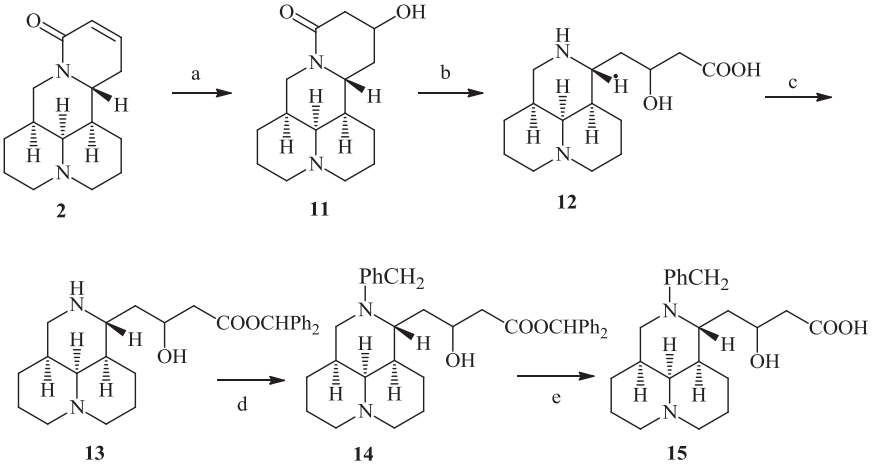


Figure 1 The chemical structures of marine (**1**), sophocarpine (**2**), (*E*)-$\Delta^{\beta\gamma}$ sophocarpinic acid (**3**) and (*E*)-$\Delta^{\alpha\beta}$ sophocarpinic acid (**4**).



Scheme 1 Synthetic routes for *N*-substituted sophocarpinic acid analogs. Reagents and conditions: (a) 10% KOH/H_2O, reflux, 7 h; then 3 mol/L HCl, pH 5–6; (b) diphenyldiazomethane, MeOH/petroleum ether(boiling range 30–60 °C), overnight; (c) (1) substituted benzyl chloride/bromine or benzenesulfonyl chloride, CH_2Cl_2 or MeCN, K_2CO_3, r.t., overnight; (2) flash column chromatography; (d) *m*-cresol, 80 °C, 8–9 h.



Scheme 2 Synthetic routes for 12-*N*-benzyl-β-hydroxylmatrinic acid (**15**). Reagents and conditions: (a) 5% KOH/H_2O, reflux, 8 h; then 3 mol/L HCl, pH 5–6; (b) 10% KOH/H_2O, reflux, 7 h; then 3 mol/L HCl, pH 5–6; (c) diphenyldiazomethane, MeOH/petroleum ether (boiling range 30–60 °C), overnight; (d) benzyl bromide, MeCN, K_2CO_3, r.t., overnight; (e) *m*-cresol, 80 °C, 8–9 h.

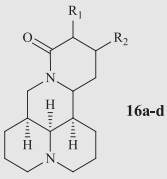
double bond on the side chain, specifically, (*E*)-12-*N*-substituted-$\Delta^{\beta\gamma}$-sophocarpinic acids (**9a–k**) and their isomers (*E*)-12-*N*-substituted -$\Delta^{\alpha\beta}$-sophocarpinic acids (**10a–f**), were evaluated in this study. First, the influence of the substituents on the 12-nitrogen atom in the (*E*)-$\Delta^{\beta\gamma}$-sophocarpinic acids was examined. The results showed that 12-*N*-benzyl derivatives **9a–c** showed anti-HCV activities similar to or lower than compound **1**, and most of the compounds in the 12-*N*-benzenesulfonyl series (**9d–k**) had low activities against HCV, except that compound **9h** and **9j** showed significantly higher activity than compound **1** with SI values of 70.3 and 30.9, respectively. The results suggested that the introduction of CF_3 or CN on the sulfonylphenyl ring might be beneficial for the anti-HCV activity.

In the case of the (*E*)-$\Delta^{\alpha\beta}$-sophocarpinic acids derivatives **10a–f**, most showed decreased or similar anti-HCV activities as compared to compound **1**, regardless of the electron-donating or electron-withdrawing groups on the phenyl ring, which hinted that a $\Delta^{\beta\gamma}$ analog could do better than its $\Delta^{\alpha\beta}$ isomer in anti-HCV area. The introduction of OH at the double bond could decrease anti-HCV activity, as compared with product **15** and compound **9b**.

All together, it appeared that a D-meso 3-ring structure scaffold was more favorable than a 4-ring scaffold, and the introduction of

an electron-withdrawing group on the phenyl ring in 12-*N*-benzenesulfonyl $\Delta^{\beta\gamma}$-sophocarpinic acid derivatives was beneficial for anti-HCV activity.

Table 1 SAR for anti-HCV activity of matrine analogues.



16a-d

No	R_1	R_2	TC_{50} (μg/mL) [a]	EC_{50} (μg/mL)[b]	SI[c]
1	H	H	>1000	98.04	>10.2
16a	H	OCH_2CH_3	>1000	104.62	>9.6
16b	OCH_2Ph	OCH_2Ph	49.87	5.60	8.9
16c	OH	$OCOCH_2Cl$	54.90	>37.04	<1.5
16d	H	$NHCH_3$	543.70	31.07	17.5
RBV			2000	292.46	6.84

[a]Cytotoxic concentration required to inhibit Vero cell growth by 50%.
[b]Concentration required to inhibit CVB3 growth by 50%.
[c]Selectivity index values equaled to TC_{50}/EC_{50}.

3. Conclusions

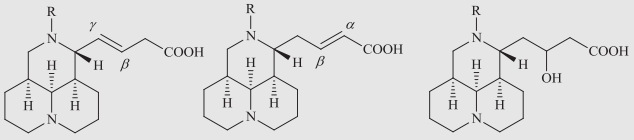
In searching for novel anti-HCV agents, 22 sophocarpinic acid or matrine derivatives were synthesized and evaluated for their anti-HCV activities *in vitro* with **1** as the lead. Among these derivatives, compounds **9h** and **9j** exhibited the most potent antiviral activities against HCV with SI values of 70.3 and 30.9, respectively. SAR revealed that (i) sophocarpinic acids with a D-seco 3-ring structure scaffold are more favorable than matrines with a 4-ring scaffold; (ii) introduction of an electron-withdrawing group on the phenyl ring in 12-*N*-benzenesulfonyl substituted $\Delta^{\beta\gamma}$-sophocarpinic acid derivatives is beneficial for activity. In addition, compounds **9h** and **9j** showed satisfactory activity against coxsackievirus type B3 (CVB3) and CVB6 in our earlier study, plus good pharmacokinetic profiles with low toxicity *in vivo*[19]. All together, **9h** and **9j** are highly recommended to be further developed as broad-spectrum antiviral drug candidates.

4. Experimental

4.1. Chemistry

Melting point (mp) was obtained with an MPA 100 OptiMelt automated melting point system (Stanford Research Systems, California, USA) and uncorrected. ^1H NMR spectra was

Table 2 SAR for anti-HCV activity of sophocarpinic acid derivatives.



9a-k **10a-f** **15**

Compd.	R	TC_{50} (μg/mL)[a]	EC_{50} (μg/mL)[b]	SI[c]
1		>1000	98.04	>10.2
9a	$-CH_2PhCH_3$-*o*	726.98	155.66	4.7
9b	$-CH_2Ph$	>1000	49.3	20.3
9c	$-CH_2PhBr$- *p*	357.41	24.36	14.7
9d	$-SO_2PhNO_2$-*p*	380.7	74.0	5.14
9e	$-SO_2Ph$	>1000	257.55	>3.9
9f	$-SO_2PhCH_3$-*p*	>1000	112.06	>8.9
9g	$-SO_2PhOCH_3$-*p*	>1000	165.21	> 6.1
9h	$-SO_2PhCF_3$-*p*	530.07	7.54	70.3
9i	$-SO_2PhCl$-*o*	876.08	134.58	6.5
9j	$-SO_2PhCN$-*m*	123.08	3.98	30.9
9k	$-SO_2Ph(NHCOCH_3)$-*p*	65.77	2.80	23.5
10a	$-CH_2PhNO_2$- *p*	>1000	52.82	>18.9
10b	$-SO_2Ph$	>1000	59.65	>16.8
10c	$-SO_2PhCH_3$-*p*	171.29	25.02	6.8
10d	$-SO_2PhCl$-*o*	404.64	241.27	1.68
10e	$-SO_2Ph(NHCOCH_3)$-*p*	552.03	222.06	2.5
10f	$-SO_2PhOCH_3$-*p*	700.4	291.8	2.4
15	$-CH_2Ph$	>1000	>333.3	3
RBV		2000	292.46	6.84

[a]Cytotoxic concentration required to inhibit Vero cell growth by 50%.
[b]Concentration required to inhibit CVB3 growth by 50%.
[c]Selectivity Index values equaled to TC_{50}/EC_{50}.

performed on a Varian Inova 400 MHz spectrometer (Varian, San Francisco, CA) or 500 MHz spectrometer (AV500-III, Brvker, Swiss), with Me_4Si as internal standard. ESI high-resolution mass spectra (HRMS) were recorded on an AutospecUItima-TOF mass spectrometer (Micromass UK Ltd., Manchester, UK). Flash chromatography was performed on CombiflashRf 200 (Teledyne, Nebraska, USA), particle size 0.038 mm.

Compound **2** with purity over 98.5% was purchased from the Yanchi Dushun Biological and Chemical Co., Ltd. (Shanxi, China) and the Ningxia Zijinghua Pharmacy Co., Ltd. (Ningxia, China). Target compounds **9b-c**, **9e–k** and **10b–f** were prepared as a family of anti-CVB3 inhibitors[19].

4.1.1. General procedures for *9* and *10*

Compound **2** (12.3 g, 50 mmol, 1 equiv.) was added to a solution of KOH (33.6 g, 600 mmol) in H_2O (300 mL). The reaction mixture was heated and refluxed for 7 h and then stirred at room temperature overnight. The reaction solution was cooled with an ice–water bath and was acidified with HCl (3 mol/L) to pH 5–6. The solvent was removed *in vacuo*, and the residue was recrystallized in methanol to give an isomer mixture of **3** and **4**.

A mixture of diphenylmethanone hydrazone (14.7 g, 75 mmol, 1.5 equiv.) and electrolytic manganese dioxide (13.04 g, 150 mmol, 3 equiv.) in petroleum ether (boiling range 30–60 °C) was heated at reflux for 0.5 h, and the mixture was filtered. The filtrate was added to the solution of **3** and **4** in methanol, and the mixture was then stirred overnight at room temperature. A corresponding isomer mixture of **5** and **6** was obtained and was used directly in the next reaction without further purification.

Anhydrous K_2CO_3 (3.5 equiv.) and benzyl bromine, or sulfonyl chloride (1 equiv.) were added to the solution of **5** and **6** in dichloromethane or MeCN (50 mL), and the reaction solution was then stirred at room temperature until TLC analysis showed completion of the reaction. The reaction mixture was filtered and the filtrate was evaporated to afford a mixture of **7** and **8**, which were obtained by splitting of the mixture with flash column chromatography on silica gel with ethyl acetate and cyclohexane as the eluents. Compound **7** was then dissolved in *m*-cresol (10 mL), and the reaction mixture was stirred at 80 °C for 8–9 h. It was then cooled, and methylisobutylketone (30 mL) was added. The resulting solution was extracted with H_2O (50 mL × 3), and the combined extracts were evaporated to afford the crude compound, which was purified by flash column chromatography on silica gel with dichloromethane and methanol as the eluents, to afford **9**. Compound **10** was obtained from compound **8** by the same method.

(*E*)-12-*N*-(*o*-Methylbenzyl)-$\Delta^{\beta\gamma}$- sophocarpinic acid (**9a**): white solid (0.6 g, 6.5%), mp 98–100 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 7.13 − 7.31 (m, 4H), 5.95 − 6.06 (m, 1H), 5.30 (dd, 1H, J = 9.2, 15.2 Hz), 4.46 (m, 1H), 3.03 − 3.10 (m, 3H), 2.99 (d, 2H, J = 7.2 Hz), 2.82 − 2.93 (m, 2H), 2.42 − 2.61 (m, 3H), 2.31 − 2.33 (m, 3H), 2.19 (s, 1H), 1.87 − 2.03 (m, 3H), 1.41 − 1.79 (m, 7H); HRMS: calcd. for $C_{23}H_{33}N_2O_2$ $(M+H)^+$ 369.2542, found 369.2529.

(*E*)-12-*N*-(*p*-Nitrobenzenesulfony)-$\Delta^{\beta\gamma}$-sophocarpinic acid (**9d**): white solid (2.2 g, 9.8%), mp 176 − 179 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 8.36 (d, 2H, J = 8.8 Hz), 7.99 (d, 2H, J = 8.8 Hz), 5.44 − 5.47 (m, 2H), 3.76 (dd, 1H, J = 5.0, 11.2 Hz), 3.42 − 3.55 (m, 1H), 3.29 − 3.37 (m, 1H), 3.07 − 3.15 (m, 1H), 2.93 (d, 2H, J = 5.0 Hz), 2.79 (d, 1H, J = 11.2 Hz), 2.68 − 2.69 (m, 1H), 2.50 − 2.54 (m, 1H), 1.90 − 2.11 (m, 3H), 1.30 − 1.83 (m, 8H); HRMS: calcd. for $C_{21}H_{28}N_3O_6S$ $(M+H)^+$ 450.1693, found 450.1710.

(*E*)-12-*N*-(*p*-Nitrobenzyl)-$\Delta^{\alpha\beta}$-sophocarpinic acid (**10a**): white solid (0.95 g, 9.5%), mp 139 − 141 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 8.12 − 8.15 (m, 2H), 7.51 − 7.57 (m, 2H), 6.97 − 7.04 (m, 1H), 5.85 (d, 1H, J = 15.0 Hz), 4.23 (d, 1H, J = 14.8 Hz), 3.27 − 3.47 (m, 4H), 2.90 − 3.01 (m, 3H), 2.79 − 2.85 (m, 1H), 2.47 − 2.78 (m, 3H), 2.19 − 2.22 (m, 1H), 1.93 − 2.08 (m, 2H), 1.65 − 1.88 (m, 7H); HRMS: calcd. for $C_{22}H_{30}N_3O_4$ $(M+H)^+$ 400.2236, found 400.2217.

4.1.2. Procedures for 12-N-benzyl-β-hydroxyl sophocarpinic acid (*15*)

To a solution of KOH (33.6 g, 0.6 mol) in water (600 mL) was added **2** (12.3 g, 0.05 mol) with stirring at room temperature. The reaction mixture was refluxed for 8 h, then cooled down to 0 °C and neutralized with 3 mol/L HCl. After concentrated *in vacuo*, MeOH (150 mL) was added, insoluble solid was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2/MeOH as eluent to give **11** (6.2 g, 47%) as white solid.

Compound **11** (1 equiv.) was added to a solution of KOH (10%) in water. The reaction mixture was refluxed for 9 h, and then stirred at room temperature overnight. The reaction solution was cooled in ice–water bath, and acidified with HCl (3 mol/L). The solvent was removed *in vacuo* and the residue was dissolved in methanol to give a corresponding solution of crude **12**.

A mixture of diphenylmethanone hydrazone (14.7 g, 75 mmol, 1.5 equiv.) and electrolytic manganese dioxide (13.04 g, 150 mmol, 3 equiv.) in petroleum ether (boiling range 30–60 °C) was heated at reflux for 0.5 h, and the mixture was filtered. The filtrate was added to the solution of **13** in methanol, and the mixture was then stirred at room temperature until the purple color disappeared, and then filtered. The resulting filtrate was evaporated under reduced pressure to dryness. The residue was washed with petroleum ether to afford crude compound **13** which was used directly in the next step without further purification.

To the mixture of crude **13** and anhydrous K_2CO_3 (3 equiv.) in MeCN was added benzyl bromide (1 equiv.). The reaction mixture was stirred at room temperature till the reaction was complete (checked by TLC), then filtered. The filtrate was evaporated *in vacuo* to give the crude product **14** as an oily residue, which was then dissolved in *m*-cresol (10 mL), and the reaction mixture was stirred at 80 °C for 8–9 h. It was then cooled, and methylisobutylketone (30 mL) was added. The resulting solution was extracted with H_2O (50 mL × 3), and the combined extracts were evaporated to dryness, and the residue was purified through flash chromatography over silica gel to give 1.5 g of compound **15**. White solid (1.5 g, 16.1%), mp 115 − 117 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 7.26 − 7.44 (m, 5H), 4.38 − 4.47 (m, 1H), 4.08 − 4.13 (m, 1H), 3.72 − 3.74 (m, 1H), 3.15 − 3.25 (m, 2H), 2.76 − 3.18 (m, 3H), 2.59 − 2.70 (m, 1H), 2.32 − 2.49 (m, 3H), 1.85 − 2.25 (m, 6H), 1.39 − 1.72 (m, 7H); HRMS: calcd. for $C_{22}H_{33}N_2O_3$ $(M+H)^+$ 373.2491, found 373.2493.

4.2. Biological methods

4.2.1. Cell culture

Human liver cell line Huh7.5 cells (kindly provided by Vertex Pharmaceuticals, Inc., Boston, MA) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10%

inactivated fetal bovine serum and 1% penicillin–streptomycin (Invitrogen). Cells were digested with 0.05% trypsin–ethylene diamine tetraacetic acid (EDTA) and split twice a week.

4.2.2. *Anti-HCV effect in vitro*

Huh7.5 cells were seeded into 96-well or 6-well plates (Costar) at a density of 3×10^4 cells/cm^2. After 24 h incubation, the cells were infected with HCV viral stock (45 IU/cell) and simultaneously treated with the test compounds at various concentrations or solvent as control. The culture medium was removed after 72 h inoculation, the intracellular total RNA (in 96-well plates) was extracted with RNeasy Mini Kit (Qiagen), and total intracellular proteins (in 6-well plates) were extracted with Cyto-Buster Protein Extraction Reagent added with 1 mmol/L protease inhibitor cocktail. The intracellular HCV RNA was quantified with a real time one-step reverse-transcription polymerase chain reaction (RT-PCR).

4.2.3. *Cytotoxicity assay*

Huh7.5 cells were seeded into 96-well plates (Costar) at a density of 3×10^4 cells/cm^2. After incubated for 24 h, fresh culture medium containing test compounds at various concentrations were added. Seventy-two hours later, cytotoxicity was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Acknowledgments

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