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Synthesis and Anti-HCV Activity of 4-Hydroxyamino α-Pyranone Carboxamide Analogues

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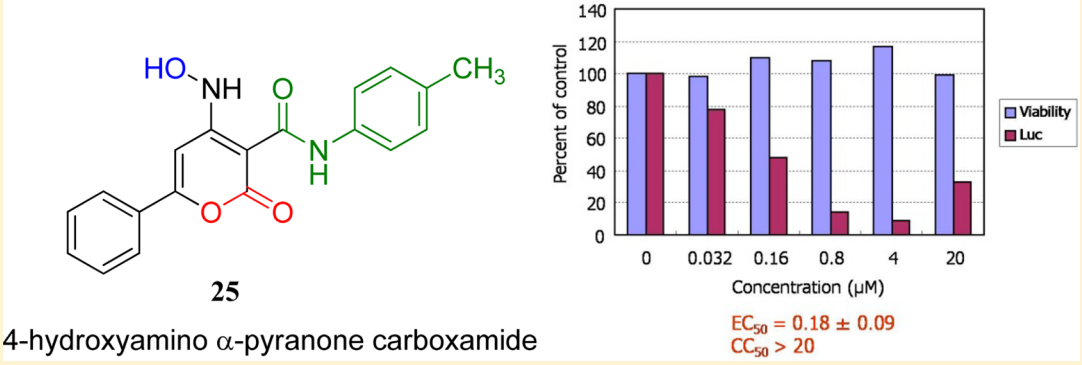
Supporting Information



ABSTRACT: High genetic variability in hepatitis C virus (HCV), emergence of drug resistant viruses and side effects demand the requirement for development of new scaffolds to show an alternate mechanism. Herein, we report discovery of new scaffold **I** based on 4-hydroxyamino α-pyranone carboxamide as promising anti-HCV agents. A comprehensive structure−activity relationship (SAR) was explored with several newly synthesized compounds. In all promising compounds (**17**−**19**, **21**−**22**, **24**−**25**, and **49**) with EC_{50} ranging 0.15 to 0.40 μM, the aryl group at C-6 position of α-pyranone were unsubstituted. In particular, **25** demonstrated potential anti-HCV activity with EC_{50} of 0.18 μM in cell based HCV replicon system with lower cytotoxicity (CC_{50} > 20 μM) and provided a new scaffold for anti-HCV drug development. Further investigations, including biochemical characterization, are yet to be performed to elucidate its possible mode of action.

KEYWORDS: *Hepatitis C virus, non-nucleoside inhibitor, α-pyranone carboxamide*

Hepatitis C virus (HCV) is a single stranded 9.6 kb RNA virus of the *Flaviviridae* family predominantly infecting hepatocytes. HCV infection is silent and in long-term may lead to serious liver disease, including fibrosis and cirrhosis followed by hepatocellular carcinoma.[1,2] The prophylactic treatment has been of very limited success,[3] and so far no vaccine is available.[4] A serious health threat has been realized, and a series of investigations are underway to discover direct acting antivirals (DAAs) as promising anti-HCV agents.[5]

The potential molecules **1**−**4** (Figure 1) belonging to DAAs target mainly three viral enzymes: (i) protease NS3/4A, (ii) replicase factor NS5A, and (iii) HCV RNA-dependent RNA polymerase (HCV RdRp) NS5B.[6] The medicinal chemistry approaches with breakthrough exploration in HCV biology[7] were translated recently into first generation DAAs, boceprevir[8] and telaprevir[9] (protease inhibitors) approved by US FDA. Several new generation protease inhibitors are under serious investigations to combat the challenges associated with anti-HCV therapy.[10,11]

The current standard of care (SoC) for HCV treatment includes one of the approved protease inhibitors combined with pegylated interferon-α (PegIFN) and ribavirin (RBV).

The new regimen improves sustained viral response (SVR) rates to ∼75%;[8,9] however, it also adds side effect burden to patients. The use of PegIFN/RBV is associated with severe side effects followed by treatment discontinuation and contra-indication.[12] Overall, drug-resistance, drug−drug interactions, and side effects are major concerns,[13] which demand effective PegIFN/RBV free regimen. In addition to recent success in protease inhibitors,[10,11] parallel research efforts are in progress to discover nucleoside inhibitors (NIs) to find novel DAAs to target HCV RdRp.[14,15] Similarly, several non-nucleoside allosteric inhibitors are in development phase, which may provide a choice of combination with other DAAs to discover

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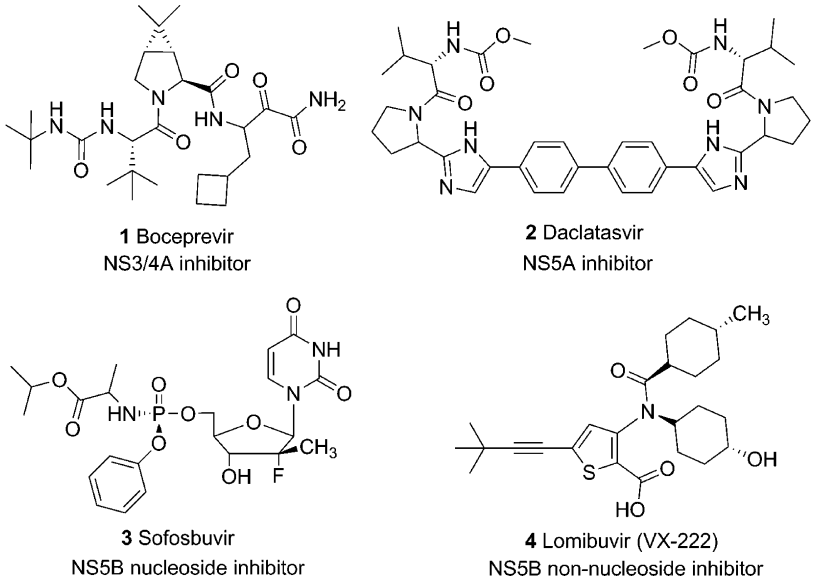

Figure 1. Chemical structures of potential scaffold as DAAs for HCV infection.

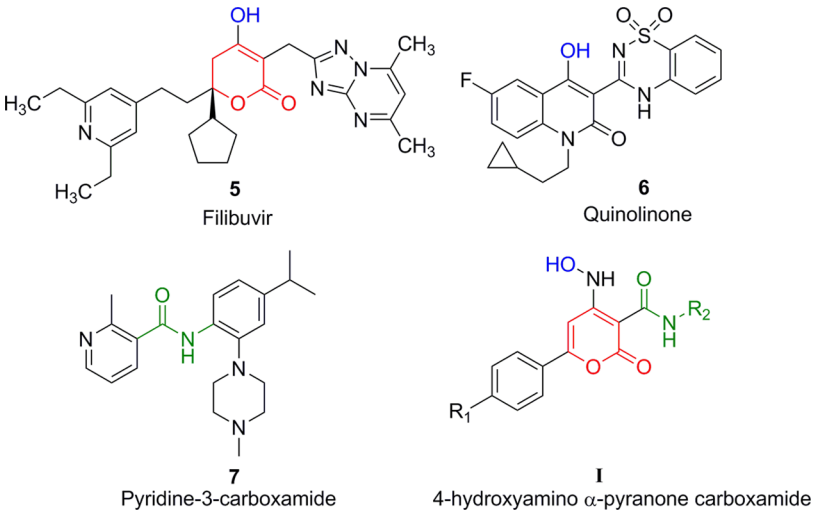

Figure 2. Structures of promising non-nucleosides (**5**−**7**) DAAs against HCV infection. Hybridization of α-pyranone motif (red), hydroxyl (blue), and carboxamide motif (green) in prototypes I.

PegIFN/RBV free regimen in future.[16] Recently several non-nucleosides (Figure 2) had shown promising results and opened a scope for the development of new anti-HCV drug candidates.[17]

The presence of α-pyranone motif[18] (red) in **5** (Figure 2) was considered as a major core in our molecular design process. Further, hydroxyl (blue, **5** and **6**)[19] and carboxamide motif (green)[20] in **7** and in several other promising anti-HCV molecules[16] prompted us to combine these motifs on core α-pyranone ring for synthesis of prototypes **I** to access their potential as possible anti-HCV candidates. In continuation to our antiviral discovery effort based on pyranone carboxamide series,[21] here, we describe the identification of a new series based on pyranone scaffold having excellent activity against HCV.

Versatile ketene dithioacetals (**9**)[22] were synthesized from appropriate acetophenones (**8**). Ketene intermediates (**9**) were converted into key intermediates α-pyranone acids (**10**) followed by coupling with appropriate amines using HATU [(dimethylamino)-*N*,*N*-dimethyl(3*H*-1,2,3-triazolo[4,5-b]-pyridine-3-yloxy)methaniminium hexafluorophosphate] as a coupling reagent yielding α-pyranone carboxamides (**11**) in good yield as per our previous reported procedure.[21] The methylthio group at C-4 of amide derivatives (**11**) was replaced with a hydroxyamino to yield target compounds I (**12**−**53**) as shown in Scheme 1 and Table 1.

All the synthesized compounds were adequately characterized by spectroscopic methods. One of the potential compounds (**25**) was further confirmed by single crystal X-ray diffraction analysis. Figure 3 represents an ellipsoidal plot of **25** as an ORTEP diagram with 30% probability level. One chloroform molecule (crystallization solvent) within the asymmetric unit of crystal structure may be attributed due to the presence of strong intermolecular H-bonding between chloroform and compound.

The anti-HCV assay of **12**−**53** was carried out in cells harboring subgenomic HCV RNA replicons (genotype 1b) with a luciferase reporter (LucNeo#2).[23] The carboxamide motifs in **12**−**29** are aromatic, **30**−**48** are aliphatic, and **49**−**53** are benzyl. Anti-HCV activity was determined by their ability to

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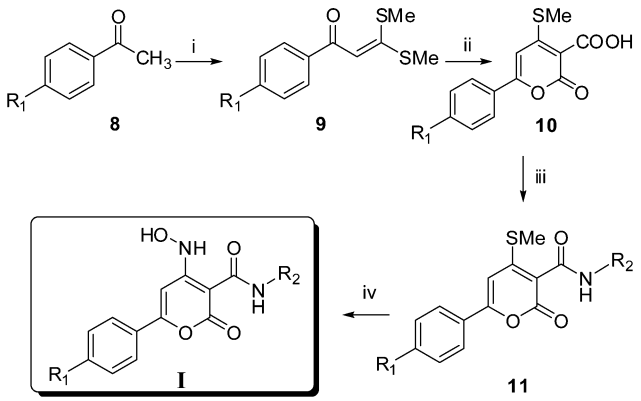
Scheme 1. Synthesis of 4-Hydroxyamino α-Pyranone Carboxamide-Based Analogues I[a]



[a]Reagents and conditions: (i) NaH, CS_2, CH_3I, THF, 0 °C to rt, 4 h; (ii) NaH, diethylmalonate, dioxane, 0−110 °C, 6 h; (iii) R_2NH_2, HATU, DMF, rt, 1 h; (iv) $NH_2OH.HCl$, $NaHCO_3$, ethanol, 80 °C, 4 h.

reduce luciferase activity in HCV RNA replicons. The cytotoxicity was evaluated by the reduction in the number of viable cells using a tetrazolium dye method. EC_{50} and CC_{50} were calculated, and results are summarized in Table 1.

The compounds **17**−**19**, **21**−**22**, **24**−**25**, and **49** (highlighted in Table 1) were significantly active with their EC_{50}s ranging between 0.15 to 0.40 μM along with low cytotoxicity (CC_{50} > 20 μM). Interestingly, R_1 = H in all of them, and except for **49**, all are aromatic carboxamides. Telaprevir, a US FDA approved NS3 protease inhibitor and nesbuvir (HCV-796), a NS5B non-nucleoside inhibitor, were used as reference for comparison. The selectivity index (SI), which is a parameter of preferential antiviral activity of a compound in relation to its cytotoxicity (CC_{50}/EC_{50}), of **12**−**53** are also tabulated in Table 1. The SIs of **21** and **25** were >110, while for telaprevir and nesbuvir, they were 44.4 and 57.1, respectively, which makes them suitable candidates for further optimization. The anti-HCV activity of **25** is shown in Figure 4.

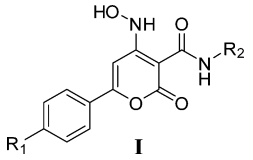
The ability of **25** to inhibit HCV RNA was evaluated by an intracellular HCV RNA quantitative assay at various concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the housekeeping genes expressed at a relatively constant level in experimental conditions, was used as an index of cell viability. HCV RNA and GAPDH RNA were quantitatively measured simultaneously by real-time PCR. The results are shown in Figure 5.

Levels of GAPDH RNA were almost constant at concentrations of **25** up to 10 μM. In contrast, the EC_{50} and EC_{90} of **25** to inhibit HCV RNA were 0.077 and 0.45 μM, respectively.

In conclusion, a new series of 4-hydroxyamino α-pyranone carboxamide analogues were prepared, and their structure−activity relationships (SAR) as inhibitors of HCV replication were studied. Eight out of 42 synthesized compounds showed significant anti-HCV activity with lower cytotoxicity. Among them, **21** and **25** had significantly higher SI (>110) in comparison to recently approved drug telaprevir (SI = 44.4), which reveals the promising discovery of novel scaffold **I** for anti-HCV drug development. Further, biochemical investigations are needed to elucidate its possible mode of action.

X-ray Crystal Structure Data for Compound 25. $C_{38}H_{32}N_4O_8.CHCl_3$, M = 792.04, triclinic, space group: $P\bar{1}$, a

Table 1. Anti-HCV Activity of Synthesized Compounds I



I	R_1	R_2	CC_{50}[a] in μM	EC_{50}[b] in μM	SI[c]
12	H	Ph	11.6	0.35	33.1
13	F	Ph	10.3	1.06	9.7
14	Cl	Ph	11.0	0.56	19.6
15	CH_3	Ph	> 20	1.45	13.8
16	OCH_3	Ph	16.2	0.56	28.9
17	H	4-F-Ph	> 20	0.32	62.5
18	H	4-Cl-Ph	> 20	0.4	50.0
19	H	4-Br-Ph	> 20	0.30	66.7
20	H	4-OH-Ph	> 20	1.00	20.0
21	H	4-OMe-Ph	> 20	0.17	117.6
22	H	4-COOMe-Ph	> 20	0.33	60.6
23	H	2-Me-Ph	> 20	0.54	37.0
24	H	3-Me-Ph	> 20	0.27	74.1
25	H	4-Me-Ph	> 20	0.18	111.1
26	F	4-Me-Ph	> 20	1.95	10.2
27	Cl	4-Me-Ph	> 20	1.43	14.0
28	CH_3	4-Me-Ph	> 20	0.46	43.5
29	OCH_3	4-Me-Ph	> 20	1.60	12.5
30	H	Me	> 20	0.60	33.3
31	F	Me	> 20	1.75	11.4
32	Cl	Me	> 20	0.78	25.6
33	CH_3	Me	> 20	1.42	14.1
34	OCH_3	Me	> 20	0.70	28.6
35	H	Et	> 20	1.00	20.0
36	H	Pr	> 20	0.45	44.4
37	H	i-Pr	> 20	0.45	44.4
38	H	C_2H_4OH	> 20	1.48	13.5
39	F	C_2H_4OH	> 20	2.98	6.7
40	Cl	C_2H_4OH	> 20	4.00	5.0
41	CH_3	C_2H_4OH	> 20	2.58	7.7
42	OCH_3	C_2H_4OH	> 20	1.41	14.2
43	H	C_3H_6OH	> 20	0.45	44.4
44	H	CH_2COOMe	> 20	1.84	10.9
45	F	CH_2COOMe	> 20	1.93	10.3
46	Cl	CH_2COOMe	> 20	3.06	6.5
47	CH_3	CH_2COOMe	> 20	3.99	5.0
48	OCH_3	CH_2COOMe	> 20	2.96	6.7
49	H	CH_2Ph	> 20	0.29	69.0
50	F	CH_2Ph	> 20	1.18	16.9
51	Cl	CH_2Ph	> 20	0.68	29.4
52	CH_3	CH_2Ph	> 20	0.91	21.9
53	OCH_3	CH_2Ph	> 20	0.57	35.1
Telaprevir (NS3 Protease inhibitor)			> 20	0.45	44.4
Nesbuvir (HCV-796) (NS5B inhibitor)			> 20	0.35	57.1

[a]The 50% cytotoxic concentration, determined by the reduction of viable cell number. [b]The 50% effective concentration, determined by the inhibition of luciferase activity. [c]Ratio of CC_{50} to EC_{50}.

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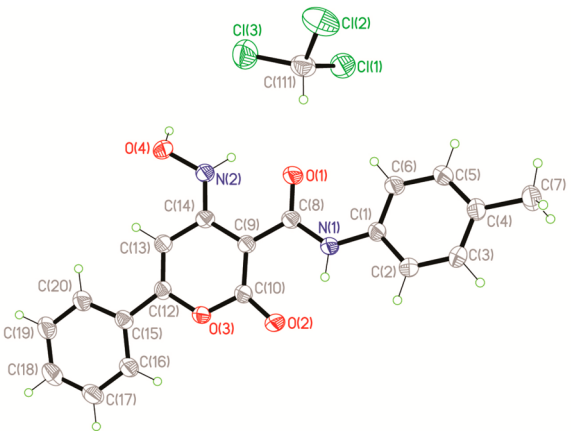

Figure 3. ORTEP diagram of **25** along with solvent of crystallization ($CHCl_3$) at 30% probability level.

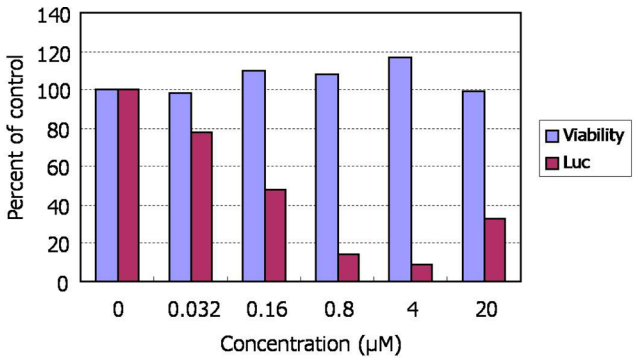

Figure 4. Anti-HCV activity of **25** in subgenomic HCV RNA replicon cells (LucNeo#2). The cell viability and luciferase activity are shown in bars with blue and brown colors, respectively.

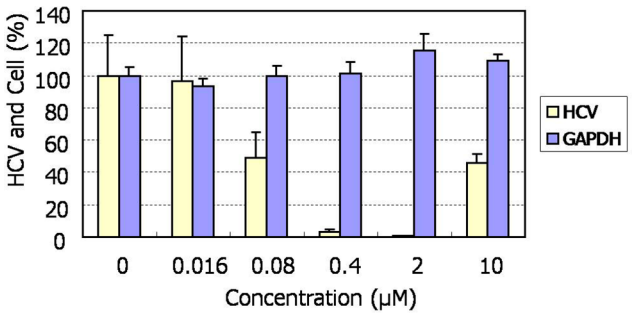

Figure 5. HCV and GAPDH RNA quantitative assays of compound **25**. The HCV and GAPDH RNA levels in full genome HCV RNA replicon cells (NNC#2) are shown in bars with yellow and blue colors, respectively. EC_{50}, EC_{90}, and CC_{50} were 0.077, 0.45, and >10 μM, respectively.

= 10.547 (1), b = 14.464 (2), c = 14.485 (2) Å, α = 62.635 (15), β = 70.780 (10), γ = 86.674 (9), V = 1841.8 (4) Å^3, T = 150 (2) K, Z = 2, μ = 0.31 mm^{-1}, $F(000)$ = 820.0, Dc = 1.428 Mg m^{-1}, yellow rectangular crystal, crystal size, 0.32 × 0.28 × 0.24 mm, 14276 reflections measured, 6483 unique, R1 = 0.0604 for 4032 $F_o > 4\sigma(F_o)$, and 0.1015 for all 6483 data and 493 parameters. Unit cell determination and intensity data collection (2θ = 50°) was performed with 99.8% completeness at 150 (2) K. Structure solutions by direct methods and refinements by full-matrix least-squares methods on F_2.

Anti-HCV Activity. Cells harboring subgenomic HCV RNA replicons with a luciferase reporter (LucNeo#2)[24] (50,000 cells/mL) were suspended in Dylbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, antibiotics, and 1 mg/mL of G418 (a protein synthesis inhibitor). After an incubation of 24 h, the cells were further incubated for 3 days in fresh culture media containing various concentrations of test compounds but not containing the G418. For a luciferase assay, the cells were washed with PBS, followed by treatment with a lysis buffer. The cell lysate (25 μL) was transferred to a microtiter plate, and luciferase assay reagent (100 μL) was dispensed into each well. Luciferase activity was measured with a luminometer. For cell viability assay, a viable cell detection reagent (Tetra Color ONE, Seikagaku Corporation) (10 μL) containing a tetrazolium salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt: WST-8) was dispensed into each well. After an incubation of 1 h at 37 °C, specific absorbance (at 450 nm) was measured with a microplate reader.

HCV RNA Quantitative Assay. Full genome HCV RNA replicon cells (NNC#2) (30,000 cells/mL) were suspended in DMEM supplemented with 10% FBS and 1 mg/mL of G418 for 24 h.[25] The cells were incubated in fresh culture media containing various concentrations of **25** but not containing the G418 for another 3 days. The cells were washed with PBS and treated with a lysis buffer containing deoxyribonuclease. The cell lysate (10 μL) was subjected to a reverse transcription reaction, followed by quantitative determination of HCV RNA and GAPDH RNA simultaneously by real-time PCR.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, characterization data, and NMR-mass spectrum for all new compounds. X-ray structure cif data for compound **25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; NNI, non-nucleoside Inhibitor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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