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Tetrazole and triazole as bioisosteres of carboxylic acid: Discovery of diketo tetrazoles and diketo triazoles as anti-HCV agents

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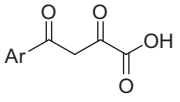
ABSTRACT

A series of diketo tetrazoles and diketo triazoles were designed and synthesized as bioisosteres of α,γ-diketo acid, the active site inhibitor of HCV (Hepatitis C virus) polymerase NS5B. Among the synthesized compounds, 4-(4-fluorobenzyloxy)phenyl diketo triazole (**30**) exhibited anti-HCV activity with an EC_{50} value of 3.9 μM and an SI value more than 128. The reduction of viral protein and mRNA levels were also validated, supporting the anti-HCV activity of compound **30**. These results provide convincing evidence that the diketo tetrazoles and diketo triazoles can be developed as bioisosteres of α,γ-diketo acid to exhibit potent inhibitory activity against HCV.

The world health organization estimates that 150 million people worldwide are chronically infected with Hepatitis C virus (HCV) and 350,000 people each year die of HCV related diseases.[1] Protective vaccination is not yet available and pegylated-interferon combined with ribavirin, the current standard therapy, is often difficult for patients to tolerate and results in a sustained viral response (SVR) in only 50% of patients infected with the predominant genotype 1.[2,3] Although two new drugs boceprevir and telaprevir as NS3 protease inhibitors were approved by FDA for the treatment of genotype 1 chronic hepatitis C recently, due to the potential of drug resistant strains[4] and widespread infection, the development of novel anti-HCV agents is still urgent.[5]

Aryl α,γ-diketo acids (DKAs, **1**, Fig. 1) were identified as specific, and reversible inhibitors of NS5B polymerase, a promising and validated target for HCV therapies, in the low micromolar range.[6] Mechanistic studies showed that, as pyrophosphate (PPi, **2**, Fig. 1) mimetic inhibitors, DKAs act as product-like analogues and chelate the two divalent cations (Mg^{2+} ions) at the active site of NS5B.[6,7] Due to the chemical and biological instability and poor membrane permeability of diketo acid group, several drug-like scaffolds were designed as analogues or mimics, such as the monoethyl ester of meconic acid (**3**, Fig. 2),[8] dihydroxypyrimidine carboxylic acid (**4**, Fig. 2),[9,10] multihydroxyl flavonoids,[11,12] etc. Unfortunately, most of these compounds were inactive in cell-based antiviral assay because of the poor cellular permeability. Since the active site of NS5B is high conserved across all HCV genotypes[13] and the mutations at the active site (e.g., S282T) significantly reduce replication capacity,[14] the active site inhibitors have the potential advantage to be active against all genotypes of the virus and the drug-resistant variants.[15]

To develop DKAs analogues with higher potential of active site inhibitors of NS5B and better cellular permeability, we replaced the free carboxylic acid of DKAs with their bioisosteres triazoles or tetrazoles and used the cell-based HCV replication system to test if a series of diketone triazoles and diketone tetrazoles could overcome the physiochemical and pharmacokinetic problems of DKAs. Although a similar replacement of carboxylic acid with triazole or tetrazole was successful in the research of integrase (IN)



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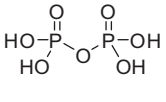

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Figure 1. Structures of aryl α,γ-diketo acids (DKAs, **1**) and pyrophosphate(**2**).

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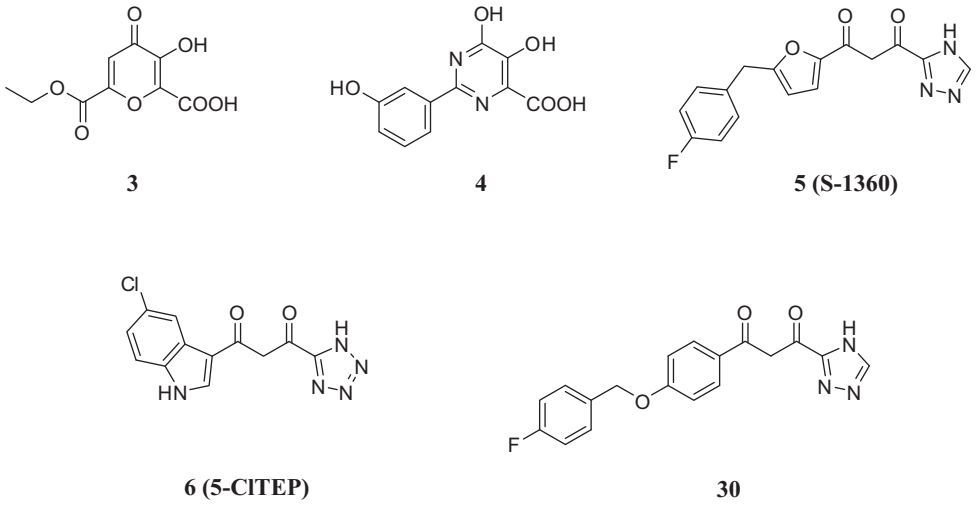


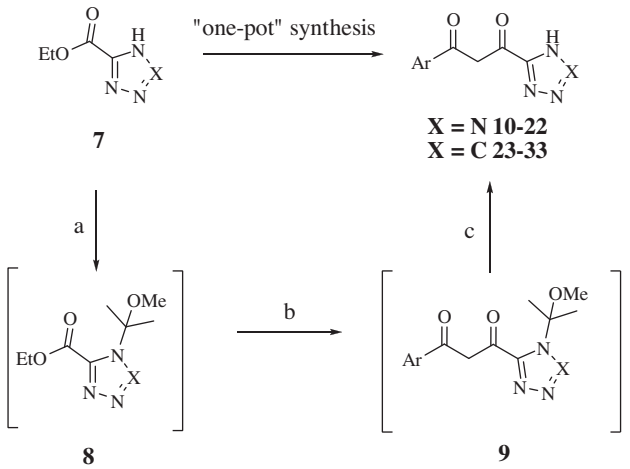
Figure 2. Structures of aryl diketo acid mimics such as monoethyl ester of meconic acid (**3**), dihydroxypyrimidine carboxylic acid (**4**), S-1360 (**5**), 5-CITEP (**6**) and our most active compound **30**.

inhibitors as anti-HIV agents leading to the discovery of S-1360 (**5**, Fig. 2) and 5-CITEP (**6**, Fig. 2),[16] related homologues of DKAs have not been tested for anti-HCV activity to our knowledge.

As shown in scheme 1, the designed compounds tetrazole derivatives **10**–**22** and triazole derivatives **23**–**33** were synthesized via a facile 'one-pot' reaction as previously reported.[17,18] Reaction of the starting material, 1*H*-tetrazol-5-ethyl formate (**7**), with 2-methoxypropene in the presence *p*-TSA and followed by Claisen condensation with various substituted aryl methyl ketone catalyzed by sodium ethoxide afford diketo tetrazole and triazole intermediate **9** which was then deprotected by 4N hydrochloric acid to obtain target compounds **10**–**33**.

All synthesized diketo triazole and tetrazole derivatives were initially evaluated for their anti-HCV activities and cytotoxicity at the single concentration of 50 μM in an authentic HCV infection/replication system in the human hepatoma cell lines Huh-7, using cell counting kit-8 (CCK8) as previously reported.[12]

The preliminary results of antiviral effect and cytotoxicity effect are shown in Table 1, respectively. RG7128 was used as positive control, which is a nucleoside clinical candidate in phase 2b.[19] A total of nine compounds exhibited more than 50% inhibition and all of the compounds showed low cytotoxic activity (cell viability ratio >50%) at 50 μM. The brief structure activities relationships (SARs) were summarized as follows. The substitute of benzyloxy in the aryl ring is essential for the biological activity. The introduction of chloride atom at the opposite position of benzyloxy can increase the anti-HCV activities, when the benzyloxy was at 4-position of phenyl ring, in both tetrazole and triazole derivatives. But the effect of the fluoride atom on the HCV activities was more complicated. For the tetrazole derivatives, the introduce fluoride atom can increase the activities, when the benzyloxy was at 3-position of phenyl ring. In comparison, the fluoride atom was more appropriate when the benzyloxy was at 3-position of phenyl ring in the triazole series. Moreover, HIV integrase inhibitors **5** and **6** were also synthesized according to the reference and their anti-HCV activities were evaluated. Unfortunately, both compounds possess much weaker anti-HCV activities with less than 50% inhibition at 50 μM, which indicated that the substituents and sort of the aryl group played a critical role for the antiviral activities, even though all the compounds contained aryl diketo tetrazole or aryl diketo triazole groups.

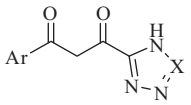


Scheme 1. Reagents and conditions: (a) 2-methoxypropene, *p*-TSA, THF, rt, 1 h; (b) aryl methyl ketone, NaOEt, 60 °C, 3 h; (c) 4 N HCl(aq), rt to 0 °C, 2 h; 62–89% overall.

Further, three of the nine active compounds (**14, 30**, and **33**) were selected to determine the EC_{50} values of their anti-HCV activities and to test their cytotoxicity in higher concentration (500 μM). The results as shown in Table 2 suggested that the triazole derivative **30** was the most potent molecule with an EC_{50} value of 3.9 μM. Additionally, compound **30** did not show clear cytotoxicity at 500 μM. Therefore, we further clarified the inhibitory effect of compound **30** on the synthesis of viral protein and the replication of viral genome. Cell lysates were subjected to western blot analysis with the antibody of viral non-structural protein NS5A, in which the level of tubulin served as a loading control. As shown in Fig. 3, the synthesis of HCV NS5A proteins was inhibited by compound **30** in a dose-dependent manner. Moreover, quantitative RT-PCR was also employed to examine the RNA level of HCV genome, which was normalized by cellular GAPDH mRNA. A dose-dependent reduction of HCV RNA levels by compound **30** was also observed (Fig. 4), which confirmed compound **30** as a promising lead with anti-HCV activity.

In conclusion, we designed and synthesized diketo tetrazole and triazole derivatives to take advantage of known bioisosteres of carboxylic acids, starting from diketo acid, known active site inhibitors of NS5B. Among the synthesized compounds, 4-(4-fluorobenzyloxy)phenyl diketo triazole (**30**) exhibited anti-HCV activity with an EC_{50} of 3.9 μM and a selectivity index greater than 128. Moreover, to confirm the antiviral activity of compound **30**,

Table 1
Anti-HCV activities for the 24 synthesized compounds



Compound	Ar	X	Inhibition ratio@[a] 50 μM (%)
10	Phenyl	N	−1.3
11	Furan-2-yl	N	0.9
12	4-Nitrophenyl	N	14.74
13	4-Methoxyphenyl	N	28.70
14	**2-(Benzyloxy)phenyl**	**N**	**67.6**[c]
15	3-(Benzyloxy)phenyl	N	11.1
16	4-(Benzyloxy)phenyl	N	54.9
17	2-(4-Fluorobenzyloxy)phenyl	N	6.8
18	3-(4-Fluorobenzyloxy)phenyl	N	62.7
19	4-(4-Fluorobenzyloxy)phenyl	N	−10.7
20	2-(4-Chlorobenzyloxy)phenyl	N	36.2
21	3-(4-Chlorobenzyloxy)phenyl	N	43.6
22	4-(4-Chlorobenzyloxy)phenyl	N	64.9
23	Phenyl	C	24.8
24	Furan-2-yl	C	14.5
25	2-(Benzyloxy)phenyl	C	34.4
26	3-(Benzyloxy)phenyl	C	48.2
27	4-(Benzyloxy)phenyl	C	12.1
28	2-(4-Fluorobenzyloxy)phenyl	C	55.0
29	3-(4-Fluorobenzyloxy)phenyl	C	43.4
30	**4-(4-Fluorobenzyloxy)phenyl**	**C**	**69.7**[c]
31	2-(4-Chlorobenzyloxy)phenyl	C	53.8
32	3-(4-Chlorobenzyloxy)phenyl	C	29.2
33	**4-(4-Chlorobenzyloxy)phenyl**	**C**	**70.2**[c]
5	(5-Chloroindol)-3-yl	N	22.4
6	(4-Fluorobenzyl)furan-2-yl	C	24.3
RG7128[b]		75.3% @ 2 μM	

[a] The anti-HCV assay was evaluated in an authentic HCV infection/replication system by measured the EGFP autofluorescence in Huh-7 cell lines. The values represent a means of triplicate results.
[b] RG7128 was used as a reference positive control.
[c] The best three compounds which were further selected to determine the EC_{50} values.

Table 2
Anti-HCV antivities (EC_{50}) and cytotoxicities (CC_{50}) for selected 3 compounds

Compound	EC_{50}[a,b] (μM)	CC_{50}[c] (μM)	SI[d]
14	9.2	>500	>54
30	3.9	>500	>128
33	6.5	>500	>77

[a] The anti-HCV assay was evaluated in an authentic HCV infection/replication system by measured the EGFP autofluorescence in Huh-7 cell lines.
[b] The EC_{50} value of each compound was the concentration required to inhibit HCV RNA replication by 50% and was estimated by linear interpolation from inhibition of 5 concentrations. The values represent average of triplicate results.
[c] The CC_{50} value of each compound means concentration required to reduce cell proliferation by 50%.
[d] Selectivity index(SI): ratio of CC_{50} to EC_{50}.

the reductionof the viral protein and mRNA levels were also tested by western blot and Real-time PCR. This study discovered a new lead compound to design more potent anti-HCV agents. Further structural modification of compound **30** as an anti-HCV candidate is currently in progress.

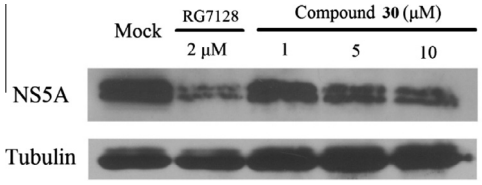


Figure 3. Western blotting showed that HCV protein expression was inhibited by compound **30** in a dose-dependent manner. RG7128 (2 μM) served as positive control and tubulin was loading control.

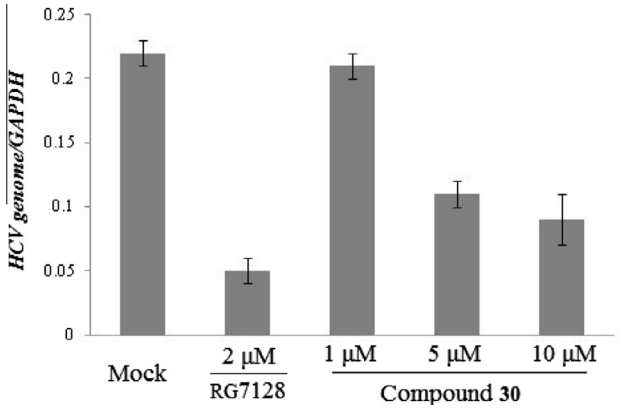


Figure 4. QRT-PCR showed that HCV replication was inhibited by compound **30** in a dose-dependent manner. RG7128 (2 μM) served as positive control and relative HCV genome levels were calculated as a ratio of the HCV genome levels to GADPH mRNA levels.

Acknowledgments

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