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Pyrazolo[1,5-*a*]pyrimidine-based inhibitors of HCV polymerase

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ABSTRACT

The present paper describes a novel series of HCV RNA polymerase inhibitors based on a pyrazolo[1,5-*a*]pyrimidine scaffold bearing hydrophobic groups and an acidic functionality. Several compounds were optimized to low nanomolar potencies in a biochemical RdRp assay. SAR trends clearly reveal a stringent preference for a cyclohexyl group as one of the hydrophobes, and improved activities for carboxylic acid derivatives.

The hepatitis C virus (HCV) was identified in 1989[1] and has been recognized as a major human pathogen associated with chronic hepatitis leading to cirrhosis and, in some cases, to hepatocellular carcinoma.[2] It is estimated that globally over 170 million people are chronically infected with HCV, and no vaccine is currently available to prevent hepatitis C.[3] The current standard therapy is pegylated interferon (IFN) in combination with Ribavirin, which has yielded modest sustained viral response (SVR) rates (40–50%) particularly in genotype 1-infected patients, the majority of the hepatitis C population in the US, Europe and Japan. Additionally this therapy is often associated with side effects, thus treatment of the chronic HCV infection represents an unmet medical need.[4]

HCV is a single stranded RNA virus in the *Flaviviridae* family. Its genome encodes for a polyprotein consisting of both structural core and envelope proteins, as well as non-structural (NS) proteins.[5] Among the NS proteins the NS5B RNA dependent RNA polymerase (RdRp) is essential for viral replication, and represents an ideal target for the development of small molecule anti-HCV compounds.[6] Inhibition of NS5B can be achieved through binding at the active site, or at one of the several allosteric sites, and several nucleoside and non-nucleoside NS5B inhibitors have been described in the literature.[7]

Our initial optimization efforts revealed compound **1** with a '5,7-pyrazolo[1,5-*a*]pyrimidine' scaffold[8a] (Fig. 1) with low micromolar biochemical activity. Compound **1** was obtained as a result of scaffold rigidification aimed at improving activity of a previously explored pyrazole chemotype.[8b] Literature HCV polymerase inhibitors **2**[9] and **3**[10] published concurrent to our synthetic efforts shared common pharmacophore features: heterocyclic hydrophobic compounds that contained an acidic functionality and hydrophobe positioning adjacent to one another, preserving cyclohexyl as one of the key hydrophobes. Our comparison of **1** with **2** and **3** resulted in the design of a new '6,7-pyrazolo[1,5-*a*]pyrimidine' scaffold **4** where by shifting the C-5 hydrophobe over to C-6 (and replacing it with *p*-benzyloxy phenyl), and by substituting the C-7 aromatic ring with a cyclohexyl moiety and adjusting the carboxylic acid position to C-3, compound **4** was obtained with improved biochemical potency. A related approach has recently been published by our group.[11] Herein, we would like to report the synthesis and optimization of novel HCV polymerase (HCV pol) inhibitors based on the '6,7-pyrazolo[1,5-*a*]pyrimidine' scaffold.

Two synthetic routes were developed to probe various sites of the scaffold and enable efficient analog synthesis. Scheme 1 describes a general sequence that allows simultaneous exploration of the distal hydrophobe at C-6, modification of the cyclohexyl group at C-7 and exploration of the carboxylic acid and derivatives at C-3 from a common intermediate **9**. Thus, starting from methyl 2-(4-(benzyloxy)phenyl)acetate or methyl 2-(4-iodophenyl)acetate, alkylation with carbonyl chlorides gave β-keto ester **6**, which in turn was saponified and decarboxylated to afford ketone **7**. Upon treatment with methoxy bis(dimethylamino)methane **7** was converted to the corresponding enamino-ketone **8**, which upon cyclization with 5-amino-1*H*-pyrazole-4-carbonitrile or ethyl 5-amino-1*H*-pyrazole-4-carboxylate in acetic acid afforded intermediate **9**. Using intermediate **9** with substituent R^1 as benzyloxy, simple treatment with boron trichloride converted R^1 to hydroxy group, which can be manipulated to afford other suitably substituted benzyloxy or phenoxy analogs via simple transformations,[12]

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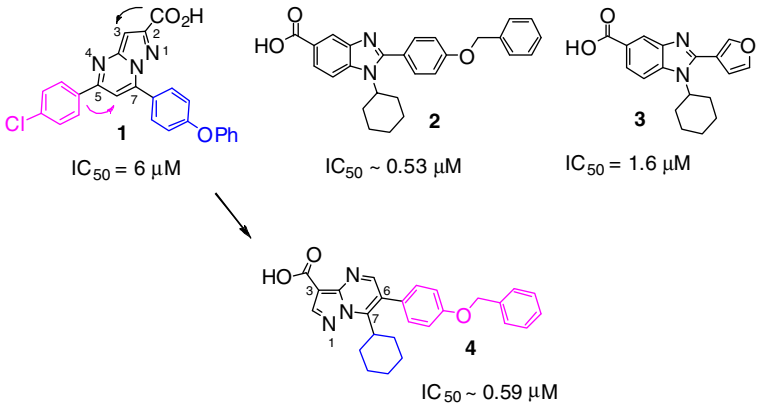
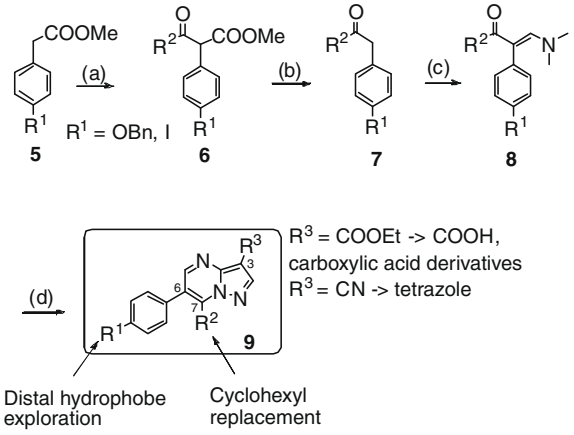
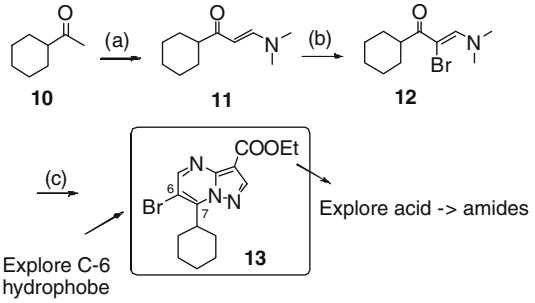


Figure 1. Design of the '6,7-pyrazolo[1,5-*a*]pyrimidine' scaffold **4**.



Scheme 1. Synthesis of advanced intermediate **9**. Reagents and conditions: (a) diisopropylamine, THF, *n*-butyllithium, methyl 2-(4-(benzyloxy)phenyl)acetate or methyl 2-(4-iodophenyl)acetate, R^2-carbonyl chloride, −78 °C to rt; ~80%; (b) DMSO, NaCl, water, 150 °C, 96%; (c) methoxy bis(dimethylamino)methane, toluene, 70 °C, quant.; (d) 5-amino-1*H*-pyrazole-4-carbonitrile or ethyl 5-amino-1*H*-pyrazole-4-carboxylate, acetic acid, reflux, 55–62%.



Scheme 2. Synthesis of advanced intermediate **13**. Reagents and conditions: (a) Bredereck's reagent (*t*-butoxybis(dimethylamino) methane), 60 °C, 93%; (b) bromine, DCM, triethylamine, 0 °C to rt; (c) 5-amino-1*H*-pyrazole-4-carboxylate, ethanol, 30% hydrogen bromide in acetic acid, reflux, 27% (steps (b) and (c)).

while with R^1 as iodo, directly attached distal hydrophobes can be introduced via Suzuki coupling.[13] The versatility of intermediate **9** was further utilized to prepare a variety of carboxylic acid derivatives and tetrazoles at C-3 simply by the use of pyrazole nitrile or carboxylate in the cyclization step (d). With R^3 as ethyl carboxylate simple hydrolysis using aqueous lithium hydroxide in tetrahydrofuran (THF) afforded the carboxylic acid which was further converted to a variety of derivatives using standard organic transformations, while with R^3 as nitrile, treatment with triethylamine hydrochloride and sodium azide in toluene/dimethylformamide (DMF) generated the corresponding tetrazole (e.g., **9**, R^3 = tetrazole).

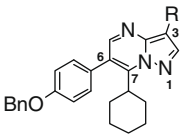
An alternative synthetic route was then developed to rapidly access analogs with fixed cyclohexyl moiety at C-7 while varying the C-6 hydrophobe and exploring carboxylic acid derivatives at C-3 from common intermediate **13**. This approach is depicted in Scheme 2 and started from the commercially available 1-cyclohexylethanone **10**. Treatment of **10** with Bredereck's reagent afforded enamino-ketone **11**, which was then brominated to generate compound **12**. Finally, cyclization of **12** with ethyl 5-amino-1*H*-pyrazole-4-carboxylate in ethanol with 30% hydrogen bromide in acetic acid gave intermediate **13** which was subsequently used in structure–activity relationships (SAR) development.

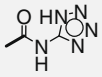
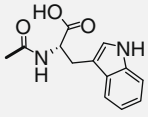
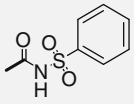
To measure the efficacy of these compounds a scintillation proximity assay (SPA)—based RNA polymerase assay was performed using radiolabeled GTP, a poly C/oligo G template/primer and the Δ-21 construct of NS5B according to a modified literature procedure.[7e,14]

The SAR development began with an investigation of the acidic functionality at C-3 and it's replacement with carboxylic acid derivatives and isosteres.[15] For this study we maintained the hydrophobes at C-6 and C-7 fixed as 4-benzyloxy-phenyl and cyclohexyl, and the results are depicted in Table 1. Comparing to the parent compound **4** reduction of the acid group to the primary alcohol (**14a**) decreased the activity dramatically, while replacement of the acid group with the tetrazole isostere gave **14b** which was equipotent to **4**. The methylated tetrazole derivative **14c** and the triazole **14d** showed a significant potency loss, however the hydroxamic acid analog **14e** retained some potency and had only a threefold loss compared to parent compound **4**. Conversion of **4** to the primary amide **14f** yielded an inactive compound, however the tetrazole carboxamide **14g** displayed similar biochemical potency to **4** and **14b**. The most potent compound in this subset was obtained by converting compound **4** to the L-tryptophan carboxamide **14h** which was active at 90 nM in the biochemical assay, and other more potent amino acid carboxamides were revealed in a subsequent investigation and their SAR will be discussed later on in the manuscript. Finally, a small set of acyl sulfonamides was synthesized (compounds **14i**–**14j**), but no significant potency gain was achieved with these analogs.

Having discovered that carboxylic acid replacement at C-3 with tetrazole is tolerated, and considering the tetrazoles' enhanced physicochemical and pharmacological properties compared to carboxylic acids,[16] we next focused our attention to the study of cyclohexyl modification and replacement at C-7, holding the tetrazole moiety fixed at C-3. (Table 2) Briefly, replacement of cyclohexyl

Table 1
Carboxylic acid and isosteres (C-3)



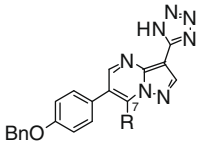
Compd	R	HCV NS5B Δ-21 IC_{50} (μM)
4		0.59
14a		12.5
14b		0.45
14c		50
14d		5.7
14e		1.9
14f		na
14g		0.67
14h		0.09
14i		1.6
14j		0.76

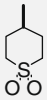
na = not active.

group with phenyl (**15a**) or other smaller acyclic groups (**15e**, **15f**, **15h**, **15i**), or modification of cyclohexyl to a pyran (**15b**) or thiopyran ring (**15c**) resulted in completely inactive or less potent compounds with micromolar biochemical activities. Therefore, we decided to maintain the cyclohexyl group fixed at C-7 for subsequent analogs.

Continuing the SAR development of the pyrazolo[1,5-*a*]pyrimidine scaffold, substitution patterns around the distal hydrophobe and linkage to the proximal hydrophobe at C-6 were also investigated, and the results are summarized in Table 3. Starting from the parent benzyloxy compound **14b**, replacement of phenyl ring with 4-pyridine (**16a**) or addition of *meta* or *para* substituents to the ring (**16b**, **16c**, **16d**, **16e**, **16f**) afforded compounds of comparable or slightly improved potency (twofold improvement for **16d** and **16e**). The most active compound identified in the benzyloxy sub-series was **16f**, bearing a carboxylic acid group *para* on the distal ring, with 42 nM in the biochemical assay. The binding mode of **16f** was investigated, and the compound was docked into the structure of NS5B using an induced-fit docking procedure[17] at

Table 2
Modification of cyclohexyl group (C-7)



Compd	R	HCV NS5B Δ-21 IC_{50} (μM)
14b		0.45
15a		na
15b		12
15c		3.4
15d		na
15e		12.5
15f		8.6
15g		na
15h		6
15i		4.6

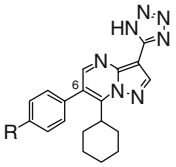
na = not active.

the finger-loop binding site. The three 'anchors' of compound **16f** in the NS5B finger-loop binding site–the tetrazole as a carboxylate mimic, and neighboring phenyl and cyclohexyl groups–overlay closely with their counterparts in the model template as well as other reported indoles and benzimidazoles[18] despite differences in scaffold and substitution (Fig. 2). As a result, compound **16f** hydrogen bonds (H-bonds) to Arg-503 with the tetrazole and makes a salt bridge interaction with Lys-491 through its benzoate moiety.

Shortening the linkage to the proximal ring by one carbon as shown in the phenoxy analogs **16g** and **16h** afforded equipotent compounds to **14b**, irrespective of the nature of the distal ring (phenyl vs cyclohexyl), while adding *meta* substituents to the distal ring (**16i** and **16j**) resulted in analogs in the same potency range as **14b**. Both benzyloxy and phenoxy linkages were slightly improved compared to the biaryl linkage (**16k** and **16l**) which afforded low micromolar analogs, and by completely removing the distal ring and adding fluorine atoms on the proximal hydrophobe, compound **16m** was obtained and found to be only slightly less potent than the parent compound **14b**.

Replacement of the distal hydrophobe with a fluorine atom enabled us to reduce the molecular weight of the scaffold, while focusing next on amide derivatives of the carboxylic acid at C-3 (Table 4), which were previously reported in the HCV literature[19] to produce inhibitors with significantly improved biochemical

Table 3
Distal hydrophobe and linkage



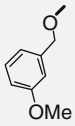
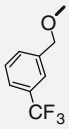
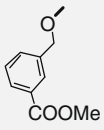
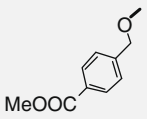
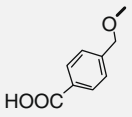
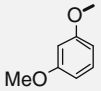
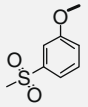
Compd	R	HCV NS5B Δ-21 IC_{50} (μM)
14b		0.45
16a		0.71
16b		0.6
16c		0.4
16d		0.22
16e		0.19
16f		0.042
16g		0.4
16h		0.4
16i		0.4

Table 3 (*continued*)

Compd	R	HCV NS5B Δ-21 IC_{50} (μM)
16j		0.2
16k		2.8
16l		0.93
16m	3,4-DiF	0.7

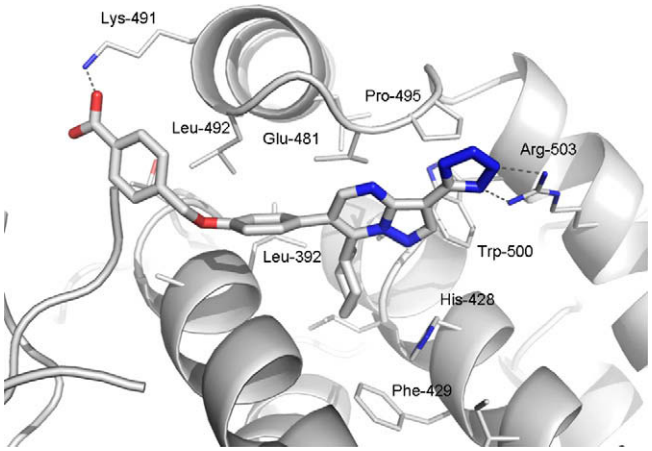
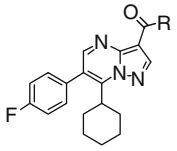
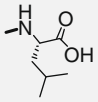
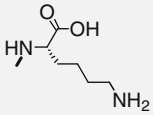
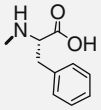
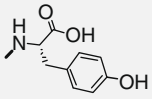
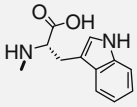
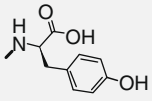
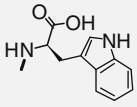
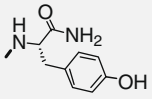
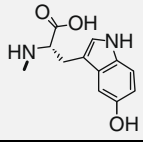


Figure 2. Induced-fit docking of **16f** into NS5B. The compound is shown is stick representation. The H-bonds to Arg-503 and Lys-491 are indicated. The figure was produced using PyMOL (Warren L. DeLano The PyMOL Molecular Graphics System; DeLano Scientific: Palo Alto, CA, USA).

activity. For this exploration we used 4-fluorophenyl ring as the C-6 hydrophobe and this gave the baseline carboxylic acid analog **17a** with an IC_{50} of 0.93 μM in the biochemical assay. Coupling this carboxylic acid with aliphatic amines generated the amides with complete loss of activity (data not shown). However, by synthesizing carboxamides of commercially available α-amino acids via 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) mediated coupling, several analogs with improved biochemical activity were obtained and the results are depicted in Table 4. With L-leucine and L-lysine side chains (**17b** and **17c**) the biochemical activity showed a 2–3-fold improvement compared to the parent **17a**. When switching to L-phenylalanine (**17d**) the potency dropped slightly to 2 μM, but simple addition of a *para* hydroxy group in L-tyrosine **17e** afforded a significant 100-fold potency improvement. The L-tyrosine amide (**17i**) and L-tryptophan analog (**17f**) were equipotent to **17e**, however the corresponding D-tyrosine and D-tryptophan analogs (**17g** and **17h**) exhibited a 25–50-fold potency loss. The most potent compound synthesized in this sub-series was 5-hydroxy L-tryptophan derivative **17j**, which had an IC_{50} of 11 nM in the biochemical assay. The extra potency of compound **17j** can be rationalized using modeling. The docking pose of **17j** presents a H-bond between the carboxylate and Arg-503 and a H-bond interaction between the 5-hydroxyl

Table 4
Carboxylic acid amides (C-3)



Compd	R	HCV NS5B Δ-21 IC_{50} (μM)
17a	OH	0.93
17b		0.29
17c		0.48
17d		2.0
17e		0.02
17f		0.02
17g		0.96
17h		0.45
17i		0.024
17j		0.011

group and Ser-431 (Fig. 3). The H-bond to Ser-431 can only be optimally reached with a 5-hydroxy L-tryptophan group.

Some of the sub-micromolar inhibitors generated during this optimization study (**4**, **14b**, **17a**) were subsequently tested in a HCV cell-based replicon assay of RNA replication.[20] However; these inhibitors displayed only modest potencies in this assay, potentially due to the higher molecular weight of the compounds.

In summary a novel series of HCV RNA polymerase inhibitors based on a '6,7-pyrazolo[1,5-*a*]pyrimidine' scaffold has been described. Several compounds were optimized to low nanomolar potencies in a biochemical RdRp assay. This series contributes to further insights into the field of HCV pol inhibition.

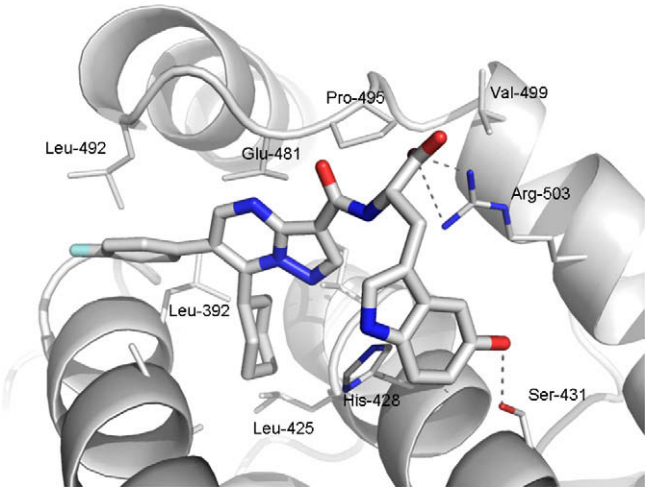


Figure 3. Docking model of compound **17j** in NS5B. The compound is shown in stick representation and the H-bonds to Arg-503 and Ser-431 are indicated with dotted lines.

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12. When R^1 = OH, conversion to various substituted phenoxy analogs was done via copper promoted arylation of phenol with aryl boronic acids following a procedure by: Evans, D. A.; Katz, J. L.; West, T. R. *Tetrahedron Lett.* **1998**, *39*, 2937, while synthesis of other aryl substituted benzyloxy analogs was accomplished either via alkylations with benzyl bromides and cesium carbonate in DMF, or Mitsunobu reactions with 'benzyl type' alcohols, triphenylphosphine and diisopropyl azodicarboxylate (DIAD).
13. Suzuki cross couplings were done using $PdCl_2$(dppf), aryl boronates and potassium phosphate in 1,4-dioxane. This procedure was also used to explore aryl hydrophobes at C-6 as depicted in Scheme 2.
14. Briefly, 50 μL reactions containing 20 mM HEPES (pH 7.3), 7.5 mM DTT, 1 unit RNasIN, 0.25 μg polyC/0.025 μg oligoG_{12}, 5 μM GTP, 1 μCi/mL [^{3}H]-GTP, 10 mM $MgCl_2$, 121 mM NaCl, 10 mM $MgCl_2$, 2% DMSO, 0.05% glycerol, 100 μg/mL BSA, and 0.05 μM NS5B (Δ-21) were incubated at room temperature for 3 hours in 96-well plates with or without test compounds. Assay was terminated by the addition of 0.5 mg streptavidin-coated SPA beads supplemented with 50 mM EDTA, and the incorporation of labeled GTP was determined by a TopCount Scintillation Counter. IC_{50} values were calculated from single experiments using 11 serial twofold dilutions (0.05–50 μM), and data was considered reliable only when the IC_{50} value of a positive internal control was within standard deviation range.
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17. The X-ray structure of an indole-based inhibitor that binds to the finger-loop region of NS5B (PDB access code: 2brk; Di Marco, S., Volpari, C., Tomei, L., Altamura, S., Harper, S., Narjes, F., Koch, U., Rowley, M., De Francesco, R., Migliaccio, G., Carfi, A., *J. Biol. Chem.* **2005**, *280*, 29765) was utilized as the frame of reference. In preparation for docking, all ligand and water molecules were deleted and hydrogen atoms were added and minimized using Macromodel as implemented in Maestro 8.5 (*Schrödinger, LLC, New York, NY, 2009*). Docking of **16f** to NS5B was performed using Glide XP (Halgren, T. A.; Murphy, R. B.; Friesner, R. A.; Beard, H. S.; Frye, L. L.; Pollard, W. T.; Banks, J. L. *J. Med. Chem.* **2004**, *47*, 1750; Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. *J. Med. Chem.* **2004**, *47*, 1739.) An inner box region (the ligand midpoint remains within this box during docking) of 10 Å and outer box region of 24 Å were used. The induced-fit docking protocol (Sherman, W.; Day, T.; Jacobson, M. P.; Friesner, R. A.; Farid, R. *J. Med. Chem.* **2006**, *49*, 534), IFD, was used to perturb the initial NS5B model in the presence of the docked ligand **16f**, while allowing a flexible adaptation of the protein environment upon compound binding. All the options used during IFD were standard, as provided by the python interface in Maestro 8.5.
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