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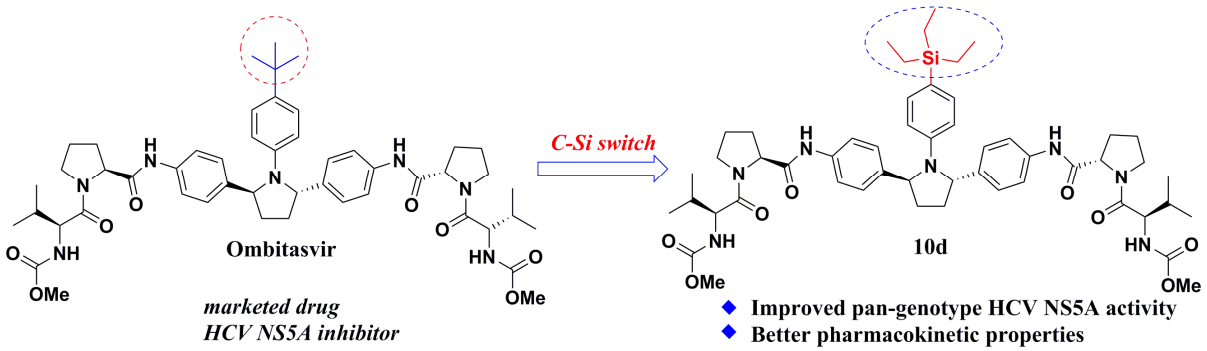

Design, synthesis and identification of silicon-containing HCV NS5A inhibitors with pan-genotype activity

Baomin Liu, Kuo Gai, Hui Qin, Xushi Liu, Yuan Cao, Qin Lu, Dandan Lu, Deyang Chen, Hengqiao Shen, Wei Song, Yang Zhang, Xiaojin Wang, Hongjiang Xu, Yinsheng Zhang*

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Abstract

Modification of a HCV NS5A inhibitor, ombitasvir, led to the identification of **10d** with improved pan-genotype NS5A inhibition and better pharmacokinetic properties. The key structural changes to ombitasvir include bioisosteric replacement of carbon with silicon atom. Compared with ombitasvir, the activity of anti-HCV genotypes (GT 1 to 6) of **10d** is increased to some extent, especially the inhibitory activity against genotype 3a and 6a is increased by more than seven times, and the dog's *in vivo* pharmacokinetics properties were also superior to ombitasvir. Further drug evaluation showed that **10d** was similar to ombitasvir on plasma protein binding and liver distribution profiles, with no cytotoxicity and no inhibitory effect on both CYP 450 and hERG ligand binding. However, permeability assay results indicated that **10d** was not the substrate of P-gp or BCRP transporter, which is different from that of ombitasvir. The results of a 14-day repeat-dose toxicity study identified no toxicity with **10d**. Our findings in preclinical tests suggest that the silicon-containing compound **10d** could be worthy of continued study as a potential drug candidate.

Keywords

Hepatitis C virus ; NS5A inhibitor; Ombitasvir; Silicon atom; Anti-HCV

1. Introduction

Chronic infection caused by hepatitis C virus (HCV) is a liver disease, which can lead to cirrhosis of the liver and hepatocellular carcinoma. It is estimated that more than 177 million adults have been infected with HCV worldwide [1].

HCV is a single-stranded, positive-sense RNA virus. It is responsible for encoding a total of three thousand amino acids [2]. At least 10 structural and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B, and protein p7, derived from E2 cleavage) are translated from HCV RNA [3]. Given their involvement in the viral life cycle, several nonstructural (NS) proteins have been treated as targets for HCV therapy [4]. In particular, the nonstructural viral protein NS5A is an attractive target for small-molecule inhibition for its relevant role in viral replication, reorganized membrane assembly, and complex interactions with cellular functions [5-7].

Traditional treatment options for HCV such as interferon and ribavirin are poorly tolerated and produces low sustained virological response (SVR) rates (42-46%)[8]. Fortunately, direct-acting antivirals (DAAs) approved by the Food and Drug Administration (FDA), such as the NS3/4A protease inhibitors simeprevir [9] and paritaprevir [10], the NS5B inhibitors sofosbuvir [11] and dasabuvir [12], the NS5A inhibitors daclatasvir [13], ledipasvir [14], elbasvir [15], ombitasvir [16], velpatasvir [17] and pibrentasvir [18] have led to significant improvement in treating chronic hepatitis C (CHC). However, most of the existing approved drugs showed different potency against various HCV genotypes (GT 1 to 6), demonstrating a clear unmet medical need for high potency, pan-genotype activity drugs.

Small-molecule NS5A inhibitors are important components of several combination therapies such as Harvoni®, Zepatier®, Technivie® & Eplcusa®, and have been considered as the most promising direct acting antiviral agents [19]. Ombitasvir (ABT-267) is a NS5A inhibitor recently approved by the FDA for use in the complicated combination with paritaprevir, ritonavir and dasabuvir in the product Viekira Pak for the treatment of HCV genotype 1 [20], and with paritaprevir and ritonavir in the product Technivie ® for the treatment of HCV genotype 4 [21]. In an effort to develop better NS5A inhibitors, we choose ombitasvir (OMBITASVIR) as a lead to identify inhibitors with more potent activity against all genotypes. Herein, we describe our efforts to investigate the impact of introducing a silicon atom into the ombitasvir molecule.

Incorporation of a silicon atom into drug molecules has attracted attention as a drug design principle that can have the potential to affect and, ideally, improve both the bioactivity and ADMET (absorption, distribution, metabolism, excretion and toxicity) profiles of compounds [22]. The changes in biological and pharmaceutical properties due to sila-substitution may be attributed to the different properties of silicon from carbon, such as atomic size, electronegativity and hydrophobicity[22]. We adopted a carbon-silicon (C-Si) switch strategy to design a series of novel silicon-containing compounds with the goal of identifying novel inhibitors that possess both pan-genotype activity and improved pharmacokinetic (PK) properties over the known drug, ombitasvir. The strategy focused on replacing the tert-butyl group of ombitasvir (ABT-267) with several silyl groups to give its novel silicon analogs (Figure 1). All the silicon analogues were assessed against various of HCV genotypes. The most potent compound **10d** was identified as a promising candidate after an in depth investigation of its ADMET profiles.

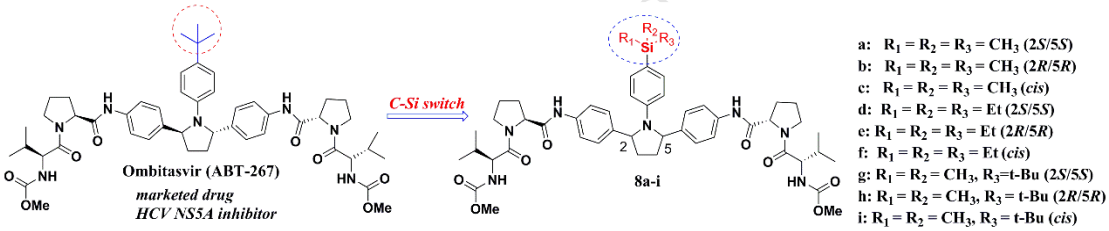


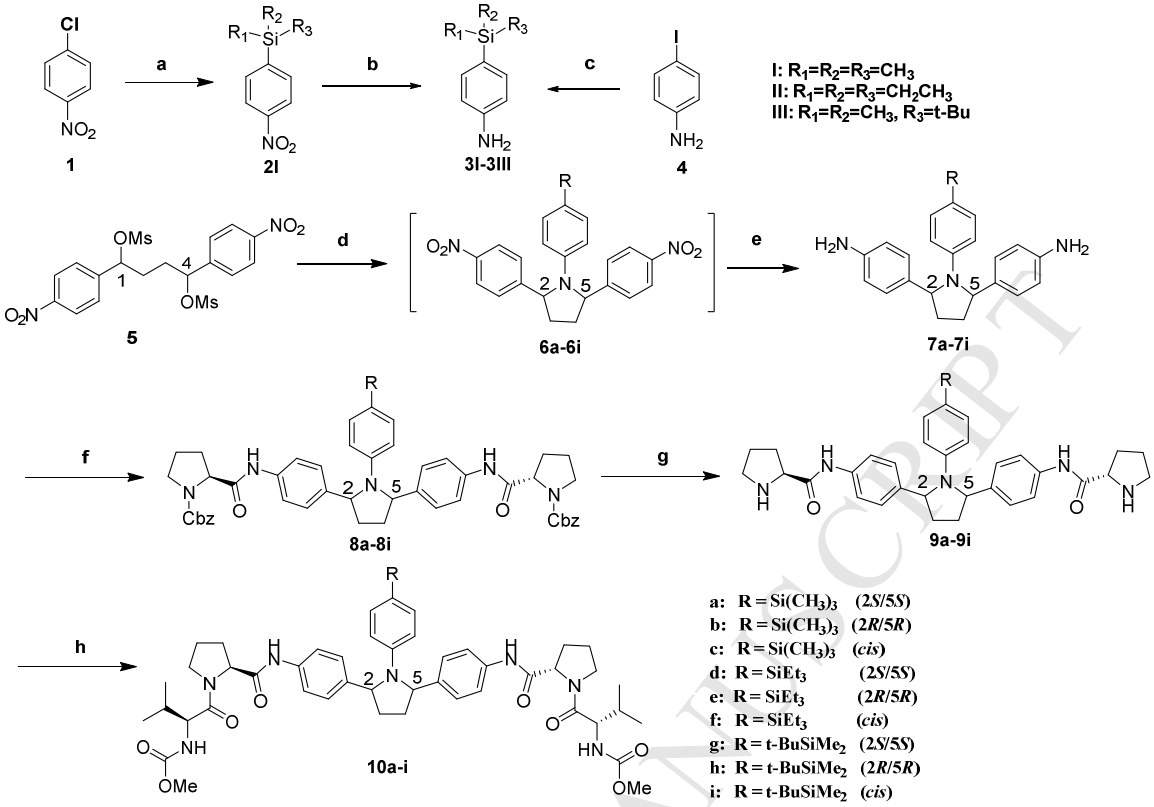
Figure 1. Design of silicon incorporated ombitasvir analogs.

2. Results and Discussion

2.1. Chemistry

The synthesis of the silicon derivatives **10a-10i** was carried out as shown in Scheme 1. The silyl-aniline **3I** was synthesized in a two-step process with good yields by following Kimes' procedures [23]. Availability of triethylsaline and t-butyldimethylsaline allowed us to prepare the silyl-amines (**3II** & **3III**) in one C-Si cross-coupling step according to the reported method [24] which used $Rh(cod)_2BF_4$ as a catalyst and K_3PO_4 as a base.

Scheme 1. Synthesis of silicon-containing HCV NS5A inhibitors[a]



[a]Reagents and conditions: (a) $Pd(PPh_3)_4$, hexamethyldisilane, xylene 170°C; (b) 10% Pd/C, H_2, EtOH, rt; (c) $Rh(cod)_2BF_4$, hydrosilanes, *N*-methylpyrolidinone, K_3PO_4, rt; (d) Compounds **3I-3III**, Et_3N, DMF, 60°C; (e) PtO_2, H_2, rt; (f) (phenoxycarbonyl)-*L*-proline, EDCI, HOBT, *N*-methylmorpholine, DMF; (g) H_2, 10% Pd/C, EtOH/MeOH, 10 atm; (h) (methoxycarbonyl)-*L*-valine, EDCI, HOBT, *N*-methylmorpholine, DMF.

The di-mesylates **5** (three stereoisomers, 1,4-SS, RR, SR) were then treated with the silyl-aniline (**3I-3III**) of choice in the presence of triethylamine to provide pyrrolidine derivatives **6a-6i** as a crude. Without further purification, di-nitro compounds **6a-6i** were reduced to di-aniline compounds **7a-7i** under PtO_2 catalyzed hydrogenation condition. Compounds **7a-7i** as a three-stereoisomer mixture can be readily separated into racemic *trans*-pyrrolidine (**7a/7b, 7d/7e, 7g/7h**) and *cis*-pyrrolidine (**7c,7f,7i**) isomers by silica gel chromatography. The proline amide side chains were attached to pyrrolidine derivatives (**7a-7i**) by peptide coupling with cbz-proline to give **8a-8i**, followed by deprotection and further peptide coupling with (methoxycarbonyl)-*L*-valine to afford the final products **10a-10i**. Final compounds prepared using racemic *trans*-pyrrolidine isomers were further separated into enantiomerically pure *trans*-pyrrolidine analogues on a CHIRALART Amylose-SA

column. The *cis*-pyrrolidine intermediates gave the *cis*-pyrrolidine analogues (**10c,10f,10i**) as final compounds directly. A single stereoisomer (1R,4R) of **5** was also prepared according to the literature method [25] and applied to prepare **10a**, **10d** and **10g** as a single enantiomer.

2.2. In *vitro* anti-HCV activity

To determine whether the compounds are effective HCV inhibitors, we investigated the inhibitory activities of the synthesized compounds using the GT1a and GT1b subgenomic replicon assays. As indicated in Table 1, most of the compounds showed similar potency with ombitasvir against GT1b and three compounds (**10a**, **10d**, **10g**) demonstrated higher potency than ombitasvir against GT1a. It's noted that the 2*S*,5*S* isomers (**10a**, **10d** & **10g**) exhibited increased potency against GT1a compared to their 2*R*,5*R* isomers (**10b**, **10e** & **10h**), while the *cis*-isomers showed no obvious potency against GT1a. In addition, the 50% cytotoxic concentration (CC_{50}) values of all the compounds tested in an HCV genotype 1a replicon cell line was more than 1 nM.

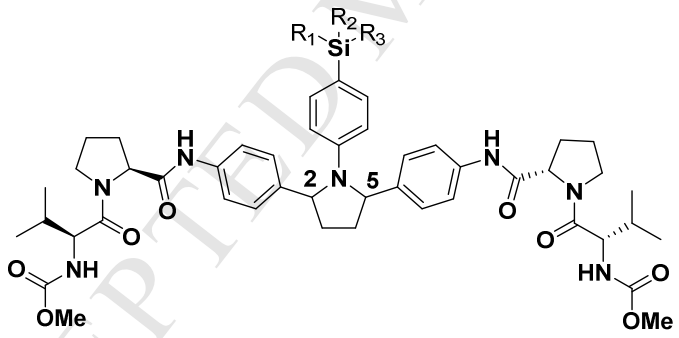


Table 1. In *vitro* activities of compounds in the replicon assay.

Compounds	Configuration	R_1	R_2	R_3	Replicon (nM)[a]	EC_{50}	Cytotoxicity CC_{50} (nM)[a]
					GT1a	GT1b	
10a	2*S*,5*S*	Me	Me	Me	0.014	0.004	>1
10b	2*R*,5*R*	Me	Me	Me	0.118	0.007	>1
10c	*cis*	Me	Me	Me	>0.5	0.008	>1
10d	**2*S*,5*S***	**Et**	**Et**	**Et**	**0.007**	**0.003**	**>1**
10e	2*R*,5*R*	Et	Et	Et	0.041	0.006	>1
10f	*cis*	Et	Et	Et	>0.5	0.007	>1
10g	2*S*,5*S*	Me	Me	t-Bu	0.011	0.003	>1
10h	2*R*,5*R*	Me	Me	t-Bu	nd[b]	nd[b]	nd[b]
10i	*cis*	Me	Me	t-Bu	nd[b]	nd[b]	nd[b]
Ombitasvir	-	-	-	-	0.03	0.005	>1

[a]Mean of triplicate well values. All experiments were performed at least twice. EC_{50} stands for 50% effective concentration; CC_{50} stands for 50% cytotoxic concentration in an HCV genotype 1a replicon cell line.[b] Not determined.

As a result of their picomolar (pM) potency, compound **10a, 10d** and **10g** were selected for further in *vitro* virologic evaluation. As shown in Table 2, the selected compounds demonstrated picomolar potency not only against GT1a and GT1b, but also against GT2 through GT6. It is interesting to note that the three selected compounds showed more potent inhibitory activity against GT3a and GT6a in comparison to ombitasvir, with 2.3-, 8.6- and 7.2-fold increased activity against GT3a and 2.4-, 7.6- and 3.7-fold increased activity against GT6a, respectively.

Table 2. Antiviral activity of 10a, 10d & 10g in HCV replicon cell lines containing NS5A from GT1-6.

	Replicon EC_{50} (pM)[a]						
Compound	GT1a	GT1b	GT2a	GT3a	GT4a	GT5a	GT6a*
10a	14	4	2.5	38	0.1	1	1411
10d	**7**	**3**	**0.8**	**10**	**0.1**	**1**	**445**
10g	11	3	1.1	12	0.1	1	925
Ombitasvir	30	5	2.6	86	0.3	4	3392

[a]Mean of triplicate well values. All experiments were performed at least twice. EC_{50} stands for 50% effective concentration.

* EC_{50} data for ombitasvir was reported in 2015[26]. The activity against GT6a from the published data (55 pM) for ombitasvir is quite different from our own data (3392 pM). Differences observed between published data and our data might result from differences in laboratory and cell source.

2.3. Pharmacokinetic studies

Based on their broad genotype activity, pharmacokinetic studies were performed for the selected compounds (**10a,10d,10g**). In *vitro* metabolic stability in mouse, rat and human microsomes (MLM, RLM and HLM, respectively) for the set of compounds appear in Table 3. Mouse, rat and human microsomal stability was generally similar for the compounds. All compounds were stable when it was incubated for 60 min in the presence of 0.2 mg/ml concentration of mouse, rat or human liver microsomal fractions.

Table 3. In vitro metabolic stability data.

Compound	MLM (% remaining[a])	RLM (% remaining[a])	HLM (% remaining[a])
10a	93.3	100.9	99.2

10d	92.7	98.0	85.6
10g	92.1	87.0	84.3
Ombitasvir	88.7	100.8	96.2

[a] Percentage of parent compound remaining after incubation for 60 min in the presence of 0.2 mg/ml mouse, rat or human liver microsomal fractions.

Oral pharmacokinetic data in mouse were obtained using solutions in organic solvents in order to aid in dissolution and absorption of the compounds. The selected compounds showed longer half-life but lower plasma concentrations (AUC) compared to ombitasvir (Table 4). Especially for compound **10d** and **10g**, plasma half-life were longer 2.9- and 4.5-fold than ombitasvir, respectively.

Table 4. In *vivo* pharmacokinetic parameters of selected compounds in mouse.

Compound	**10a**	**10d**	**10g**	Ombitasvir
T_{max} (h)	3	3	3	3.0
C_{max} (ng/mL)	262.1	198.2	211.4	583.3
$t_{1/2}$(h)	8.8	22.5	34.7	7.7
$AUC_{(0-t)}$ (ng*h/mL)	2914	3041	3516	6480

Considering its better broad genotype activity and pharmacokinetic properties, compound **10d** was selected for further pharmacokinetic properties studies in dog. In comparison to ombitasvir, it is very interesting that compound **10d** showed an improved plasma exposure (604.7 *VS* 187.5) with similar half-life (12.7h *VS* 11.1h) in dog following oral dosing (Table 5). The pharmacokinetic data in dog are very different from that in mouse. The results support this canine setting for further evaluation of compound **10d**.

Table 5. In vivo pharmacokinetic parameters of 10d in dog.

Compound	**10d**	Ombitasvir
T_{max}(h)	10.5	8.3
C_{max} (ng/mL)	29.18	15.68
$t_{1/2}$(h)	12.7	11.1
$AUC_{(0-t)}$ (ng*h/mL)	604.7	187.5

2.4. Plasma protein binding

Studies were performed to evaluate plasma protein binding to compound **10d** and ombitasvir and species differences (Table 6) as well. Equilibrium dialysis studies demonstrated that compound **10d** is highly bound (>99.9%) to human, dog, rat and

mouse albumin, similar to ombitasvir. High plasma protein binding limits the partitioning of the testing compounds from the blood into the tissues where they could be metabolized. The results are consistent with the highly lipophilic property of both compounds.

Table 6. Plasma protein binding profiles of 10d and ombitasvir.

		10d	Ombitasvir
Rat	1 μM	99.991%	99.998%
	10 μM	99.983%	99.999%
Mice	1 μM	99.992%	99.996%
	10 μM	99.989%	99.999%
Dog	1 μM	99.996%	99.998%
	10 μM	99.994%	99.999%
Human	1 μM	99.992%	99.997%
	10 μM	99.992%	99.996%

2.5. Apparent permeability in human Caco-2 cells

To obtain information about their apparent permeability (P_{app}), **10d** and ombitasvir were studied in a human Caco-2 cell model (Table 7). For the apical-to-basolateral (**A→B**) transport, the apparent permeability was low for both **10d** (P_{app}= 0.7 nm/s) and ombitasvir (P_{app}= 0.8 nm/s). The efflux ratio was low for **10d** (0.8) and high for ombitasvir (6.7). These data suggest **10d** is not the substrate of P-gp or BCRP transporter, while P-gp or BCRP or both may played roles in the absorption of ombitasvir.

Table 7. Apparent permeability (P_{app}) and efflux ratio (ER) of 10d and ombitasvir.

compound	**P_{app} (nm/s) (N=2)**		**Efflux ratio**
	A→B	**B→A**	
10d	0.7	0.5	0.8
Ombitasvir	0.8	5.4	6.7

2.6. Liver distribution

The liver distribution of **10d** and ombitasvir were evaluated in adult male Sprague-Dawley(SD) rats. The mean concentrations of **10d** and ombitasvir in rat plasma and liver after oral administration are shown in Table 8. The concentrations of **10d** in plasma and liver were lower than ombitasvir till the 8 hours after oral dosing, but similar at 24 hour. The plasma and liver concentrations of both compounds displayed a similar changing tendency, which the concentration of the liver was much higher than the plasma at same time, and the maximum concentration occurred at

about 2 hours in plasma and liver, implicating that the **10d** and ombitasvir might be distributed in rats with a higher speed. The maximum LPR of **10d** at 8 h and ombitasvir at 0.5 h were 52.9 and 43.4, respectively, suggesting that the two compounds can distribute into liver easily.

Table 8. Liver distribution of 10d and ombitasvir in rat (mean ± SD, n=3).

Time(h)	Ombitasvir			**10d**		
	Plasma	Liver	LPR [a]	Plasma	Liver	LPR [a]
0.5	50.5 ± 19	2198 ± 864	43.4 ± 1.95	22.3 ± 7.84	160 ± 72.8	7.22 ± 1.67
2	129 ± 9.17	3134 ± 573	24.6 ± 6.34	35.4 ± 2.06	982 ± 288	28.1 ± 9.59
8	50.8 ± 6.48	1704 ± 414	33.5 ± 6.36	11.2 ± 1.22	592 ± 191	52.9 ± 16.4
24	10 ± 4.58	199.8 ± 22	22.3 ± 8.01	5.86 ± 3.37	160 ± 37.7	31.9 ± 12.2

[a] Liver concentration/Plasma concentration ratio (LPR).

2.7. Cytotoxicity

Several experiments were conducted to assess the safety of compound **10d**. First, we investigated the effect of the compound **10d** and ombitasvir in human umbilical-vein endothelial cells (HUVEC) from healthy donors. Both compounds at 50 μM level showed no cytotoxicity (16.6% and 11.8% inhibition) to HUVEC (Table 9), compared to HCV replicon cells (EC_{50} value in pM level).

Table 9. Inhibition of compound 10d toward HUVEC [a] (mean± 3, n = 3).

Compound	Concentration (μM)	Inhibition (%)
10d	50	16.6 ± 2.3
Ombitasvir	50	11.8 ± 2.1

[a]Human umbilical vein endothelial cells.

2.8. CYP inhibition

Then, the affinity of compound **10d** and ombitasvir toward several human liver CYP450 enzymes were tested. As shown in Figure 2, both compounds did not inhibit any of the studied human liver CYP450 enzymes at concentrations of 1 μM and 10 μM. The results suggest the absence of clinically significant interactions between **10d** and other drugs metabolized by the above mentioned human cytochromes.

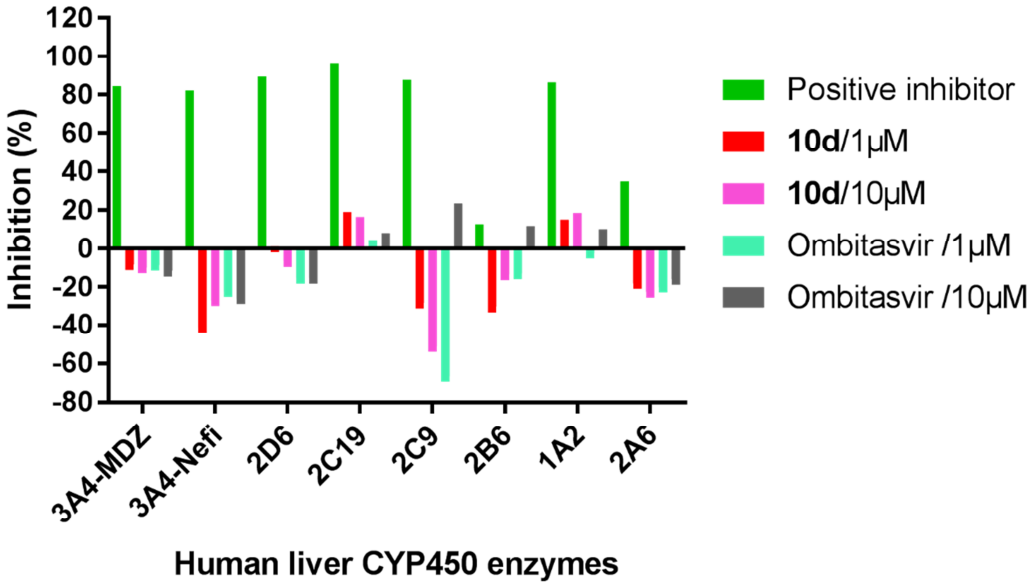


Figure 2. The inhibition of compound **10d** and ombitasvir toward human liver CYP450 panel.

2.9. hERG activity

To assess the potential heart related safety issues of compound **10d**, the inhibitory effect of **10d** at the hERG potassium ion channel was tested at 3 and 10 μM concentration respectively (Table 10). At both test concentrations, the inhibitory effect of **10d** did not exceed 10%, suggesting a relatively low risk of QT interval prolongation.

Table 10. Results of hERG ligand binding assay (mean ± SD, n = 3).

Compound	Concentration (μM)	Inhibition (%)
10d	3	6.9 ± 1.6
	10	8.7 ± 1.9
Ombitasvir	3	1.1 ± 1.2
	10	1.8 ± 3.1

2.10. Repeat-dose toxicity study

A 14-day repeated oral dose toxicity study of **10d** was performed. ICR mice, each group consisting of 5 males and 5 females, were administered **10d** once daily by gavage at dose of 150 mg/kg body weight. Mice were killed after the 14th administration. Animals in all groups were observed daily and detailed clinical signs evident. Their body weights and food intake were monitored on day 1 of administration and thereafter twice per week. As demonstrated in figure 3, all animals

continued to gain weight, no significant difference with the control group. Food consumption were no difference between the two groups (data not shown).

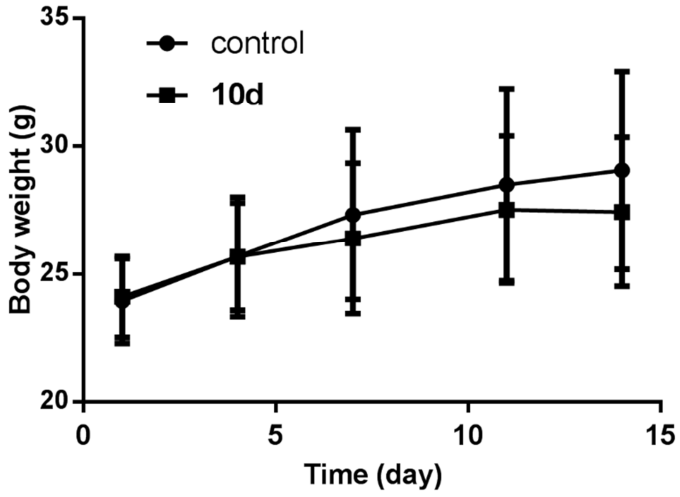


Figure 3. Body weights for mice orally treated with **10d** for14 days.

At necropsy, animals were weighed and sacrificed by exsanguination from the abdominal aorta under ether anesthesia. Before killing, blood samples were collected from the abdominal aorta to use for hematology, blood chemistry and measurement of serum hormone levels. Hematologically, decrease in the RET and reticulocyte ratio was observed from **10d**, statistically significant ($p < 0.05$) (Table 11). Blood chemistry data indicated a statistically significant increase of direct bilirubin in **10d** ($P < 0.01$) (Table 12).

Table 11. Hematology data for mice orally treated with 10d for 14 days (Mean±SD, n = 10).

	Control	**10d**
WBC [a] (10^9/L)	7.03 ± 1.56[a]	6.20 ± 1.75
RBC [b] (10^12/L)	9.04 ± 0.80	9.00 ± 0.76
HGB [c] (g/L)	157.40 ± 15.50	154.60 ± 11.61
PLT [d] (10^9/L)	1260.30 ± 137.87	1263.40 ± 382.86
NEUT# [e] (10/μL)	184.30 ± 102.75	212.60 ± 150.72
LYMPH# [f] (10/μL)	412.20 ± 162.4	318.80 ± 121.87
MONO# [g] (10/μL)	93.90 ± 47.09	76.60 ± 26.90
EO# [h] (10/μL)	11.90 ± 5.11	12.00 ± 7.80
BASO# [i] (10/μL)	0.20 ± 0.42	0.10 ± 0.32
NEUT% [j] (%)	27.74 ± 16.27	33.12 ± 16.45
LYMPH% [k] (%)	57.65 ± 13.53	52.54 ± 15.94

MONO% [l] (%)	12.93 ± 3.61	12.44 ± 3.27
EO% [m] (%)	1.66 ± 0.54	1.88 ± 0.92
BASO% [n] (%)	0.02 ± 0.04	0.02 ± 0.06
RET# [o] (10^9/L)	427.84 ± 64.70	348.97 ± 72.01*
RET% [p] (%)	4.73 ± 0.56	3.88 ± 0.76*

*Significantly different from the control value at the levels of P< 0.05.

[a] White blood cell count ; [b] Red blood cell count; [c] Hemoglobin; [d] Platelet count; [e] Neutrophil count; [f] Lymphocyte count; [g] Monocyte count; [h] Eosinophil count; [n] Basophil count; [j] Neutrophil percentage; [k] Lymphocyte percentage; [l] Monocyte percentage; [m] Eosinophil percentage; [n] Basophil percentage; RET# [o] Reticulocyte count; [p] Reticulocyte percentage.

Table 12. Blood chemistry data for mice orally treated with 10d for 14 days (Mean ± SD, n = 10).

	Control	**10d**
ALT [b] (U/L)	28.33 ± 9.89[a]	25.30 ± 3.68
AST [c] (U/L)	111.44 ± 32.76	110.60 ± 37.17
ALB [d] (g/l)	25.87 ± 1.67	25.38 ± 1.45
TP [e] (g/l)	53.97 ± 1.56	52.31 ± 2.29
GGT [f] (U/L)	0.00 ± 0.01	0.03 ± 0.09
ALP [g] (U/L)	114.44 ± 21.78	105.70 ± 23.42
BUN [h] (mmol/l)	10.44 ± 1.77	10.16 ± 2.02
CR [i] (μmol/l)	25.22 ± 3.42	23.20 ± 2.25
GLU [j] (mmol/l)	3.64 ± 0.79	4.18 ± 1.18
TB [k] (μmol/L)	9.95 ± 2.18	11.24 ± 2.70
DB [l] (μmol/L)	7.60 ± 0.59	8.57 ± 0.72**
TBA [m] (μmol/L)	5.67 ± 2.45	10.50 ± 11.52
TG [n] (mmol/l)	1.67 ± 0.32	1.53 ± 0.39
CHO [o] (mmol/l)	0.93 ± 0.31	0.85 ± 0.21
GLDH [p] (U/L)	13.78 ± 2.86	15.10 ± 5.84
CK [q] (U/L)	705.78 ± 330.84	953.30 ± 1014.67

[a] Mean ± SD.

*,**Significantly different from the control value at the levels of P< 0.05, P< 0.01, respectively.

[b] Alanine aminotransferase; [c] Aspartate aminotransferase; [d] Albumin; [e] Total protein; [f] Glutamyl transpeptidase; [g] Alkaline phosphatase; [h] Blood urea nitrogen; [i] Creatinine; [j] Glucose; [k] Total bilirubin; [l] direct bilirubin; [m] Total bile acid; [n] Triglyceride; [o] Cholesterol; [p] Glutamate dehydrogenase; [q] Creatine kinase.

Absolute and relative weights were non-significant statistically between control and

10d (Table 13). Moreover, no abnormality was observed on the organs, when the animal sacrificed. Pathological examination showed that the lesions were spontaneous and non drug-induced. Although some of the indicators were abnormal in the repeat-dose toxicity study, the pathological examination and other related indicators were normal. In summary, animal studies identified no toxicity with **10d**.

Table 13. Body and organ weights (g, Mean ± SD, n = 10).

group	BW	heart	liver	spleen	kidneys	Thymus	brain	testis	Ovary
control	25.8	0.539	4.365	0.355	1.510	0.289	1.686	0.881	0.079
	±3.5[a]	±0.094	±0.222	±0.100	±0.211	±0.081	±0.241	±0.037	±0.007
10d	24.0	0.567	4.346	0.345	1.579	0.240	1.807	0.789	0.097
	±2.5	±0.100	±0.305	±0.065	±0.167	±0.088	±0.185	±0.174	±0.017

[a] Mean ± SD.

3. Conclusion

In summary, we have designed and synthesized several silicon-containing derivatives of ombitasvir and identified a few of HCV NS5A inhibitors with pan-genotype activity. Among them, compound **10d** demonstrated the most promising pan-genotypic HCV inhibitory activity, with an EC_{50} range of 0.1-10 pM against genotypes 1a, 1b, 2a, 3a, 4a, and 5a, and 445 pM against genotype 6a. As compared to the marketed anti-HCV drug, ombitasvir, the activity of anti-HCV genotypes (GT 1 to 6) of **10d** is increased to some extent, especially the inhibitory activity against genotype 3a and 6a is increased by more than seven times. Similarly to ombitasvir, compound **10d** showed high stability in mouse, rat and human liver microsomes and did not inhibit most of human liver CYP450 enzymes. In addition, compound **10d** exhibited excellent pharmacokinetic properties in dog with good liver specificity in rat. Permeability assay results indicated that **10d** was not the substrate of P-gp or BCRP transporter. Several experiments were conducted to assess the safety of compound **10d**. Our data suggest that compound **10d** has no cytotoxicity in HUVEC and no inhibition activity detected in hERG ligand binding inhibition assay. Moreover, no toxicity with **10d** was observed in 14-day repeat-dose toxicity study. Based on these encouraging results, further studies are being pursued.

4. Experimental section

4.1 Chemistry. General Procedures. Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. ^{1}H NMR

spectra (300 MHz) were collected on a Bruker-300 FT NMR spectrometer with tetramethylsilane (TMS) as internal standard. High resolution mass spectra (HRMS) were recorded on an AB SCIEX Triplet TOF 4600 Mass Spectrometer. All final compounds were purified to > 96% purity as determined by HPLC analyse. Reverse phase HPLC purity determinations were performed on an Agilent 1260 HPLC system. Compounds **3I**, **3II** and **3III** were prepared according to the reported methods [27]. Compound **5** was purchased from Nanjing Ally Chemical S& T Co., Ltd. Ombitasvir was synthesized according to the reported methods with 99% purity [25].

4,4'-(*trans*-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)dianiline (7a /7b) and 4,4'-(*cis*-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)dianiline (7c). To a solution of **5** (4.5 g, 9.2 mmol) in anhydrous *N*,*N*-dimethylformamide (24 mL) was added **3I** (10.66 g, 64.47 mmol), followed by dropwise addition of triethylamine (9.32 g, 92.1 mmol). The resulting mixture was stirred at 60 °C for 12 h. The cooled mixture was poured into water (50 mL), extracted with ethyl acetate (50 mL× 3), and the combined organic layers were dried over Na_2SO_4. The drying agent was filtered off, and the solvent was evaporated to give 2,5-bis(4-nitrophenyl)-1-(4-(trimethylsilyl)phenyl)pyrrolidine (**6a/6b, 6c**, 12 g) as a crude product which was used without further purification.

A mixture of **6a/6b & 6c** (12 g, crude product) and platinum (IV) oxide (4.0g, 17.61 mmol) in tetrahydrofuran (80 mL) was stirred under hydrogen gas at 10 atm for 48 h. The resulting mixture was filtered and concentrated in vacuo to give a crude product which was purified by column chromatography on silica gel using a solvent gradient of 10-50% ethyl acetate in petrol. The first eluting component was the *trans*-pyrrolidine isomers **7a/7b** (560 mg, 15.2% yield, two steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.03-7.00 (d, *J* = 8.2 Hz, 2H), 6.83-6.80 (d, *J* = 8.1 Hz, 4H), 6.48-6.46 (d, *J* = 8.1 Hz, 4H), 6.28-6.26 (d, *J* = 8.3 Hz, 2H), 5.00-4.98 (d, *J* = 6.3 Hz, 2H), 4.86 (s, 4H), 2.38-2.36 (m, 2H), 1.57-1.55 (d, *J* = 5.7 Hz, 2H), 0.08 (s, 9H). HRMS (ESI) m/z: Anal.calcd.for $[M+H]^+$ $C_{25}H_{31}N_3Si$: 402.2365; found 402.2363. The second eluting component was the *cis*-pyrrolidine isomer **7c** (860 mg, 23.3%, two steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.12-7.08 (m, 6H), 6.56-6.54 (d, 4H), 6.44-6.41 (d, *J* = 8.0 Hz, 2H), 4.92 (s, 4H), 4.53-4.50 (m, 2H), 2.40-2.20 (m, 2H), 1.81-1.74 (m, 2H), 0.10 (s, 9H). HRMS (ESI) m/z: Anal.calcd.for $[M+H]^+$ $C_{25}H_{31}N_3Si$: 402.2365; found 402.2362.

Dibenzyl 2,2'-((((*trans*-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene)) bis(azanediyl))bis(carbonyl))(2*S*,2'*S*)-bis(pyrrolidine-1-carboxylate) (8a/8b). A mixture of **7a/7b** (450 mg, 1.12 mmol), ((benzyloxy)carbonyl)-*L*-proline (1.34g, 5.6 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (869.34 mg, 5.6 mmol), 1-hydroxybenzotriazole (756.67 mg, 5.6 mmol) and *N*-methylmorpholine (566.44 mg, 5.6 mmol) in *N*,*N*-dimethylformamide (15 mL) was stirred at room temperature for 12 h. The mixture was poured into water (50 mL), extracted with dichlormethane (50 mL× 3), and the combined organic layers were dried with Na_2SO_4. The drying agent was filtered off, and the solvent was concentrated in vacuo. The residue was purified by column chromatography on silica gel, eluting with a solvent gradient of 60% ethyl acetate in petrol to give compound **8a/8b** (790 mg, 81.4%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.99 (s, 2H), 7.53-7.50 (d, *J* = 7.2 Hz, 4H), 7.40-7.25 (m, 4H), 7.20-7.01 (m, 12H), 6.34-6.25 (m, 2H), 5.22 (s, 2H), 5.10-5.06 (m, 3H), 4.94-4.90 (d, *J* = 13.0 Hz, 1H), 4.45-4.20 (m, 2H), 3.48-3.44 (m, 4H), 2.88 (s, 1H), 2.73 (s, 1H), 2.35-2.10 (m, 2H), 1.90-1.88 (m, 6H), 1.67 (s, 2H), 0.08 (s, 9H). HRMS (ESI) m/z: Anal.calcd.for $[M+H]^+$ $C_{51}H_{57}N_5O_6Si$: 864.4156 ; found 864.4096.

(2*S*,2'*S*)-*N*,*N*'-((*trans*-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(pyrrolidine-2-carboxamide) (9a/9b). A mixture of **8a/8b** (790 mg, 0.914 mmol) and 10% Pd/C (97.2 mg) in a solution of ethanol (30 mL) and methanol (5mL) was stirred under hydrogen gas at 10 atm for 30 h. The resulting mixture was filtered and concentrated in vacuo to give compound **9a/9b** (410 mg, 75.9%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.80 (s, 2H), 7.50-7.47 (d, *J* = 8.1 Hz, 4H), 7.06-7.04 (d, *J* = 8.1 Hz, 4H), 6.97-6.94 (d, *J* = 7.9 Hz, 2H), 6.20-6.17 (d, *J* = 8.0 Hz, 2H), 5.13-5.12 (d, *J* = 5.1 Hz, 2H), 3.60-3.55 (m, 2H), 2.81-2.77 (m, 5H), 2.65 (s, 1H), 2.43-2.37 (m, 2H), 1.97-1.88 (m, 2H), 1.70-1.52 (m, 8H), 0.0009 (s, 9H). HRMS (ESI) m/z: Anal.calcd.for $[M+H]^+$ $C_{35}H_{45}N_5O_2Si$ 596.3421; found 596.3409.

Dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*S*,5*S*)-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10a) dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*R*,5*R*)-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-meth

yl-1-oxobutane-1,2-diyl))dicarbamate (10b). Compound **9a/9b** (400 mg, 0.67 mmol) was dissolved in *N*,*N*-dimethylformamide (15 mL) and cooled to 0°C, followed by addition of (methoxycarbonyl)-*L*-valine (586.85 mg, 3.35 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (520 mg, 3.35 mmol), 1-hydroxybenzotriazole (452.65 mg, 3.35 mmol) and *N*-methylmorpholine (338.85 mg, 3.35 mmol). The resulting mixture was stirred at room temperature for 12 h. The mixture was poured into water (50 mL), filtered and the solvent was concentrated in vacuo. The residue was purified by column chromatography on silica gel, eluting with a solvent gradient of 60% ethyl acetate in petrol to give a 1:1 mixture of trans-pyrrolidine isomers (**10a/10b**, 358 mg, 58.7%). The mixture was separated on a CHIRALART Amylose-SA column (250*30mm, 5μm), eluting with a mixture of (35% water) and (65% acetonitrile). Compound **10a** was the first of two stereoisomers to elute (116 mg, 99% ee by chiral HPLC). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.96 (s, 2H), 7.51-7.48 (d, *J* = 8.2 Hz, 4H), 7.29-7.26 (d, *J* = 8.0 Hz, 2H), 7.14-7.11 (d, *J* = 8.2 Hz, 4H), 7.06-7.03 (d, *J* = 8.0 Hz, 2H), 6.27-6.24 (d, *J* = 8.0 Hz, 2H), 5.19 (s, 2H), 4.45-4.40 (m, 2H), 4.06-4.00 (m, 2H), 3.90-3.70 (m, 2H), 3.65-3.55 (m, 2H), 3.52 (s, 6H), 2.49-2.45 (m, 2H), 2.13-1.88 (m, 10H), 1.70-1.60 (m, 2H), 0.94-0.87 (m, 12H), 0.07 (s, 9H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{49}H_{67}N_7O_8Si$: 910.4899; found 910.4865.

Compound **10b** was the second of two stereoisomers to elute (103 mg, 99% ee by chiral HPLC). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.96 (s, 2H), 7.51-7.48 (d, *J* = 8.6 Hz, 4H), 7.28-7.25 (d, *J* = 7.9 Hz, 2H), 7.13-7.11 (d, *J* = 8.2 Hz, 4H), 7.06-7.03 (d, *J* = 8.3 Hz, 2H), 6.27-6.24 (d, *J* = 8.3 Hz, 2H), 5.19 (s, 2H), 4.45-4.40 (m, 2H), 4.05-4.00 (m, 2H), 3.78-3.76 (m, 2H), 3.63-3.58 (m, 2H), 3.52 (s, 6H), 2.49-2.45 (m, 2H), 2.12-1.88 (m, 10H), 1.65-1.64 (m, 2H), 0.92-0.85 (m, 12H), 0.07 (s, 9H). Anal. calcd. for $[M+H]^+$ $C_{49}H_{67}N_7O_8Si$: 910.4899; found 910.4896.

Dibenzyl 2,2'-(((((2*S*,5*R*)-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))(2*S*,2'*S*)-bis(pyrrolidine-1-carboxylate) (8c). Compound **8c** was synthesized according to the method to prepare compound **10a**. Compound **7c** (860mg, 2.14 mmol) was treated with ((benzyloxy)carbonyl)-*L*-proline under coupling condition to give compound **8c** (1.42 g ,76.5%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.05 (s, 2H), 7.60-7.58 (d, *J* = 6.5 Hz, 4H), 7.45-7.42 (d, *J* = 7.9 Hz, 4H), 7.36-7.10 (m, 12H), 6.42-6.40 (m, 2H), 5.12-4.92 (m, 4H), 4.73 (s, 2H),

4.38-4.33 (m, 2H), 3.49-3.45 (m, 4H), 2.45-2.35 (m, 2H), 2.26-2.23 (m, 2H), 1.89-1.85 (m, 8H), 0.10 (s, 9H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{51}H_{57}N_5O_6Si$: 864.4156 ; found 864.4088.

(2*S*,2'*S*)-*N*,*N*'-(((2*S*,5*R*)-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(pyrrolidine-2-carboxamide) (9c). Compound **9c** was synthesized according to the method to prepare compound **9a/9b**. Compound **8c** (1.42 g, 1.64 mmol) was treated using hydrogen gas with catalyst to give compound **9c** (758 mg, 77.4%).

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.06 (s, 2H), 7.65-7.63 (d, *J* = 8.3 Hz, 4H), 7.43-7.40 (d, *J* = 8.4 Hz, 4H), 7.14-7.11 (d, *J* = 8.3 Hz, 2H), 6.40-6.37 (d, *J* = 8.3 Hz, 2H), 4.72 (s, 2H), 4.18 (br, 2H), 3.80-3.75 (m, 2H), 3.30-2.91 (m, 4H), 2.45-2.35 (m, 2H), 2.14-2.02 (m, 2H), 1.82-1.65(m, 8H), 0.10(m, 9H). HRMS (ESI) m/z: Anal.calcd.for $[M+H]^+$ $C_{35}H_{45}N_5O_2Si$ 596.3421; found 596.3399.

Dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*S*,5*R*)-1-(4-(trimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10c). Compound **10c** was prepared according to the method to synthesize the mixture of compound **10a** and **10b**. Compound **9c** (758 mg, 1.27 mmol) reacted with (methoxycarbonyl)-*L*-valine (586.85 mg, 3.35 mmol) under peptide coupling condition to give compound **10c** (622 mg ,53.7%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.03 (s, 2H), 7.60-7.57 (d, *J* = 8.3 Hz, 4H), 7.43-7.40 (d, *J* = 8.4 Hz, 4H), 7.32-7.30 (d, *J* = 7.3 Hz, 2H), 7.14-7.11 (d, *J* = 8.1 Hz, 2H), 6.39-6.36 (d, *J* = 8.3 Hz, 2H), 4.70 (s, 2H), 4.52-4.35 (m, 2H), 4.06-4.00 (m, 2H), 3.83-3.70 (m, 2H), 3.69-3.62 (m, 2H), 3.53 (s, 6H), 2.45-1.90 (m, 14H), 0.94-0.88 (m, 12H), 0.10 (m, 9H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{49}H_{67}N_7O_8Si$: 910.4899; found 910.4851.

4,4'-(*trans*-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)dianiline (7d/7e) and 4,4'-(*cis*-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)dianiline (7f).

2,5-bis(4-nitrophenyl)-1-(4-(triethylsilyl)phenyl)pyrrolidine (**6d/6e, 6f**) was synthesized according to the method to prepare compounds **6a/6b & 6c**. The crude product was used without further purification.

Compound **7d/7e** and **7f** were synthesized according to the method to prepare compounds **7a/7b** and **7c**. **7d/7e (568mg)**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.00-6.98 (d, *J* = 8.2 Hz, 2H), 6.84-6.81 (d, *J* = 8.1 Hz, 4H), 6.49-6.46 (d, *J* = 8.1 Hz,

4H), 6.30-6.27 (d, *J* = 8.2 Hz, 2H), 5.00-4.98 (d, *J* = 6.0 Hz, 2H), 4.86 (s, 4H), 2.42-2.31 (m, 2H), 1.56-1.54 (m, 2H), 0.93-0.63 (m, 15H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{28}H_{37}N_3Si$: 444.2835; found 444.2829. **7f (830 mg)**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.14-7.11 (d, *J* = 8.2 Hz, 4H), 7.08-7.06 (d, *J* = 8.2 Hz, 2H), 6.58-6.55 (d, *J* = 8.2 Hz, 4H), 6.46-6.44 (d, *J* = 8.2 Hz, 2H), 4.92 (s, 4H), 4.54 (s, 2H), 2.38-2.26 (m, 2H), 1.82-1.74 (m, 2H), 0.91-0.77 (m, 9H), 0.70-0.66 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{28}H_{37}N_3Si$: 444.2835; found 444.2831.

Dibenzyl 2,2'-((((*trans*-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))(2*S*,2'*S*)-bis(pyrrolidine-1-carboxylate) (8d/8e). Compound **8d/8e** (979 mg, 84.3%) was synthesized according to the method to prepare compound **8a/8b**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.99 (s, 2H), 7.54-7.51 (d, *J* = 6.4 Hz, 4H), 7.42-7.18 (m, 12H), 7.05-7.03 (m, 4H), 6.33-6.30 (m, 2H), 5.22 (s, 2H), 5.11-5.07 (m, 3H), 4.95-4.90 (d, *J* = 13 Hz, 1H), 4.35 (s, 2H), 3.50-3.44 (m, 4H), 2.88 (s, 2H), 2.73 (s, 2H), 2.35-2.10 (m, 2H), 1.98-1.84 (m, 6H), 1.66 (s, 2H), 0.93-0.79 (m, 9H), 0.63-0.61 (m, 4H), 0.58-0.49 (m, 2H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{54}H_{63}N_5O_6Si$: 906.4626; found 906.4670.

(2*S*,2'*S*)-*N*,*N*'-((*trans*-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(pyrrolidine-2-carboxamide) (9d/9e). Compound **9d/9e** (535 mg, 77.1%) was synthesized according to the method to prepare compound **9a/9b**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.93 (s, 2H), 7.57-7.54 (d, *J* = 8.3 Hz, 4H), 7.16-7.13 (d, *J* = 8.2 Hz, 4H), 7.02-6.99 (d, *J* = 8.2 Hz, 2H), 6.29-6.26 (d, *J* = 8.3 Hz, 2H), 5.21-5.20 (d, *J* = 5.6 Hz, 2H), 3.73-3.68 (m, 2H), 2.92-2.89 (m, 4H), 2.50-2.45 (m, 2H), 2.07-1.98 (m, 2H), 1.82-1.63 (m, 8H), 1.05-1.01 (m, 2H) , 0.84-0.79 (m, 9H), 0.63-0.55 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{38}H_{51}N_5O_2Si$: 638.3890; found 638.3875.

Dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*S*,5*S*)-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10d) and dimethyl((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*R*,5*R*)-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10e). Compound **10d** and **10e** was synthesized according to the methods to prepare compound **10a** and **10b**. Compound **9e/9d** was treated with (methoxycarbonyl)-*L*-valine under peptide coupling condition

to give a 1:1 mixture of compound **10d** and **10e** as trans-pyrrolidine isomers (490 mg, 61.4%). The mixture was separated on a CHIRALART Amylose-SA column to give compound **10d** (176 mg, 99% ee by chiral HPLC) and **10e** (98mg, 99% ee by chiral HPLC). **10d:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.98 (s, 2H), 7.52-7.49 (d, J = 8.19 Hz, 4H), 7.31-7.29 (d, J = 8.2 Hz, 2H), 7.15-7.12 (d, J = 8.2 Hz, 4H), 7.03-7.00 (d, J = 8.1 Hz, 2H), 6.28-6.25 (d, J = 8.1 Hz, 2H), 5.19-5.17 (d, J = 5.1 Hz, 2H), 4.44-4.41 (m, 2H), 4.05-4.00 (m, 2H), 3.84-3.77 (m, 2H), 3.65-3.58 (m, 2H), 3.52 (s, 6H), 2.48-2.45 (m, 2H), 2.20-1.87 (m, 10H), 1.63-1.61 (m, 2H), 0.94-0.89 (m, 12H), 0.87-0.79 (m, 9H), 0.62-0.54 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{52}H_{73}N_7O_8Si$: 952.5368; found 952.5343.

10e: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.98 (s, 2H), 7.51-7.49 (d, J = 8.2 Hz, 4H), 7.31-7.28 (d, J = 8.3 Hz, 2H), 7.14-7.12 (d, J = 8.2 Hz, 4H), 7.03-7.00 (d, J = 8.1 Hz, 2H), 6.27-6.25 (d, J = 8.1 Hz, 2H), 5.19-5.18 (d, J = 3.6 Hz, 2H), 4.44-4.40 (m, 2H), 4.05-3.99 (m, 2H), 3.84-3.78 (m, 2H), 3.65-3.58 (m, 2H), 3.52 (s, 6H), 2.48-2.45 (m, 2H), 2.17-2.12 (m, 2H), 2.04-1.86 (m, 8H), 1.63-1.62 (m, 2H), 0.92-0.79 (m, 21H), 0.62-0.55 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{52}H_{73}N_7O_8Si$: 952.5368; found 952.5343.

Dibenzyl 2,2'-(((((2*S*,5*R*)-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))(2*S*,2'*S*)-bis(pyrrolidine-1-carboxylate) (8f). Compound **8f** (1.34 g, 79.2%) was synthesized according to the method to prepare compound **8c**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 10.03 (s, 2H), 7.60-7.58 (d, J = 7.4 Hz, 4H), 7.46-7.43 (d, J = 8.2 Hz, 4H), 7.31-7.10 (m, 12H), 6.45-6.43 (m, 2H), 5.12-5.08 (m, 4H), 4.74 (s, 2H), 4.39-4.34 (m, 2H), 3.50-3.45 (m, 4H), 2.50-2.33 (m, 2H), 2.31-2.10 (m, 2H), 1.98-1.85 (m, 8H), 0.91-0.81 (m, 9H), 0.65-0.58 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{54}H_{63}N_5O_6Si$: 906.4626; found 906.4670.

(2*S*,2'*S*)-*N*,*N*'-(((2*S*,5*R*)-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(pyrrolidine-2-carboxamide) (9f). Compound **9f** (738 mg, 78.2%) was synthesized according to the method to prepare compound **9c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.97 (s, 2H), 7.65-7.63 (d, J = 8.5 Hz, 4H), 7.44-7.41 (d, J = 8.3 Hz, 4H), 7.11-7.08 (d, J = 8.3 Hz, 2H), 6.42-6.39 (d, J = 8.3 Hz, 2H), 4.73 (s, 2H), 3.74-3.71 (m, 2H), 2.93-2.89 (m, 4H), 2.45-2.35 (m, 2H), 2.11-2.00 (m, 2H), 1.90-1.61(m, 8H), 0.86-0.81(m, 9H), 0.65-0.58 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{38}H_{51}N_5O_2Si$: 638.3890; found 638.3876.

Dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*S*,5*R*)-1-(4-(triethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10f). Compound **10f** (665 mg, 60.4%) was synthesized according to the method to prepare compound **10c**.

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.03 (s, 2H), 7.60-7.57 (d, *J* = 8.4 Hz, 4H), 7.44-7.41 (d, *J* = 8.3 Hz, 4H), 7.32-7.30 (d, *J* = 7.9 Hz, 2H), 7.11-7.08 (d, *J* = 8.2 Hz, 2H), 6.41-6.38 (d, *J* = 8.0 Hz, 2H), 4.71 (s, 2H), 4.48-4.44 (m, 2H), 4.06-4.01(m, 2H), 3.83-3.78 (m, 2H), 3.69-3.62 (m, 2H), 3.59 (s, 6H), 2.48-2.45 (m, 2H), 2.20-2.10 (m, 2H), 2.04-1.78 (m, 10H), 0.95-0.80 (m, 21H), 0.65-0.57 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{52}H_{73}N_7O_8Si$: 952.5368; found 952.5335.

4,4'-(*trans*-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl)dianiline (7g/7h) and 4,4'-(*cis*-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl)dianiline (7i) . 1-(4-(tert-butyldimethylsilyl)phenyl)-2,5-bis(4-nitrophenyl)pyrrolidine (**6g/6h, 6i**) was synthesized according to the method to prepare compound **6a/6b & 6c**. The crude product was used without further purification.

Compound **7g/7h** and **7i** was synthesized according to the method to prepare compound **7a/7b** and **7c**. **7g/7h** (310 mg)**:** ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.02-6.99 (d, *J* = 8.4 Hz, 2H), 6.84-6.81 (d, *J* = 8.3 Hz, 4H), 6.49-6.46 (d, *J* = 8.3 Hz, 4H), 6.29-6.27 (d, *J* = 8.5 Hz, 2H), 5.00-4.98 (d, *J* = 6.4 Hz, 2H), 4.87 (s, 4H), 1.56-1.54 (d, *J* = 5.8 Hz, 2H), 1.25-1.17 (m, 2H), 0.75 (s, 9H), 0.08-0.07 (m, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{28}H_{37}N_3Si$: 444.2835; found 444.2820.

7i (660 mg)**:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.13-7.04 (m, 6H), 6.57-6.54 (d, *J* = 8.3 Hz, 4H), 6.48-6.43 (m, 2H), 4.99-4.97 (m, 4H), 4.65-4.45 (m, 2H), 2.35-2.18 (m, 2H), 1.82-1.74 (m, 2H), 0.77 (s, 9H), 0.10 (s, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{28}H_{37}N_3Si$: 444.2835; found 444.2825.

Dibenzyl 2,2'-((((*trans*-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))(2*S*,2'*S*)-bis(pyrrolidine-1-carboxylate) (8g/8h). Compound **8g/8h** (516 mg, 81.6%) was synthesized according to the method to prepare compound **8a/8b**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.98 (s, 2H), 7.52-7.50 (d, *J* = 7.5 Hz, 4H), 7.39-7.36 (m, 6H), 7.17-7.12 (m, 6H), 7.05-7.01 (m, 4H), 6.31-6.29 (d, *J* = 7.9 Hz, 2H), 5.23 (s, 2H), 5.10-4.99 (m, 4H), 4.35-4.32 (m, 2H),

3.59-3.56 (m, 4H), 2..45-2.35 (m, 2H), 2.33-1.55 (m, 10H), 0.74 (s, 9H), 0.08-0.06 (d, *J* = 7.9 Hz, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{54}H_{63}N_5O_6Si$: 906.4626; found 906.4684.

(2*S*,2'*S*)-*N*,*N*'-((*trans*-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis (4,1-phenylene))bis(pyrrolidine-2-carboxamide) (9g/9h). Compound **9g/9h** (304 mg, 83.7%) was synthesized according to the methods to prepare compound **9a/9b**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.93 (s, 2H), 7.57-7.55 (d, *J* = 8.4 Hz, 4H), 7.13-7.10 (d, *J* = 7.6 Hz, 4H), 7.04-7.01 (d, *J* = 8.3 Hz, 2H), 6.29-6.26 (d, *J* = 8.4 Hz, 2H), 5.21-5.19 (d, *J* = 5.9 Hz, 2H), 3.72-3.67 (m, 2H), 2.91-2.87 (m, 4H), 2.45-2.35 (m, 2H), 2.14-1.97 (m, 2H), 1.90-1.62 (m, 8H), 0.74 (s, 9H), 0.08-0.06 (d, *J* = 5.4 Hz, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{38}H_{51}N_5O_2Si$: 638.3890; found 638.3901.

Dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*S*,5*S*)-1-(4-(tert-butyldimethylsilyl) phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl)) bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10g) and dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*R*,5*R*)-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2, 5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bi s(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10h). Compound **10g** and **10h** were synthesized according to the method to prepare compound **10a** and **10b**. A 1:1 mixture of trans-pyrrolidine isomers was obtained (350 mg, 77.1%). **10g** (108 mg): ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.96 (s, 2H), 7.51-7.49 (d, *J* = 8.2 Hz, 4H), 7.29-7.27 (d, *J* = 8.2 Hz, 2H), 7.15-7.12 (d, *J* = 8.2 Hz, 4H), 7.05-7.02 (d, *J* = 8.1Hz, 2H), 6.27-6.25 (d, *J* = 8.0 Hz, 2H), 5.19 (s, 2H), 4.44-4.43 (m, 2H), 4.05-4.00 (m, 2H), 3.85-3.75 (m, 2H), 3.62-3.60 (m, 2H), 3.52 (s, 6H), 2.45-2.35 (m, 2H), 2.12-2.08 (m, 2H), 1.99-1.89 (m, 8H), 1.63-1.61 (m, 2H), 0.94-0.82 (m, 12H), 0.73 (s, 9H), 0.08-0.06 (d, *J* = 5.4 Hz, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{52}H_{73}N_7O_8Si$: 952.5368; found 952.5405. **10h** (110 mg**):** ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.96 (s, 2H), 7.51-7.48 (d, *J* = 8.3 Hz, 4H), 7.29-7.26 (d, *J* = 7.9 Hz, 2H), 7.14-7.11 (d, *J* = 8.3 Hz, 4H), 7.05-7.02 (d, *J* = 8.2 Hz, 2H), 6.27-6.24 (d, *J* = 8.3 Hz, 2H), 5.19 (s, 2H), 4.44-4.33 (m, 2H), 4.05-3.99 (m, 2H), 3.85-3.75 (m, 2H), 3.63-3.60 (m, 2H), 3.52 (s, 6H), 2.45-2.35 (m, 2H), 2.12-2.08 (m, 2H), 2.00-1.75 (m, 8H), 1.64-1.62 (m, 2H), 0.92-0.85 (m, 12H), 0.73 (s, 9H), 0.08-0.06 (d, *J* = 5.4 Hz, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{52}H_{73}N_7O_8Si$: 952.5368; found 952.5404.

Dibenzyl 2,2'-(((((2S,5R)-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))(2S,2'S)-bis(pyrrolidine-1-carboxylate) (8i). Compound **8i** (1.07 g, 79.4%) was synthesized according to the method to prepare compound **8c**. ^{1}HNMR (300 MHz, DMSO-d_6) δ 10.04 (s, 2H), 7.63-7.58 (m, 4H), 7.45-7.43 (m, 4H), 7.36-7.09 (m, 12H), 6.44-6.42 (d, *J* = 6.5 Hz, 2H), 5.13-5.08 (m, 4H), 4.74(s, 2H), 4.36-4.34 (m, 4H), 3.59-3.56 (m, 4H), 2.45-2.35 (m, 2H), 2.26-1.88 (m, 8H), 0.76 (s, 9H), 0.10 (s, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{54}H_{63}N_5O_6Si$: 906.4626; found 906.4671.

(2*S*,2'*S*)-*N*,*N*'-(((2*S*,5*R*)-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl) bis(4,1-phenylene))bis(pyrrolidine-2-carboxamide) (9i). Compound **9i** (559 mg, 74.2%) was synthesized according to the method to prepare compound **9c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.93 (s, 2H), 7.65-7.63 (d, *J* = 8.5 Hz, 4H), 7.43-7.40 (d, *J* = 8.4 Hz, 4H), 7.12-7.10 (m, 2H), 6.42-6.39 (d, *J* = 8.3 Hz, 2H), 4.73 (s, 2H), 3.70-3.63 (m, 2H), 2.90-2.86 (m, 4H), 2.45-2.35 (m, 2H), 2.07-1.98 (m, 2H), 1.84-1.62 (m, 8H), 0.76 (s, 9H), 0.09 (s, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{38}H_{51}N_5O_2Si$: 638.3890; found 638.3888.

Dimethyl ((2*S*,2'*S*)-((2*S*,2'*S*)-(((((2*S*,5*R*)-1-(4-(tert-butyldimethylsilyl)phenyl)pyrrolidine-2,5-diyl)bis(4,1-phenylene))bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(3-methyl-1-oxobutane-1,2-diyl))dicarbamate (10i). Compound **10i** (469 mg, 56.2%) was synthesized according to the method to prepare compound **10c**. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.01 (s, 2H), 7.59-7.57 (d, *J* = 8.1 Hz, 4H), 7.45-7.35 (m, 4H), 7.33-7.21 (m, 2H), 7.14-7.10 (m, 2H), 6.40-6.37 (d, *J* = 7.5 Hz, 2H), 4.80-4.60 (m, 2H), 4.50-4.40 (m, 2H), 4.05-3.95 (m, 2H), 3.85-3.73 (m, 2H), 3.63-3.55 (m, 2H), 3.52 (s, 6H), 2.45-2.35 (m, 2H), 2.12-2.08 (m, 2H), 2.00-1.70 (m, 10H), 0.93-0.87 (m, 12H), 0.75 (s, 9H), 0.08-0.06 (d, *J* = 4.0 Hz, 6H). HRMS (ESI) m/z: Anal. calcd. for $[M+H]^+$ $C_{52}H_{73}N_7O_8Si$: 952.5368; found 952.5400.

4.2 Biological studies

4.2.1. Antiviral activity assay test with HCVcc (GT2a)

4.2.1.1. Cell line and cell culture

The Huh 7.5.1 cell line and the HCVcc reporter virus were kindly provided by Wuhan Institute of Virology, Chinese Academy of Sciences. Cells were cultured in

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin at 37°C with 5% CO_2. HCVcc reporter virus (GT2a, JFH-1) is an infectious HCV virus derived from the JFH1 virus (genotype 2a). The establishment of the infectious HCVcc (GT2a, JFH-1) virus has been described previously. Virus was propagated in Huh7.5.1 cells. Briefly, Huh7.5.1 cells were infected with virus at MOI = 0.1 and cultured for 4d. Culture supernatant was collected and fltered through a 0.45 μm membrane and stored at -80ºC as virus stock. The infectious titers of cell-culture supernatants were evaluated by classical titration assay[28].

4.2.1.2 Antiviral activity test.

Huh-7.5.1 cells were infected with the HCVcc reporter virus (MOI = 0.1) and treated with the compounds at 8 concentrations (3-fold dilution, in duplicate) for 3 days. Antiviral activity was detected by measuring RLuc activity using Renilla luciferase assay buffer and substrate by following the manufacture's instructions. Cytotoxicity of the compounds was tested in uninfected Huh-7.5.1 cells in parallel. EC_{50} and CC_{50} values were calculated with the GraphPad Prism software.

4.2.2. Measurement of the anti-HCV activities of compounds using HCV replicon

4.2.2.1. Cell line and cell culture

The Huh7 cell line were kindly provided by AppTec company(America). HCV GT1b and GT1a wild type replicon, GT1b/3a, GT1b/4a, GT1b/5a and GT1b/6a NS5A chimeric replicons were provided by WuXi AppTec. Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The replicon RNAs were in *vitro* transcribed using the replicon plasmid DNAs.

4.2.2.2. Antiviral activity assay test with HCV replicon

Compounds were added to 96-well plates by Echo at 9 concentrations with a serial 3-fold dilution, in triplicate. The final concentration of DMSO in the cell culture medium was 0.5%. The Huh7 cells were transiently transfected with the replicon RNAs by electroporation and seeded at a density of 10,000 cells/well in 96-well plates. And then the cells were cultured and treated with the compounds at 37°C and 5% CO_2 for 3 days. The cell viability in GT1b replicon assay was tested using CellTiter-Fluor

following the manufacture's instruction. For antiviral activity detection, the supernatants were removed from the wells, then Bright-Glo will be added to detect luciferase luminescent signal. The raw data (RLU) will be used for calculating the antiviral activity (% inhibition) of the compounds. EC_{50} and CC_{50}values were calculated using the GraphPad Prism software.

4.2.3. In *vitro* PK assessment

Pooled mouse, rat and human liver microsomes were obtained from BD Gentest. A typical standard reaction mixture 300μL consisted of the pooled liver microsomes 0.2 mg/mL, 1mM NADPH, 5mM $MgCl_2$, 100 mM potassium phosphate buffer (pH 7.4) and 0.2 μM of test compouds. After a 5-min pre-incubation at 37 °C, the reactions were initiated by addition of NADPH and incubation proceeded for 5, 15, 30, 60 min at 37 °C in a shaking metal bath. The reaction was stopped by transferring 60 μL aliquots to the tubes on ice and adding 120 μL amounts of ice-cold acetonitrile containing internal standards. Concentration of the test compounds was measured by UPLC-MS/MS.

4.2.4. In vivo PK assessment.

Adult male Mice, provided by sino-British SIPPR/BK Lab. Animal Ltd, were housed at the centralized animal facilities with a 12 h light-dark cycle. The housing temperature and relative humidity were controlled at 22 °C and 55%, respectively. The animals fasted 12 h before drug administration had free access to water. A single dose of 10 mg/kg was administered through intragastric (i.g.) route of administration. The concentration of compounds in the plasma and liver was measured by the ultra-performance liquid chromatography-mass spectrometry analysis (UPLC-MS/MS). PK parameters were calculated using DAS 3.2.5.8 beagle dogs. They were administrated via extravascular routes with a single dose of 2 mg/kg, the methods of plasma preparation and detection were same as mice plasma samples.

4.2.5. Interaction with CYP450 enzymes

The inhibitory potencies of compound **10d** on CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2B6, CYP1A2, CYP2A6 were evaluated in pooled human liver microsomes using eight CYP substrates and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

The incubation mixtures were prepared in total volumes of 100 μL as follows: pooled human liver microsomes (0.2 mg/mL), 1 mM NADPH, 5 mM $MgCl_2$, 100 mM

potassium phosphate buffer (pH 7.4), 1 µM and 10 µM of compound 12 in dimethyl sulfoxide (DMSO, DMSO <1% v/v), and seven CYP probe substrates. The CYP substrates were used at concentrations approximating their respective Km values: 5 µM midazolam, 40 µM Nifedipine, 5 µM dextromethorphan, 100 µM (S)-mephenytoin, 100 µM tolbutamide, 75 µM bupropion hydrochloride, 20 µM phenacetin and 2 µM coumarin. After a 5-min pre-incubation at 37 °C, the reactions were initiated by addition of NADPH and incubation proceeded for 30 min at 37 °C in a shaking metal bath. The reaction was stopped by placing the tubes on ice and adding 300-µL amounts of ice-cold acetonitrile containing internal standards. Concentration of the CYP substrates and their metabolites was measured by UPLC-MS/MS.

4.2.6. Cardiotoxicity in *vitro*

Micropipette was pulled from borosilicate glass with the pipette tip resistance between 3 ~ 5 MΩ. For each experiment, a single dish of cells is removed from the incubator, washed twice with room temperature ECS and then placed on the microscope stage. A commercial patch clamp amplifier was used for the whole cell recordings.

The tail currents were evoked in room temperature once every 30 s by a 3 s -50 mV repolarizing pulse following a 2 s +50 mV depolarizing pulse with a hold voltage of -80 m V. A 50 ms depolarized pulse to -50 mV at the beginning of the voltage protocol served as a baseline for calculating the amplitude of the peak tail current. Only stable cells with recording parameters above threshold were allowed to enter the drug application procedure. The hERG currents were allowed to stabilize over a 3 minutes period in the presence of vehicle alone prior to test article application. The cells were kept in the test solution until the peak tail current was stable (< 5% change) for ~5 sweeps or for a maximum of 6 min, whichever came first.

4.2.7. Permeability and efflux in human Caco-2 cells

For the permeability studies, Caco-2 cells were seeded on 24-well Transwell inserts (Millicell Hanging Cell Culture Insert, PET 1 µm) at a cell density of 10^4 cells per well. The cells were cultured at 37°C, 5% CO_2 atmosphere and maintained in Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, and 100 U/ml and 100 g/ml penicillin and streptomycin, with replacement every 2-3 day, during 21-23 days, until the monolayer achieved minimum of 200 Ω cm^2 of transepithelial electrical resistance (TEER) values. The tightness of the Caco-2 monolayer was assessed as the TEER

values using a Millicell ERS-2 Volt-Ohm Meter and accessories (Millipore Corporation, Bedford, MA), equipped with a pair of chopstick electrodes inserted into the apical medium. Before the transport experiments, the cell monolayers were rinsed twice using warm (37 °C) HBSS (Gibco 14025-092), and then the cells were incubated using 2% BSA (with or without inhibitor) at 37°C for 30min. After preincubation, the cell monolayers were incubated with samples in 2% BSA from either the apical (AP) or basolateral (BL) side for 90 min at 37 °C. The volume of incubation media on the AP and BL sides was 0.4 mL and 1.4 mL, respectively, and a 100μL aliquot of the incubation solution was withdrawn from the receiver compartment for analysis. Each experiment was performed in duplicate. The amount of the samples was analyzed by UPLC/MS/MS. The apparent permeability (Papp) was calculated as described elsewhere[29].

4.2.8. Plasma protein binding

In vitro plasma protein binding assay was carried out by equilibrium dialysis method using HTD96b (interchim inc.USA). Plasma (pH 7.4) was preincubated for 15 min at 37 °C prior to addition of stock DMSO solutions (0.5% final concentration) containing **10d** and ombitasvir(1 and 10 μM). Drug-spiked plasma was then aliquoted into the donor chambers of the dialysis plate (100 μL per half-well). An equal volume of phosphate buffer (100 mM, pH 7.4) was placed in each corresponding receiver well. The dialysis plate was sealed with the kit adhesive (HT-Dialysis) and dialysis was conducted in an orbital shaker (100 rpm) maintained at 37 °C for 6 h. Following incubation, aliquots from the donor and receiver chambers, as well as the plasma stock solution incubated for 6 h at 37 °C were removed and added to vials containing internal standard in acetonitrile and were mixed to prevent nonspecific binding to the vial and pipette tip. All samples were analyzed by UPLC–MS/MS.

4.2.9. Liver distribution

The liver distribution of ombitasvir and **10d** were evaluated in adult male Institute of Cancer Research (ICR) mice (18.0~23.0g,4~6 weeks). After a 12h overnight fast (with free access to water), the mice were randomly divided into two groups (15 animals for each), and administrated with a single dose of 10 mg/kg of ombitasvir and **10d** respectively by intragastric (i.g.) administration. Plasma and liver samples were collected at 0.5h, 3h, 8h, 16h and 24h after oral dosing (n=3). The concentration of the two compounds was measured by the ultra-performance liquid chromatography-mass

spectrometry analysis (UPLC-MS/MS) and the concentration ratio of liver and plasma (LPR) was calculated.

4.2.10. Repeated-dose toxicity study

After acclimation, animals were randomly allocated to two groups, each containing five males and five females, and orally administered 20ml/kg body weight of **10d** (150 mg/kg body weight) or the vehicle alone once daily by gavage. Mice were killed after the 14th administration. Animals in all groups were observed daily and detailed clinical signs evident. Their body weights and food intake were monitored on day 1 of administration and thereafter twice per week. Body weights were used for calculation of the volume of the test chemical to be administered.

4.2.11. Hematology, blood biochemistry, and serum hormone measurements

At necropsy, animals were weighed and sacrificed by exsanguination from the abdominal aorta under ether anesthesia. Before killing, blood samples were collected from the abdominal aorta to use for hematology, blood chemistry and measurement of serum hormone levels. The hematological parameters included the white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, platelet (Plt) count, reticulocyte ratio and differential leukocyte counts. Blood biochemistry parameters were total protein (TP), albumin (ALB), total bilirubin (TB), indirect bilirubin (DB), total billliary acid(TBA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GGT), glutamate dehydrogenase (GLDH) and alkaline phosphatase (ALP), total cholesterol (CHO), triglyceride (TG), glucose (GLU), blood urea nitrogen (BUN), creatinine (Cre), Creatine Kinase (CK).

4.2.12. Histopathology

At autopsy, gross findings were recorded and abnormal portions were removed along with all organs/tissues. They were fixed with 10% formalin. Hearts, liver, spleen, kidneys, thymus, brain, testis and ovary were weighed before fixation. Reference to the results of blood biochemistry and hematology, the heart, liver, spleen were sent for pathological examination.

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Highlights

- A carbon-silicon switch strategy was successfully used to design HCV NS5A inhibitors.
- The silicon-containing compound showed increased pan-genotype activity and better pharmacokinetic properties.
- The silicon-containing HCV NS5A inhibitor could be a candidate with good safety profiles.