European Journal of Medicinal Chemistry 101 (2015) 163–178

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Novel benzidine and diaminofluorene prolinamide derivatives as potent hepatitis C virus NS5A inhibitors

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form
14 June 2015
Accepted 15 June 2015
Available online 24 June 2015

Keywords:
HCV
NS5A inhibitor
Benzidine
Diaminofluorene
Structure–activity relationship

ABSTRACT

Our study describes the discovery of a series of highly potent hepatitis C virus (HCV) NS5A inhibitors based on symmetrical prolinamide derivatives of benzidine and diaminofluorene. Through modification of benzidine, L-proline, and diaminofluorene derivatives, we developed novel inhibitor structures, which allowed us to establish a library of potent HCV NS5A inhibitors. After optimizing the benzidine prolinamide backbone, we identified inhibitors embedding meta-substituted benzidine core structures that exhibited the most potent anti-HCV activities. Furthermore, through a battery of studies including hERG ligand binding assay, CYP_{450} binding assay, rat plasma stability test, human liver microsomal stability test, and pharmacokinetic studies, the identified compounds **24**, **26**, **27**, **42**, and **43** are found to be nontoxic, and are expected to be effective therapeutic anti-HCV agents.

1. Introduction

Hepatitis C virus (HCV) infection is reported in almost 170 million individuals worldwide (~2.4% of the population), including approximately 5 million people in the United States with additional 3 to 4 million global population every year [1–5]. It has been estimated that around 70–80% of those infested with HCV will progress to chronic hepatitis, which, if left untreated, may result in liver cirrhosis and eventually liver cancer, with ultimately lethal consequences (1–5%) [6,7]. It is well accepted that the HCV infection is one of the main reasons for liver transplantation in patients [8]. Although vaccines are available for infection with other widespread liver viruses such as hepatitis A virus (HAV) and hepatitis B virus (HBV), no vaccines are available for HCV infection [8]. Recently, telaprevir (VX-950, Vertex Pharmaceuticals and Johnson & Johnson) and boceprevir (Victrelis, Merck) were approved as anti-HCV NS3-4A protease inhibitors by the US Food and Drug Administration (FDA). The traditional therapy for patients with HCV infection consists of oral doses of ribavirin (RBV) in combination with subcutaneous injections of PEG–IFN–α and protease inhibitor for a total duration of 24–28 weeks [9–11]. However, this interferon-centered therapy not only has serious side effects, including anemia, rash and depression, but is also associated with a restricted sustained virologic response (SVR), notably in those infected with HCV genotype 1 (G-1) [12,13]. In G-1-infected patients, favorable results have been reported after the addition of

Abbreviations: HCV, hepatitis C virus; Peg-IFNα, pegylated interferon α; SVR, sustained virological response; GT1a, hepatitis C virus genotype 1a, a subtype of hepatitis C virus genotype 1; GT1b, hepatitis C virus genotype 1b, a subtype of hepatitis C virus genotype 1; DAA, direct-acting antiviral; NS5A, hepatitis C virus nonstructural protein 5A; pM, picomolar; RNA, ribonucleic acid; Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; NFSI, N-fluorobenzenesulfonimide; LiHMDS, lithium hexamethyldisilazide; EC_{50}, concentration at which inhibition is half-maximal; μM, micromolar; nM, nanomolar; SAR, structure–activity relationship; hERG, the human ether-a -go-go-related gene; CYP_{450}, cytochrome P450; PK, pharmacokinetics; HPBCD, (2-hydroxypropyl)-β-cyclodextrin; IV, intravenous; p.o., orally; WSTs, water-soluble tetrazolium salts; DNA, deoxyribonucleic acid.

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http://dx.doi.org/10.1016/j.ejmech.2015.06.033
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either boceprevir or telaprevir to the standard of care [14]. Although promising, advantageous outcome is expected from the launching of direct acting antivirals (DAAs) into the therapeutic regimen, their conceivable limitations include a limited genetic barrier, which may be attributable to drug-resistant mutants developed through long term medical care [10,15–20]. Therefore, the discovery of safe and effective antiviral candidates aimed at diverse HCV gene targets is greatly needed [21–28].

A polyprotein of almost 3200 amino acids is encoded from the HCV genome, which contains three structural proteins (E1, E2, and core) and six nonstructural proteins (NS2, NS3, NS4A-4B, and NS5A-5B) [29–32]. Among the nonstructural proteins, NS5A has been shown to have a direct role in viral replication, virus assembly, virion production, virus persistence, and pathogenesis [33,34]. There are three domains in NS5A: domain 1 (37–213 residues), containing the zinc-binding shape essential for HCV RNA replication; domain 2 (250–342 residues), which cooperates with cellular proteins and NS5B; and domain 3 (356–447 residues), which has a role in infectious HCV assembly, however not HCV RNA replication [35–43].

In 2010, a landmark NS5A inhibitor, daclatasvir (**1**), was reported to present excellent anti-HCV activity, especially in patients with HCV G-1 infection. This new class of inhibitor was approved by the US FDA in 2014 [44–48]. The effective concentration (EC_{50}) value of daclatasvir was two-digit picomolar (pM) range in *in vitro* assay, and treatment with a single 100 mg dose in clinical trials reduced HCV RNA levels by an average of $3.3\log_{10}$ without apparent toxicity [49]. This result stimulated numerous research groups and pharmaceutical companies to focus on the development of new inhibitors targeting NS5A [50–59]. Currently, there are many candidate compounds in this series: ABT-267, ACH-2928, ACH-3102, AZD-7295, BMS-346, BMS-665, BMS-824393, EDP-239, GS-5885, GSK-2336805, IDX-719, MK-4882, MK-8742, PPI-461, and PPI-1301 (Fig. 1) [60–67]. Most recently, interferon-free multi-class drug combinations (daclatasvir and asunaprevier, Viekira Pak®, and Harvoni®) have been reported as the most optimal therapy [68]. The structure of daclatasvir is characterized by a central biaryl core unit linked to an imidazole and proline moiety, and lastly, a methyl carbamate L-valine moiety as a capping group (Fig. 2) [69]. In 2012, Schinazi et al. reported nascent NS5A inhibitors containing a part of biaryl core and some modifications on other parts through "Click" and C–C bond cross coupling reactions [70,71]. We recently developed a new class of NS5A inhibitors represented by BMK-20113, which has benzidine (I) and L-proline (II) connected as an amide functionality and a variety of capping groups (III) [72]. In this paper, we report improvements in antiviral potency through the introduction of new modifications to the backbone of BMK-20113: L-proline to other proline isosteres (area I in Fig. 2), and benzidine to substituted benzidine derivatives (area II in Fig. 2).

2. Chemistry

The symmetry in the benzidine-proline scaffold greatly streamlined our strategy of synthesizing BMK-20113. Initially, we wanted to evaluate the antiviral activity of the proline variation;

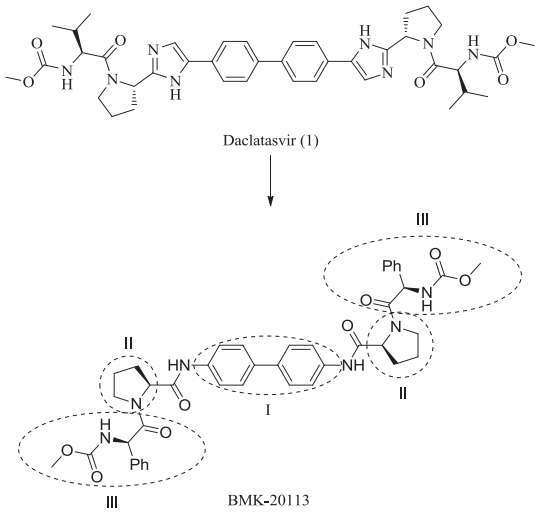


Fig. 2. Strategy for designing HCV NS5A inhibitors.

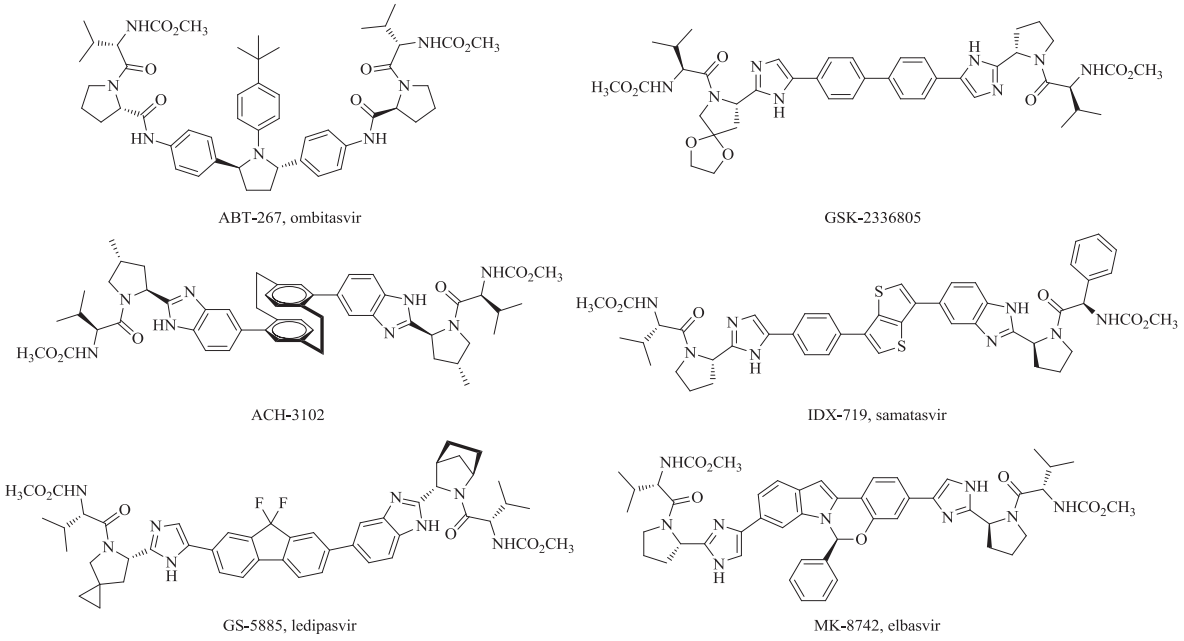


Fig. 1. Structure of NS5A inhibitors.

therefore, L-proline isosteres were employed, such as D-proline, 4-oxo-L-proline, L-thioproline, L-pipecolic acid, D-pipecolic acid, and α-methyl-L-proline, by using the standard peptide coupling protocol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) in dichloromethane (DCM). After purification of intermediates **2**–**8** through chromatographic columns, the Boc groups of the secondary amine site was removed with diluted trifluoroacetic acid (TFA) in DCM [73]. Then methoxycarbonyl phenylglycine **9** was coupled with the free amine with the aid of EDCI and *N*,*N*-diisopropylethylamine (DIPEA) in DCM. In cases where solubility was a persistent problem, we used *N*,*N*-dimethylformamide (DMF) as a solvent [74,75]. The targeted benzidine proline derivatives **10**–**16** were then prepared in low to reasonable yields (13–57%) (Scheme 1).

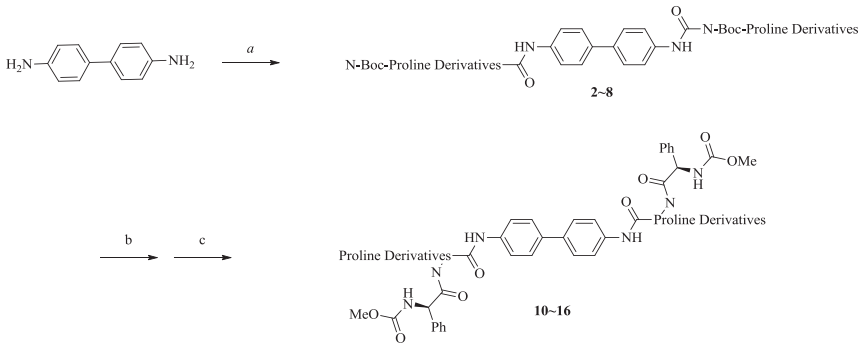
Next, structure–activity relationship studies centered on the benzidine core by employing various substituted benzidine compounds were carried out using *o*- or *m*-substituted benzidine derivatives (*o*-methyl, *m*-methyl, *m*-trifluoromethyl, *m*-fluoro, *m*-chloro, and *m*-bromo). After a coupling reaction, almost pure *N*-Boc-protected prolinamide products **17**–**22** were obtained in excellent yields (92–97%). Using the same method described above, capping groups were incorporated and the final products **23**–**28** were obtained in low to good yields (32–73%) (Scheme 2).

For the construction of the fluorene derivative core structures, we first needed to synthesize 2,7-diaminofluorene derivatives (Scheme 3). After the treatment of 2,7-dinitrofluorene with 1.00 M lithium hexamethyldisilazide (LiHMDS) followed by *N*-fluorobenzenesulfonimide (NFSI) in tetrahydrofuran (THF), we obtained 9,9-difluoro-2,7-dinitro-9*H*-fluorene **30** in 86% yield. Dimethyl derivative was synthesized likewise, treating 2,7-dinitrofluorene with NaO*t*-Bu followed by iodomethane in DMF to furnish the desired 9,9-dimethyl-2,7-dinitro-9*H*-fluorene **31** with an 80% yield [76]. The nitro groups of both **30** and **31** were efficiently reduced to amino functionality per our recent report on commercially available Fe_3O_4 nanoparticles as a heterogeneous catalyst for nitro group reduction in combination with hydrazine monohydrate [77]. Extremely high yields (99%) of the desired diamino compounds **32** and **33** were obtained. Moreover, a reasonable yield (76%) of the diamino ketone compound **35** was obtained by oxidizing the 9*H*-fluorene-2,7-diamine in the presence of a strong base (Cs_2CO_3) in DMSO in open air [78].

After preparation of various 2,7-diaminofluorene derivatives, inhibitors containing L-proline and the capping group were prepared using the method described in Scheme 1. *N*-Boc-protected fluorine prolinamides **36**–**39** were thus obtained in high yields (91–97%), obviating column chromatographic purification. Deprotected amines and coupling of the (*R*)-phenylglycine derivative gave us the desired fluorene derivatives **40**–**43** in moderate yields (50–66%) (Scheme 4).

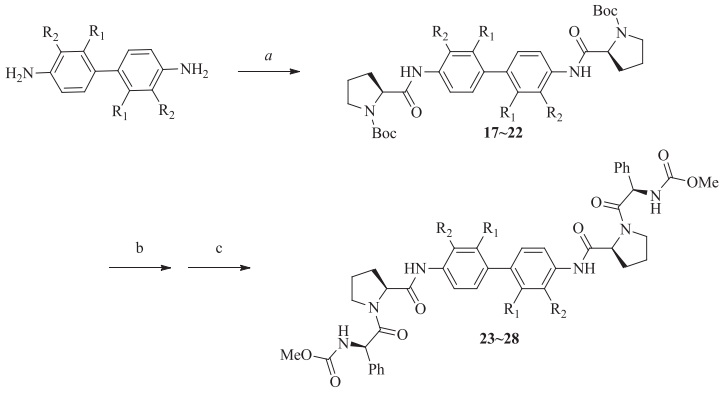
3. Results and discussion

To determine the inhibitory activities (EC_{50} values) of each compound, we used assays based on the HCV cell culture system (HCVcc) and HCV replicon system [79–81]. To determine EC_{50} values in the HCVcc system (genotype 2a), we used a modified JFH1 clone (JFH 5a-Rluc-ad34) containing the *Renilla* luciferase gene (Rluc) in the NS5A region and cell culture adaptive mutations in the E2 and p7 region [82]. Huh7.5.1 cells were infected with JFH5a-Rluc-ad34 for 4 h. After infection, Huh7.5.1 cells were incubated with compounds of interest at various concentrations for 3 d. Then, the cells were harvested and luciferase activities were measured. We determined EC_{50} values using the HCV replicon system to



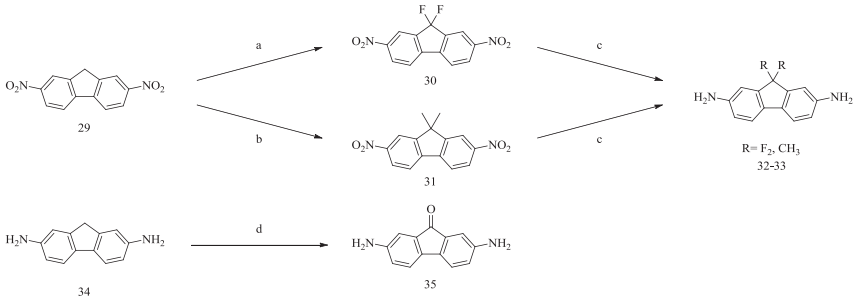
		Proline Derivatives
2	**10**	L-Proline
3	**11**	D-Proline
4	**12**	4-Oxo-L-proline
5	**13**	L-Thioproline
6	**14**	L-Pipecolic acid
7	**15**	D-Pipecolic acid
8	**16**	α-Methyl-L-proline

Scheme 1. Synthesis of benzidine and proline derivatives from an amide skeleton. Reagents and conditions: (a) *N*-Boc-proline derivatives, EDCI, DCM, 31–99%; (b) TFA, DCM; (c) **9** (Capping Group), EDCI, DIPEA, DCM or DMF, 13–57% (2 steps).

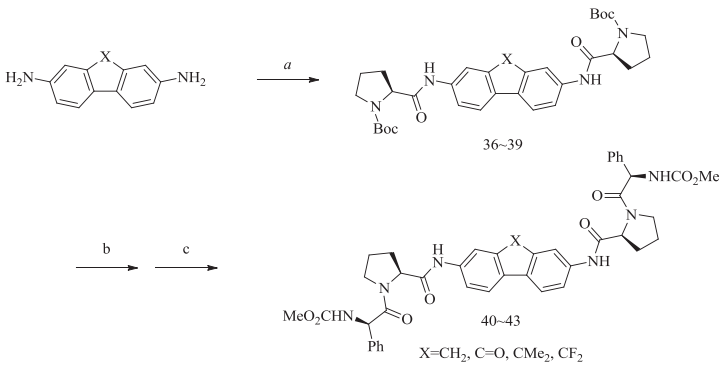


		R1	R2
17	**23**	H	CH_3
18	**24**	CH_3	H
19	**25**	CF_3	H
20	**26**	F	H
21	**27**	Cl	H
22	**28**	Br	H

Scheme 2. Synthesis of benzidine derivatives and prolinamide skeleton. Reagents and conditions: (a) *N*-Boc-L-Proline, EDCI, DCM, 92–97%; (b) TFA, DCM; (c) **9** (capping group), EDCI, DIPEA, DCM, 32–73% (2 steps).



Scheme 3. Synthesis of fluorene-2,7-diamine derivatives. Reagents and conditions: (a) *N*-fluorobenzenesulfonimide, LiHMDS in THF (1.00 M solution), THF, 86%; (b) iodomethane, NaO*t*-Bu, DMF, 80%; (c) Fe_3O_4, $NH_2NH_2{\cdot}H_2O$, DMF, 99%; (d) Cs_2CO_3, DMSO, 76%.



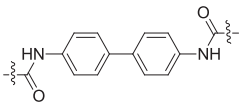
36	**40**	CH_2
37	**41**	C=O
38	**42**	CMe_2
39	**43**	CF_2

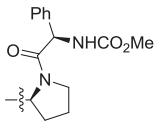
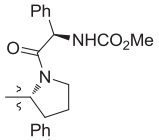
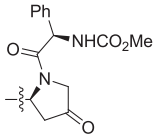
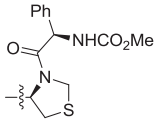
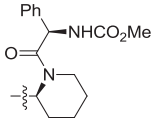
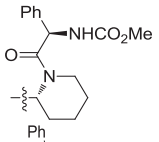
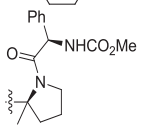
Scheme 4. Synthesis of fluorine-containing prolinamide derivatives. Reagents and conditions: (a) *N*-Boc-L-Proline, EDCI, DCM, 91–97%; (b) TFA, DCM; (c) **3** (capping group), EDCI, DIPEA, DCM 50–66% (2 steps).

evaluate the inhibitory activities of the synthesized compounds on HCV replication. HCV replicon systems (genotype 1b) containing HCV NS3–5B and the *Renilla* luciferase gene allow for productive viral RNA replication in cell culture systems. HCV replicon-containing cells were cultivated in the presence of serially diluted compounds for 3 d. Cells were harvested after treatment with inhibitors for 3 d, and luciferase activities were measured. Luciferase activities were normalized to those obtained from mock-treated cells and EC_{50} values were calculated using a Sigma Plot analysis based on luciferase activities in the cells. The antiviral activities (EC_{50}) of these compounds against the HCV GT-2a and GT-1b are listed in Tables 1–3.

The SAR data indicates that the inhibitory activities of these compounds are significantly dependent upon the structural feature of the proline derivatives (Table 1). The EC_{50}'s of our previously reported inhibitor containing L-proline, namely BMK-20113 (entry 1, compound **10**), against GT-2a and GT-1b were reported to be 260 and 28 pM, respectively. The inhibitory activities of the D-isomer **11** were 6500-fold and 2900-fold lower than those of the L-epimer, respectively, against the GT-2a and GT-1b genes (Table 1, entry 2). Installation of a ketone at the 4-position of L-proline **12** resulted in a similar GT-2a inhibitory activity (EC_{50}: 0.26 nM), and a slightly decreased GT-1b inhibitory activity (EC_{50}: 0.044 nM) compared to those of the L-proline derivatives (Table 1, entry 3). When L-thioproline was incorporated into the benzidine **13**, the activity was slightly decreased in GT-2a, but there was a similar potency in GT-1b (EC_{50} of GT-2a and GT-1b: 15.5 nM and 0.027 nM, respectively; Table 1, entry 4). The inhibitor containing a six-membered L-

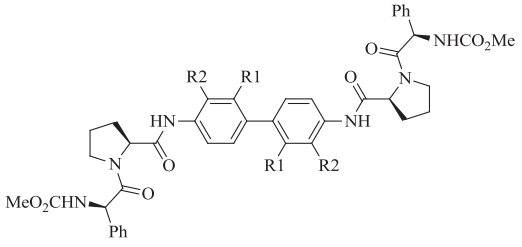
Table 1
Structure–activity relationships of inhibitors containing various proline isosteres against HCV type 2a and type 1b.



Entry	Compounds	R	Proline	HCVCC EC_{50} (type 2a) (nM)	Replicon EC_{50} (type 1b) (nM)	Cytotoxicity (μM)
1	**10**		L-proline	0.26	0.028	>25
2	**11**		D-proline	1770	83	>25
3	**12**		4-oxo-L-proline	0.26	0.044	>25
4	**13**		L-thioproline	15.5	0.027	>25
5	**14**		L-pipecolic acid	180	2.1	>25
6	**15**		D-pipecolic acid	419	370	>25
7	**16**		α-methyl-L-proline	3.3	2.2	>25

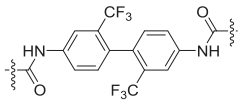
pipecolic acid moiety **14** showed lower activity than its five-membered ring counterpart (Table 1, entry 5). The opposite stereoisomeric D-pipecolic acid derivative **15** exhibited an even more pronounced decrease in inhibitory activities (EC_{50} values all in the nanomolar range; Table 1, entry 6). Lastly, the antiviral activities of the α-methyl substituted L-proline derivative **16** were lower than

Table 2
Structure–activity relationships of inhibitors containing substituted benzidine derivatives against HCV type 2a and type 1b.



Entry	Compound	R_1	R_2	HCVCC EC_{50} (type 2a) (nM)	Replicon EC_{50} (type 1b) (nM)	Cytotoxicity (μM)
1	**10**	H	H	0.26	0.028	>25
2	**23**	H	CH_3	193	7.4	>25
3	**24**	CF_3	H	0.025	0.0025	>25
4	**25**	CH_3	H	0.32	0.035	>25
5	**26**	F	H	0.16	0.005	>25
6	**27**	Cl	H	0.01	0.007	>25
7	**28**	Br	H	0.01	0.007	>25

Table 3
Kinetic resolution of compound **24**.



Entry	Compound **24**	HCVCC EC_{50} (type 2a) (pM)	Replicon EC_{50} (type 1b) (pM)	Cytotoxicity (μM)
1	Isomer 1	332	27.9	>25
2	Isomer 2	24.6	2.4	>25

those of compound **10** (EC_{50} of GT-2a and GT-1b: 3.3 nM and 2.2 nM, respectively; Table 3, entry 7).

Next, after fixing the L-proline and capping groups at both ends, we investigated the SAR studies of various benzidine-containing derivatives by varying the middle biaryl portion (area I in Fig. 2). We first tested the *o*-methyl substituted benzidine derivative **23** for inhibitory effects and observed that low activities against the HCV (EC_{50} of GT-2a and GT-1b: 193 nM and 7.4 nM, respectively; Table 2, entry 2). However, a series of *m*-substituted benzidine derivatives **24**–**28** exhibited more enhanced inhibitory activities against HCV. In detail, the EC_{50} of CF_3-substituted compound **24** in GT-2a and GT-1b were 25 and 2.5 pM, respectively (Table 2, entry 3), and the values for CH_3-substituted compound **25** were 320 and 35 pM, respectively (Table 2, entry 4). After noticing the strong inhibitory effect of the meta-substituted compound, we decided to explore a heteroatom substitution in the *m*-position of the benzidine core [83–86]. All three inhibitors containing *m*-fluoro-, *m*-chloro- and *m*-bromo-substituted benzidine cores (compounds **26, 27,** and **28**, respectively) exhibited powerful antiviral activities. EC_{50} values against GT-2a and GT-1b were 160 and 5 pM, respectively, for compound **26** (Table 2, entry 5) and 10 and 7 pM for compounds **27** and **28** (entries 6 and 7, respectively).

To see whether compound **24** has an additive or synergistic antiviral effect in combination with an NS5B polymerase inhibitor (sofosbuvir), one of the top 10 blockbuster drugs in 2014 [87–91], we treated NK/R2AN replicon cells with serially diluted compound **24** in the presence of an EC_{30} concentration (123 nM) of sofosbuvir, and the luciferase activities in the cells were measured. Our data demonstrated that the combination therapy of compound **24** and sofosbuvir has a remarkably positive effect against HCV replicon cells, probably due to the independent mechanisms of action of the dual treatment [92–95] (Fig. 3).

Since compound **24** contains a core structure that can exist as atropisomers, we were curious about the potentially different inhibitory activities of each isomer. The separation of racemic 2,2′-bis(trifluoromethyl)benzidine was successfully performed through the use of a chiral preparative column on supercritical fluid chromatography (SFC) [96]. Then, each isomer of compound **24** was synthesized using each enantiomeric diamine from the separation following the method described in Scheme 2. Its effect on HCV proliferation was measured. It was found that isomer **1** of compound **24** showed a 13-fold less potent anti-viral activity than isomer **2**, which exhibited a similar activity to that of racemic **24** (Table 3, entries 1–2, and Table 2, entry 3, respectively). Therefore, we concluded that the isomer **2** of **24** is the active inhibitor of HCV.

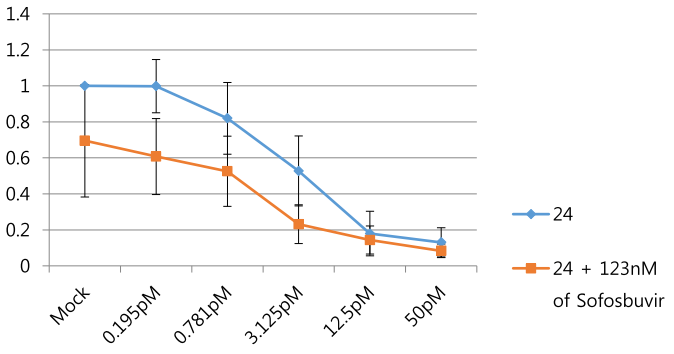


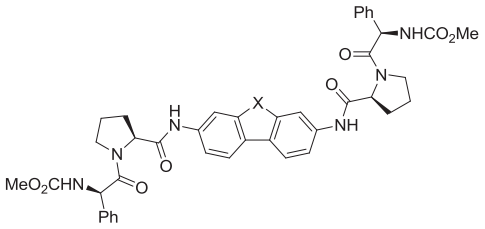
Fig. 3. Effect of compound **24** in combination with EC_{30} of NS5B polymerase inhibitor (sofosbuvir) on the HCV GT-1b replicon system. Measurements were carried out in triplicate.

When we tested the anti-viral activities of compounds **23**–**28**, meta-substitution of the benzidine prolinamides made the inhibitors highly potent HCV inhibitors. Therefore, we envisioned that the activity of *m*-,*m*-connected benzidine, i.e., fluorene derivatives would exhibit high potency in the antiviral assays. Based on the positive results of previously reported NS5A inhibitors containing a fluorenyl core structure [66], we synthesized and investigated inhibitors with a fluorenyl core. We were extremely pleased to observe that these inhibitors exhibited exceptionally high potencies, particularly in the replicon (GT-1b) assay (Table 4, entries 1–4). Inhibitory activities (EC_{50}) of compound **40** containing the parent fluorene were 5.6 and 9 pM against GT-2a and GT-1b, respectively (Table 4, entry 1). The flurorenone-containing inhibitor **41** exhibited a similar potency to compound **40;** EC_{50} of GT-2a and GT-1b were 4.3 and 4 pM, respectively (Table 4, entry 2). Furthermore, substituents at the methylene position of the fluorine core showed a dramatic increase in anti-viral activity against the GT-2a gene [97]. The inhibitory activity of compound **42** with a dimethyl-substituted fluorene moiety was 2800-fold higher against the GT-2a gene than that of compound **40** containing the parent fluorene (EC_{50} of GT-2a and GT-1b: 20 and 4 pM, respectively; Table 4, entry 3). Replacement of the dimethyl with difluorine **43** showed a similar effect (EC_{50} of GT-2a and GT-1b: 59 and 3 pM, respectively; Table 4, entry 4) [98]. All of the inhibitors listed in Table 1, Table 2, Table 3, and Table 4 were not cytotoxic at 25 μM.

Since the compounds **24**, **26**, **27**, **41**, and **43** exhibited high inhibitory activities both in the HCV GT-2a and the replicon (GT-1b) systems (Table 2, entries 3, 6, 7, and Table 3, entries 3, 4, respectively), we next focused on further evaluation of these selected compounds [99]. First, to evaluate any probable cardiac toxicity, we performed a hERG ligand binding assay, examining inhibition of the inner rectifying voltage-gated fluorescence polarization potassium ion channel which is encoded by hERG gene [100,101]. Compared to astemizole, which was used as a control (99.9% inhibition at 10 μM), the values of compounds **24**, **26**, **27**, **42**, and **43** (7.98%, 1%, 5.80%, 25.7%, 39.1% inhibition at 10 μM, respectively) suggested that these inhibitors bound poorly to the hERG membrane preparations, reflecting minimal possible cardiac toxicity (Table 5).

Next, when the compounds were tested against rat plasma, our data showed a high *in vitro* stability for compound **24**, which was unscathed after 2 h (99% of the compound remained; Table 6, entry 1), whereas after incubating for 30 min, compounds **26** and **27** had

Table 4
Structure–activity relationship studies of inhibitors containing a fluorene skeleton against HCV type 2a and type 1b.



Entry	Compound	X	HCVCC EC_{50} (type 2a) (nM)	Replicon EC_{50} (type 1b) (nM)	Cytotoxicity (μM)
1	**40**	CH_2	5.6	0.009	>25
2	**41**	C=O	4.3	0.004	>25
3	**42**	CMe_2	0.020	0.004	>25
4	**43**	CF_2	0.059	0.003	>25

Table 5
Results of hERG ligand binding assay.[a]

Entry	Compound	% Inhibition (10 μM)
1	**24**	7.98 ± 4.49
2	**26**	<1
3	**27**	5.80 ± 3.22
4	**42**	25.7 ± 4.90
5	**43**	39.1 ± 2.93
6	Control (astemizole)	99.9

[a] Fluorescence polarization assay.

Table 6
Stability in rat plasma (% remaining).

Entry	Compound	0.5 h (%)	1 h (%)	2 h (%)
1	**24**	93	99	99
2	**26**	65	61	63
3	**27**	72	75	70

degraded slightly (Table 6, entries 2 and 3). Consequently, it was necessary to carry out additional stability tests.

In the human liver microsomal stability test, compound **24** was diminished 21% in the plasma after 0.5 h (Table 7, entry 1). Compounds **26**, **27**, **42,** and **43** had lower levels of degradation after 0.5 h (Table 7, entries 2, 3, 4, and 5, respectively). These results indicate that these promising compounds had high microsomal stability in the human liver [102,103].

Next, we outlined CYP_{450} inhibitory profiles to evaluate any drug–drug interactions possible with the selected compounds **24**, **26**, **27**, **42**, and **43**. In general, high EC_{50} values for the CYP_{450} enzyme inhibitor indicate decreased liability for drug–drug interactions [104,105]. Our selected compounds exhibited low inhibition profiles for various CYP_{450} isoforms. CYP1A2, CYP2D6, CYP2C19, and CYP3A4 were rather weakly inhibited in the presence of compounds **24**, **26**, **27**, **42**, and **43**, while the inhibition was higher for CYP2C9 (0.14, 0.0877, 0.116, 0.269, and 0.277 μM respectively; Table 8). However, there should still be a large safety margin when we compare these inhibitions to their anti-viral activities [106]. In summary, compounds **24**, **26**, **27**, **42**, and **43** do not significantly inhibit these five representative CYP enzymes (Table 8) [107].

Table 7
Human liver microsomal stability (% remaining).

Entry	Compound	0.5 h (%)
1	**24**	79
2	**26**	97
3	**27**	96
4	**42**	95
5	**43**	92

Studies on the pharmacokinetics (PK) of 5 selected compounds were carried out in rats, with the vehicles consisting of 5% DMSO, 10% solutol, and 85% (2-hydroxypropyl)-β-cyclodextrin (HPBCD) (Table 9). Despite high potencies in *in vitro* studies of HCV inhibition, the selected compounds provoked some concern due to the symmetrical natures of their structures and their high molecular weights, which might lead to undesirable PK properties [108–114]. Our data showed that the highest maximum concentration in plasma (C_{max}) was obtained with compound **42** (0.98 μmol/mL) through oral administration, and compound **43** (18.64 μmol/mL) through intravenous administration (Table 9, entries 5 and 9, respectively). The half-lives ($t_{1/2}$) of these two compounds upon p.o. administration were 1.7–6.4 h, and in IV administration were 0.9–2.9 h, respectively. When we checked the oral bioavailability (F %), their low solubility and high lipophilicity contributed to a moderate oral absorption and bioavailability [115,116].

Then, we tested some of our selected inhibitors (**42** and **43**) against daclatasvir resistant mutants (L31V and Y93H) as described in Materials and Method [18]. *In vitro* transcribed resistant mutant RNAs (L31V and Y93H) were individually transfected to Huh7.5.1 cells [117,118]. After 4 h after transfection, the culture media were replaced with DMEM media containing serially diluted compounds. By comparing the luciferase activities of **42** and **43** with those of daclatasvir, we concluded that **43** had a higher activity than **42** and a similar anti-viral potency to daclatasvir, to single mutant viruses. Therefore, we concluded that **43** could be a solution to the resistance problems of current HCV drugs (Fig. 4).

Then, we tested the cytotoxicity of compound **43** against some eukaryotic cells [119]. The cell counting kit-8 assay method was used to measure the survival rate of the cells by using WST-8

Table 8
EC_{50} of compounds (μM) against various subtypes of CYP_{450}.[a]

Entry	Compound	1A2	2C9	2D6	2C19	3A4
1	**24**	78.8	0.660	>100	4.64	4.64
2	**26**	>100	0.0877	4.94	0.724	2.46
3	**27**	23.9	0.116	41.2	1.04	3.99
5	**42**	>100	0.269	>100	2.94	1.03
4	**43**	>100	0.277	>100	13.9	0.976

[a] Standard inhibitors for each CYP_{450} subzymes: 1A2: α-naphthoflavone, 2C9: sulfaphenazole, 2D6: quinidine, 2C19: miconazole 3A4: ketoconazole.

Table 9
Pharmacokinetics features of selected inhibitors in rats.[a]

Entry	Compound	Administration route	Dose (mg/kg)	C_{max} (μmol/mL)	$t_{1/2}$ (h)	F (%)
1	**24**	p.o.	10	0.44 ± 0.14	3.80 ± 0.11	11.74
2	**26**		10	0.11 ± 0.07	2.34 ± 0.37	3.12
3	**27**		10	0.17 ± 0.07	6.37 ± 3.39	4.99
5	**42**		10	0.98 ± 0.02	4.45 ± 4.02	13.8
4	**43**		10	0.39 ± 0.20	1.68 ± 0.43	7.6
6	**24**	iv	5	13.76 ± 4.1	2.90 ± 0.62	
7	**26**		5	16.63 ± 2.57	0.88 ± 0.34	
8	**27**		5	15.88 ± 1.62	1.55 ± 0.25	
10	**42**		5	13.26 ± 1.13	1.62 ± 0.78	
9	**43**		5	18.64 ± 19.4	0.90 ± 0.02	

[a] Vehicle: 5% DMSO/10% Solutol/85% HPBCD (20%, w/v), n = 3.

tetrazolium salt. Hydrophilic WST-8 turns into orange formazan dye by dehydrogenase in cell lines. The number of living cells is determined by the activated dehydrogenase, which indicates the eukaryotic cell survival rate. At the maximal concentration (100 μM) used in the experiment, compound **43** showed less than 50% inhibition (the limit) in monkey kidney, human embryonic

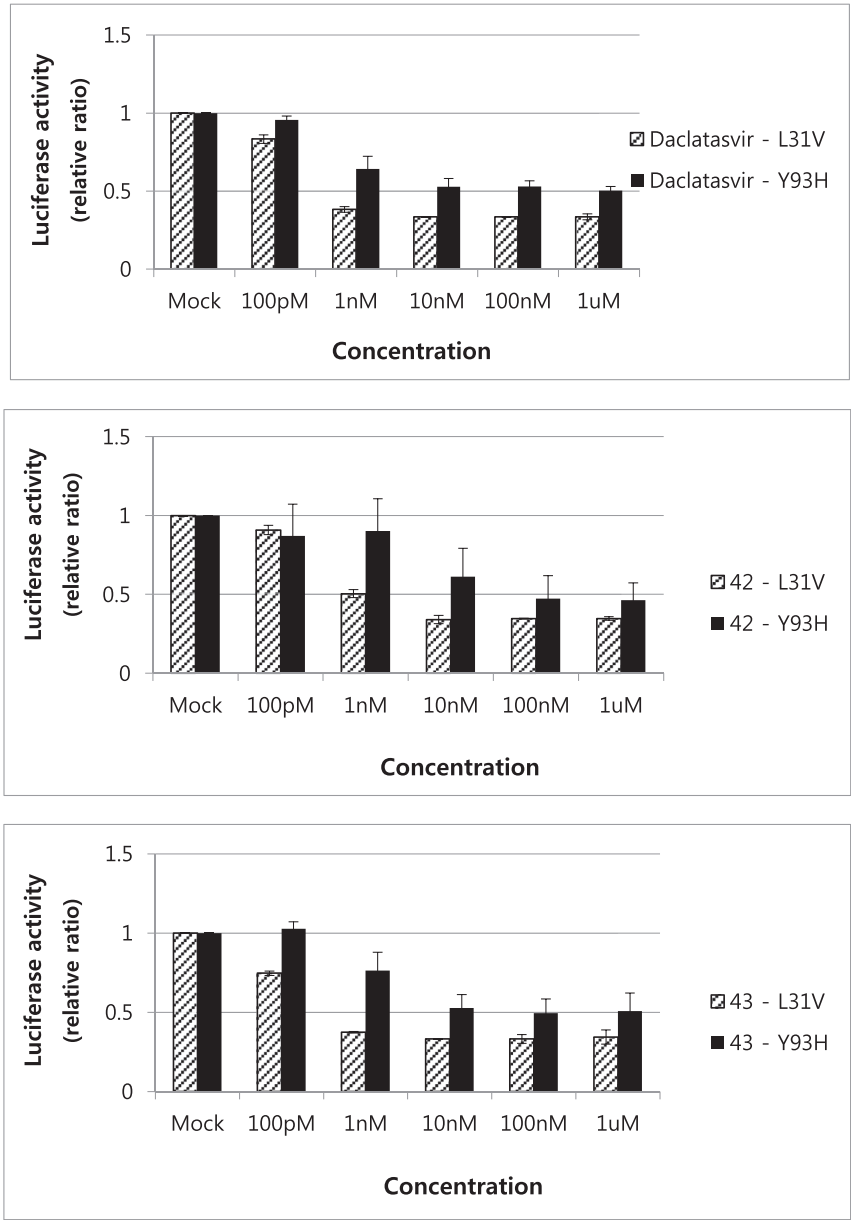


Fig. 4. Resistance profiles of inhibitors daclatasvir, **42,** and **43**. Measurements were carried out in triplicate.

lung, mouse fibroblast, and hamster ovary cell line (Table 10). Thus, we concluded that **43** is nontoxic and safe in the tested eukaryotic cell lines.

Next, we investigated the possibility that compound **43** might have genetic toxicity using the Ames test [120,121]. For this purpose, we used *Salmonella typhimurium* strains TA-98 and TA-100 that require several amino acids for growth to test whether compound **43** functions as a mutagen. Growth of TA-98 and TA-100 strains in the presence of the compound of interest in the media lacking histidine indicates that the compound is a potential mutagen inducing substitution, addition, and/or deletion of DNA. The result is considered to be positive when the number of colonies on the compound-treated plate increases double or more of those on the vehicle control plate. When we tested 200 μg/plate of **43**, the number of revertant colonies on the compound-treated plate was not increased compared with that on the vehicle-treated plate. And the number was much lower than those on 4 positive control compound-treated plates (Table 11). Therefore, compound **43** is considered to be non-genotoxic.

4. Conclusion

In conclusion, we developed a series of extremely potent HCV NS5A inhibitors using new benzidine and fluorene prolinamide derivatives as core structures. Several of them have high potencies that produce inhibition even at the single digit pM level. Through the SAR studies using a variety of benzidine, proline, and fluorene derivatives with a phenyl glycine capping group, we were able to identify inhibitors possessing excessively high inhibitory activities. Among the new inhibitors, compounds **24**, **26**, **27**, **42**, and **43** were chosen for further evaluation since they were the most potent. A synergistic effect with the NS5B polymerase inhibitor was seen with compound **24**. Moreover, subsequent studies demonstrated that these compounds have a desirable low cardiac toxicity, high human microsomal stability, and limited drug–drug interaction possibilities. Compound **43** was revealed to be non-toxic for eukaryotic cells and genetically non-toxic by the Ames test. The results of this study suggest that compound **43** is a highly potent and safe lead warranting further study as a potential HCV drug candidate. Further research on other pharmacological properties of the selected inhibitors and new structure of the HCV NS5A inhibitors is currently under progress at our laboratories.

Table 10
Cytotoxicity of **43** in eukaryotic cells.[a]

Compound	IC_{50} (μM)				
	VERO	HFL-1	L929	NIH 3T3	CHO–K1
43	>100	>100	>100	>100	>100

[a] VERO: African green monkey kidney cell line; HFL-1: human embryonic lung cell line; L929: NCTC clone 929, mouse fibroblast cell line; NIH 3T3: mouse embryonic fibroblast cell line; CHO–K1: Chinese hamster ovary cell line.

5. Experimental section

5.1. Chemical studies

5.1.1. General chemical methods

The ^{1}H and ^{13}C NMR-spectra were measured with an Agilent 400-MR DD2 Magnetic Resonance System (400 MHz) and a Varian/Oxford As-500 (500 MHz) Spectrophotometer. The signals were reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet) and chemical shifts were measured as parts per million (δ values) from tetramethylsilane as an internal standard at probe temperature in $CDCl_3$ or DMSO-D_6 for neutral compounds. Reactions that needed anhydrous conditions were carried out in flame-dried glassware under a positive pressure of dry N_2 using standard Schlenk line techniques. Evaporation of solvents was performed at reduced pressure using a rotary evaporator. TLC was performed using silica gel 60F254 coated on an aluminum sheet (E. Merck, Art.5554). Chromatogram was visualized by UV-lamp (Vilber Lournat, VL-4LC) and/or colorized with following solutions: (a) 20% ethanolic phosphomolybdic acid (PMA), (b) potassium permanganate solution, and (c) 2% ninhydrin ethanolic solution. Column chromatography was performed on silica gel (Merck. 7734 or 9385 Kiesel gel 60), and the eluent was mentioned in each procedure. High resolution mass spectra (HRMS) were recorded on a ThermoFinnigan LCQ™ Classic, Quadrupole Ion-Trap Mass Spectrometer. HPLC analyses were carried out on an Agilent HP1100 system (Santa Clara, CA, USA), composed of an auto sampler, quaternary pump, photodiode array detector (DAD), and HP Chemstation software. The separation was carried out on a C18 Vydac 218TP54 column 250 × 4.6 mm i.d. (5 μm particle size) with 0.1% TFA in water (A), acetonitrile (B), as a mobile phase at a flow rate of 1 mL/min at 20 °C. Method: 100% A and 0% B (0 min), 0% A and 100% B (10 min), 0% A and 100% B (20 min), 100% A and 0% B (22 min), 100% A and 0% B (25 min). All materials were purchased from a commercial supplier and used without further purification unless otherwise noted.

*5.1.1.1. (R)-2-((Methoxycarbonyl)amino)-2-phenylacetic acid (**9**).* Na_2CO_3 (0.55 g, 5.2 mmol) was added to an aq NaOH (10 mL of 1 M/H_2O, 10 mmol) solution of D-phenylglycine (1.500 g, 10.0 mmol) and the resulting solution was cooled in an ice-water bath. Methyl chloroformate (0.85 mL, 11.0 mmol) was added dropwise, then the cooling bath was removed and the reaction mixture was stirred at

Table 11
Bacterial reverse mutation assay of **43**.[a]

Tester strain	Compound	Dose (μg/plate)	Revertant colonies/plate (Mean ± SD) [Factor][b]	
			Without S-9 mix	With S-9 mix
TA-98	Vehicle control	0	18 ± 2	28 ± 3
	43	200	23 ± 5[1.3]	30 ± 2[1.1]
TA-100	Vehicle control	0	117 ± 10	126 ± 19
	43	200	106 ± 3[0.9]	115 ± 8[0.9]
	Positive control	Dose (μg/plate)	Without S-9 mix	With S-9 mix
TA-98	2-Nitrofluorene	1	187 ± 14[10.2]	ND
	Benzo[a]pyrene	2	ND	171 ± 10[6.1]
TA-100	Sodium azide	1	728 ± 15[6.2]	ND
	Benzo[a]pyrene	2	ND	521 ± 28[4.1]

ND: Non Determined.
[a] (Mean ± SD, n = 3).
[b] No. of revertant colonies in the treated plate/No. of revertant colonies in the vehicle control plate.

ambient temperature for 3.25 h. The reaction mixture was washed with ether (3 × 18 mL), and the aqueous phase was cooled in an ice-water bath and acidified with conc. HCl to a pH range of 1–2, and extracted with CH_2Cl_2 (3 × 18 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo, and the resulting oil residue was treated with diethyl ether/hexanes (~5:4 ratio; 10 mL) to provide a precipitate. The precipitate was filtered and washed with diethyl ether/hexanes (~1:3 ratio) and dried in vacuo to provide **9** as a fluffy white solid (1.4 g, 67%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 500 MHz): 12.79 (br s, 1H), 7.96 (d, *J* = 12, 1H), 7.40-7.29 (m, 5H), 5.13 (d, *J* = 12, 1H), 3.55 (s, 3H).

*5.1.1.2. (2R,2′R)-Di-tert-butyl 2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**3**).* A mixture of *N*-Boc-D-proline (771 mg, 3.6 mmol), EDCI (812 mg, 4.2 mmol), and benzidine (300 mg, 1.63 mmol) in CH_2Cl_2 (4 mL) was stirred at ambient temperature for 2 h. The resulting residue was divided between CH_2Cl_2 and H_2O. The organic soluble layer was washed with 1 N aq HCl solution and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Without any purification, **17** was obtained as a solid (930 mg, 99%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.08 (s, 2H), 7.69-7.59 (dd, 8H), 4.29–4.20 (m, 2H), 3.44–3.35 (m, 4H), 2.21 (m, 2H), 1.90–1.78 (m, 6H), 1.40–1.28 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.5, 153.2, 138.2, 134.4, 126.4, 119.6, 78.5, 60.4, 46.6, 31.0, 28.2, 28.0, 23.4. HRMS: Anal. calcd. for $[M+H]^+$ $C_{32}H_{42}N_4O_6$: 579.3177; found 579.3167.

*5.1.1.3. (5S,5′S)-Di-tert-butyl 5,5′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(3-oxopyrrolidine-1-carboxylate) (**4**).* Yield 320 mg (87%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.36 (d, 2H), 7.65 (m, 8H), 4.78–6.49 (m, 2H), 3.89-3.76 (m, 4H), 3.11 (m, 2H), 2.57 (m, 2H), 1.42–1.33 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 209.5, 208.9, 171.0, 170.7, 153.8, 153.4, 137.8, 134.8, 126.64, 126.58, 119.7, 79.9, 79.8, 57.8, 57.0, 53.1, 41.5, 41.2, 28.1, 28.0. HRMS: Anal. calcd. for $[M+H]^+$ $C_{32}H_{38}N_4O_8$: 607.2762; found 607.2756.

*5.1.1.4. (4R,4′R)-Di-tert-butyl 4,4′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(thiazolidine-3-carboxylate) (**5**).* Yield 2.5 g (64%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.15 (s, 2H), 7.68-7.61 (dd, 8H), 4.72 (app br s, 1H), 4.63 (d, 2H), 4.55 (app br s, 1H), 4.45 (d, 2H), 3.46 (m, 2H), 3.15 (m, 2H), 1.94–1.81 (m, 6H), 1.43–1.32 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 169.0, 152.6, 137.9, 134.7, 126.5, 119.8, 80.0, 63.0, 50.0, 35.2, 27.9. HRMS: Anal. calcd. for $[M+H]^+$ $C_{30}H_{38}N_4O_6S_2$: 615.2306; found 615.2278.

*5.1.1.5. (2S,2′S)-Di-tert-butyl 2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(piperidine-1-carboxylate) (**6**).* A mixture of *N*-Boc-L-pipecolic acid (400 mg, 1.75 mmol), EDCI (363 mg, 1.9 mmol), and benzidine (134 mg, 0.73 mmol) in CH_2Cl_2 (7 mL) was stirred at ambient temperature for 2 h. The resulting residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with 1.0 N aq HCl solution and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. A silica gel mesh was prepared from the residue and submitted to flash chromatography (silica gel: EtOAc/hexane as eluent) to provide **20** as a solid (186 mg, 42%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.99 (s, 2H), 7.67-7.58 (dd, 8H), 4.70 (m, 2H), 3.82 (d, 2H), 3.34 (m, 2H), 2.08 (app br s, 2H), 1.72–1.58 (m, 6H), 1.37 (app br s, 22H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.8, 155.3, 138.1, 134.5, 126.4, 119.7 78.9, 55.2, 53.9, 42.0, 28.0, 27.5, 24.2, 19.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{34}H_{46}N_4O_6$: 607.3490; found 607.3491.

*5.1.1.6. (2R,2′R)-Di-tert-butyl 2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(piperidine-1-carboxylate) (**7**).* Yield 190 mg (35%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.00 (s, 2H), 7.67-7.58 (dd, 8H), 4.69 (m, 2H), 3.81 (d, 2H), 3.26 (m, 2H), 2.07 (app br s, 6H), 1.64–1.58 (m, 6H), 1.37 (app br s, 22H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.8, 155.3, 138.1, 134.5, 126.4, 119.7, 78.9, 55.2, 53.9, 42.0, 28.0, 27.5, 24.2, 19.6. HRMS: Anal. calcd. for $[M+H]^+$ $C_{34}H_{46}N_4O_6$: 607.3490; found 607.3489.

*5.1.1.7. (2S,2′S)-Di-tert-butyl 2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(2-methylpyrrolidine-1-carboxylate) (**8**).* Yield 86 mg (31%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.37 (app br s, 2H), 7.74-7.62 (m, 8H), 3.67 (m, 2H), 3.41 (m, 2H), 2.15 (m, 2H), 1.90 (m, 6H), 1.51–1.26 (m, 24H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 173.0, 172.5, 153.0, 152.6, 138.4, 134.3, 126.1, 120.8, 120.6, 78.7, 78.6, 66.2, 65.9, 47.5, 47.1, 28.2, 28.0, 22.6, 22.1, 21.1. HRMS: Anal. calcd. for $[M+H]^+$ $C_{34}H_{46}N_4O_6$: 607.3490; found 607.3490.

*5.1.1.8. Dimethyl ((1R,1′R)-((2R,2′R)-2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**11**).* Compound **17** (300 mg, 0.52 mmol) in CF_3CO_2H (2 mL) and CH_2Cl_2 (2 mL) was stirred at room temperature for 4 h. The volatile component was removed in vacuo, EDCI (258 mg, 0.620 mmol) and **9** (260 mg, 1.24 mmol) were added in batches over 4 min to a solution of *i*-Pr_2NEt (455 μL, 2.6 mmol) in CH_2Cl_2 (3 mL) and the reaction mixture was stirred at room temperature for 75 min. The residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. A silica gel mesh was prepared from the residue and submitted to flash chromatography (silica gel: EtOAc/hexane as eluent) to provide **23** as a white solid (171 mg, 57%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.14 (s, 2H), 7.68-7.60 (m, 9H), 7.46–7.30 (m, 11H), 5.49 (d, 2H), 4.53 (m, 2H), 3.68 (m, 2H), 3.54 (s, 6H), 3.12 (m, 2H), 2.00–2.13 (m, 2H), 1.89–1.82 (m, 6H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.2, 168.1, 156.4, 138.2, 136.9, 134.4, 128.4, 128.4, 127.8, 126.4, 119.4, 60.6, 56.6, 51.6, 46.9, 29.4, 24.7. HRMS: Anal. calcd. for $[M+H]^+$ $C_{42}H_{44}N_6O_8$: 761.3293; found 761.3281.

*5.1.1.9. Dimethyl ((1R,1′R)-((5S,5′S)-5,5′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(3-oxopyrrolidine-5,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**12**).* Yield 48 mg (23%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.38 (s, 2H), 7.92 (d, 2H), 7.70-7.10 (m, 18H), 5.46 (d, 2H), 4.94 (d, 2H), 4.25 (d, 2H) 3.89 (d, 2H), 3.54 (s, 6H), 3.06 (m, 2H), 2.54 (m, 2H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 208.5, 170.1, 169.4, 156.1, 137.8, 136.8, 134.7, 128.6, 128.2, 128.1, 126.5, 119.7, 57.4, 56.9, 53.1, 51.7, 40.4. HRMS: Anal. calcd. for $[M+H]^+$ $C_{42}H_{40}N_6O_{10}$: 789.2879; found 789.2877.

*5.1.1.10. Dimethyl ((1R,1′R)-((4R,4′R)-4,4′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(thiazolidine-4,3-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**13**).* Yield 25 mg (14%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.89 (s, 2H), 7.93 (d, 2H), 7.73-7.59 (m, 8H), 7.46–7.13 (m, 10H), 5.64 (d, 2H), 4.87 (m, 4H), 4.53 (d, 2H), 3.58 (s, 6H), 3.36 (m, 2H), 3.19 (m, 2H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 168.3, 167.9, 156.4, 137.8, 136.3, 134.7, 128.7, 128.3, 128.1, 126.4, 119.8, 63.2, 56.8, 51.8, 49.0, 33.1. HRMS: Anal. calcd. for $[M+H]^+$ $C_{40}H_{40}N_6O_8S_2$: 797.2422; found 797.2422.

5.1.1.11. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(piperidine-2,1-diyl))bis(2-oxo-1-

*phenylethane-2,1-diyl))dicarbamate (**14**).* Compound **20** (73 mg, 0.12 mmol) in CF_3CO_2H (1 mL) and CH_2Cl_2 (1 mL) was stirred at room temperature for 5 h. The volatile component was removed in vacuo, EDCI (60 mg, 0.31 mmol) and compound **9** (60 mg, 0.29 mmol) were added in batches over 4 min to a solution of *i*-Pr_2NEt (105 μL, 0.60 mmol) in DMF (1 mL) and the reaction mixture was stirred at room temperature for 75 min. The residue was partitioned between CH_2Cl_2 and H_2O. The organic soluble layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. A silica gel mesh was prepared from the residue and submitted to flash chromatography (silica gel: EtOAc/hexane as eluent) to provide **26** as a solid (12 mg, 13%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.99-9.84 (s, 2H), 7.85–7.30 (m, 20H), 5.73–5.64 (m, 2H), 5.17/4.85 (m, 2H), 4.45/3.77 (m, 2H), 3.55 (app br s, 6H), 3.18/2.83 (m, 2H), 2.15 (m, 2H), 1.76 (m, 2H), 1.63–1.24 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.0, 169.3, 168.5, 156.2, 138.0, 137.2, 134.5, 128.6, 128.4, 128.2, 127.7, 126.5, 120.2, 119.7, 67.0, 55.5, 52.8, 51.6, 43.2, 27.5, 25.1, 24.6, 19.7. HRMS: Anal. calcd. for $[M+H]^+$ $C_{44}H_{48}N_6O_8$: 789.3606; found 789.3605.

*5.1.1.12. Dimethyl ((1R,1′R)-((2R,2′R)-2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(piperidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**15**).* Yield 23 mg (15%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.98-9.84 (s, 2H), 7.84–7.28 (m, 20H), 5.72–5.64 (m, 2H), 5.16/4.85 (m, 2H), 4.45/3.75 (m, 2H), 3.55 (app br s, 6H), 3.17/2.83 (m, 2H), 2.15 (m, 2H), 1.76 (m, 2H), 1.63–1.23 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.0, 169.3, 168.5, 156.2, 138.0, 137.2, 134.5, 128.6, 128.4, 128.2, 127.7, 126.5, 120.2, 119.7, 67.0, 55.5, 52.8, 51.6, 43.2, 27.5, 25.1, 24.6, 19.7. HRMS: Anal. calcd. for $[M+H]^+$ $C_{44}H_{48}N_6O_8$: 789.3606; found 789.3605.

*5.1.1.13. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(([1,1′-biphenyl]-4,4′-diylbis(azanediyl))bis(carbonyl))bis(2-methylpyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**16**).* Yield 77 mg (41%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.03 (s, 1H), 8.89 (s, 1H), 7.77-7.57 (m, 10H), 7.40–7.32 (m, 10H), 5.46 (m, 2H), 3.99 (m, 1H), 3.76 (m, 1H), 3.56 (s, 3H), 3.54 (s, 3H), 3.48 (m, 1H), 3.21 (m, 1H), 2.18–2.08 (m, 2H), 1.91–1.80 (m, 6H), 1.55 (s, 3H), 1.43 (s, 3H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.8, 171.7, 168.2, 167.7, 156.4, 156.2, 138.2, 138.0, 137.2, 136.5, 134.7, 134.3, 128.7, 128.4, 128.22, 128.17, 127.70, 127.68, 126.09, 126.05, 120.9, 120.3, 67.6, 67.5, 57.2, 57.0, 51.7, 51.6, 47.7, 47.5, 23.5, 23.1, 20.6, 20.5. HRMS: Anal. calcd. for $[M+H]^+$ $C_{44}H_{48}N_6O_8$: 789.3606; found 789.3600.

*5.1.1.14. (2S,2′S)-Di-tert-butyl 2,2′-(((3,3′-dimethyl-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**17**).* A mixture of *N*-Boc-L-proline (9.47 g, 44.0 mmol), EDCI (9.97 g, 52.0 mmol), and *ortho*-tolidine (4.25 g, 20.0 mmol) in CH_2Cl_2 (30 mL) was stirred at ambient temperature for 2 h. The resulting residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with 1.0 N aq HCl solution and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Without any purification, **3** was obtained as a solid (11.3 g, 93%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.35 (d, 2H), 7.51 (s, 2H), 7.48-7.42 (m, 4H), 4.34 (m, 2H), 3.45 (m, 2H), 3.55 (m, 2H), 2.17 (s, 6H), 2.16 (m, 2H), 1.94–1.81 (m, 6H), 1.42–1.37 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.4, 153.3, 138.5, 135.4, 132.2, 128.3, 125.3, 124.0, 78.5, 59.9, 46.6, 31.4, 28.1, 23.3, 17.9. HRMS: Anal. calcd. for $[M+H]^+$ $C_{34}H_{46}N_4O_6$: 607.3490; found 607.3480.

*5.1.1.15. (2S,2′S)-Di-tert-butyl 2,2′-(((2,2′-bis(trifluoromethyl)-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**18**).* Yield 4.3 g (96%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.44 (d, 2H), 8.21 (s, 1H), 8.14 (s, 1H), 7.92-7.82 (dd, 2H), 7.33 (m, 2H), 4.26 (m, 2H), 3.50–3.38 (m, 4H), 2.22 (m, 2H), 1.94–1.80 (m, 6H), 1.42 (s, 9H), 1.30 (s, 9H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.9, 171.6, 153.8, 153.2, 139.0, 132.0, 131.0, 128.19, 128.15, 127.9, 124.9, 122.1, 121.2, 121.0, 116.34, 116.28, 116.2, 116.1, 78.7, 78.6, 60.5, 60.1, 54.1, 46.6, 46.4, 31.0, 29.9, 27.8, 27.7, 24.0, 23.3. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −57.37. HRMS: Anal. calcd. for $[M+H]^+$ $C_{34}H_{40}F_6N_4O_6$: 715.2925; found 715.2919.

*5.1.1.16. (2S,2′S)-Di-tert-butyl 2,2′-(((2,2′-dimethyl-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**19**).* Yield 2.75 g (96%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.97 (s, 2H), 7.57-7.41 (m, 4H), 6.97 (app br s, 2H), 4.26 (m, 2H), 3.42–3.35 (m, 4H), 2.21 (m, 2H), 1.97 (s, 6H), 1.89–1.78 (m, 6H), 1.40–1.31 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.4, 171.0, 153.6, 153.2, 138.0, 138.0, 135.69, 135.65, 129.52, 120.48, 120.47, 120.4, 120.3, 116.7, 116.5, 78.6, 78.4, 60.3, 60.0, 46.7, 46.5, 31.3, 31.1, 30.2, 28.1, 28.0, 23.9, 23.3, 19.7. HRMS: Anal. calcd. for $[M+H]^+$ $C_{34}H_{46}N_4O_6$: 607.3490; found 607.3489.

*5.1.1.17. (2S,2′S)-Di-tert-butyl 2,2′-(((2,2′-difluoro-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**20**).* Yield 2.6 g (93%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.33 (s, 2H), 7.72-7.68 (m, 2H), 7.46–7.36 (m, 4H), 4.28–4.19 (m, 2H), 3.45–3.32 (m, 4H), 2.22 (m, 2H), 1.99–1.79 (m, 6H), 1.40–1.29 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 172.0, 171.6, 160.1, 157.7, 153.6, 153.1, 140.5, 140.4, 131.58, 131.55, 117.0, 116.9, 115.1, 115.0, 106.2, 106.0, 78.74, 78.57, 60.4, 60.1, 46.7, 46.6, 31.0, 30.2, 28.1, 28.0, 24.0, 23.4. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −113.53. HRMS: Anal. calcd. for $[M+H]^+$ $C_{32}H_{40}F_2N_4O_6$: 647.2398; found 647.2887.

*5.1.1.18. (2S,2′S)-Di-tert-butyl 2,2′-(((2,2′-dichloro-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**21**).* Yield 2.8 g (95%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.31 (app br s, 2H), 7.97-7.87 (m, 2H), 7.63–7.52 (m, 2H), 7.29–7.25 (m, 2H), 4.29–4.20 (m, 2H), 3.47–3.41 (m, 2H), 3.38–3.32 (m, 2H), 2.25–2.19 (m, 2H), 1.92–1.82 (m, 6H), 1.41–1.31 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.9, 171.5, 153.6, 153.1, 139.9, 132.5, 132.0, 131.6, 119.2, 117.6, 78.7, 78.5, 60.4, 60.1, 46.7, 46.5, 31.3, 31.0, 30.1, 28.1, 28.0, 23.9, 23.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{32}H_{40}Cl_2N_4O_6$: 647.2398; found 647.2394.

*5.1.1.19. (2S,2′S)-Di-tert-butyl 2,2′-(((2,2′-dibromo-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**22**).* Yield 990 mg (92%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.26 (s, 2H), 8.12-8.01 (m, 2H), 7.66–7.55 (m, 2H), 7.23 (app br s, 2H), 4.26–4.20 (m, 2H), 3.39–3.35 (m, 4H), 2.22 (m, 2H), 1.89–1.81 (m, 6H), 1.40–1.30 (app br s, 18H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 172.0, 171.6, 153.7, 153.1, 139.9, 135.9, 131.4, 123.0, 122.3, 122.2, 118.2, 118.1, 78.8, 78.6, 60.5, 60.2, 46.8, 46.6, 31.0, 30.2, 28.2, 28.0, 24.0, 23.4. HRMS: Anal. calcd. for $[M+H]^+$ $C_{32}H_{40}Br_2N_4O_6$: 735.1387; found 735.1373.

*5.1.1.20. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((3,3′-dimethyl-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**23**).* Compound **3** (144 mg, 0.238 mmol) in CF_3CO_2H (1 mL) and CH_2Cl_2 (1 mL) was stirred at room temperature for 5 h. The volatile component was removed in vacuo, EDCI (119 mg, 0.620 mmol), and **9** (100 mg, 0.572 mmol) were added in batches over 4 min to a solution of *i*-Pr_2NEt (208 μL, 1.192 mmol) in CH_2Cl_2 (1 mL). The reaction mixture was stirred at room temperature for 75 min. The residue was divided between CH_2Cl_2 and H_2O. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and

concentrated in vacuo. A silica gel mesh was prepared from the residue and submitted to flash chromatography (silica gel: EtOAc/hexane as eluent) to provide **11** as a white solid (77 mg, 41%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.31 (s, 2H), 7.74 (d, 2H), 7.54-7.23 (m, 16H), 5.52 (d, 2H), 4.52 (m, 2H), 3.85 (m, 2H), 3.52 (s, 6H), 3.18 (m, 2H), 2.28 (s, 6H), 2.00–1.82 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.1, 168.8, 156.1, 137.1, 136.5, 135.4, 132.2, 128.6, 128.2, 128.1, 128.0, 125.2, 123.9, 60.6, 56.8, 51.6, 46.9, 29.1, 24.3, 17.9. HRMS: Anal. calcd. for $[M+H]^+$ $C_{44}H_{48}N_6O_8$: 789.3606; found 789.3597.

*5.1.1.21. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((2,2′-bis(trifluoromethyl)-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**24**).* Yield 145 mg (32%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.29 (s, 2H), 8.21 (d, 2H), 7.83 (m, 2H), 7.75 (d, 2H), 7.43-7.05 (m, 12H), 5.51 (d, 2H), 4.41 (m, 2H), 3.85 (app br s, 2H), 3.54 (s, 6H), 3.20 (app br d, 2H), 2.06–1.82 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.26, 168.75, 156.42, 139.30, 137.16, 132.79, 131.36, 128.89, 128.35, 128.25, 125.07, 122.88, 121.81, 116.37, 61.05, 56.97, 51.87, 47.22, 29.45, 24.51. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −57.28. HRMS: Anal. calcd. for $[M+H]^+$ $C_{44}H_{42}F_6N_6O_8$: 897.3041; found 897.3046.

*5.1.1.22. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((2,2′-dimethyl-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**25**).* Yield 292 mg (73%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.84 (s, 2H), 7.73 (d, 2H), 7.59 (s, 2H), 7.48-7.14 (m, 12H), 6.87 (d, 2H), 5.51 (d, 2H), 4.42 (m, 2H), 3.84 (m, 2H), 3.55 (s, 6H), 3.20 (m, 2H), 1.98 (s, 6H), 1.97–1.78 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.2, 168.4, 156.1, 137.9, 137.1, 135.8, 135.8, 129.58, 128.62, 128.1, 127.9, 120.5, 116.7, 60.7, 56.8, 51.7, 47.0, 29.4, 24.3, 19.8. HRMS: Anal. calcd. for $[M+H]^+$ $C_{44}H_{48}N_6O_8$: 789.3606; found 789.3605.

*5.1.1.23. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((2,2′-difluoro-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**26**).* Yield 134 mg (40%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.43 (s, 2H), 7.74-7.71 (m, 3H), 7.46–7.11 (m, 15H), 5.51 (d, 2H), 4.43 (m, 2H), 3.83 (m, 2H), 3.54 (s, 6H), 3.19 (m, 2H), 2.05–1.77 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.8, 168.4, 160.0, 158.0, 156.1, 140.5, 137.2, 131.6, 128.6, 128.5, 128.1, 127.9, 127.6, 117.1, 115.1, 106.3, 106.1, 60.8, 56.7, 51.7, 47.0, 29.3, 24.3. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −73.45. HRMS: Anal. calcd. For $[M+H]^+$ $C_{42}H_{42}F_2N_6O_8$: 797.3105; found 797.3112.

*5.1.1.24. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((2,2′-dichloro-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**27**).* Yield 146 mg (44%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.14 (s, 2H), 7.97-7.91 (m, 2H), 7.75 (d, 2H), 7.65–7.55 (m, 2H), 7.43–7.12 (m, 12H), 5.51 (d, 2H), 4.39 (m, 2H), 3.84 (m, 2H), 3.55 (s, 6H), 3.20 (m, 2H), 2.06–1.79 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.8, 168.5, 156.2, 139.9, 137.1, 132.6, 132.1, 131.7, 128.6, 128.1, 127.9, 119.3, 117.7, 60.8, 56.73, 51.68, 47.0, 29.3, 24.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{42}H_{42}Cl_2N_6O_8$: 829.2514; found 829.2518.

*5.1.1.25. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((2,2′-dibromo-[1,1′-biphenyl]-4,4′-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**28**).* Yield 110 mg (42%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.13 (s, 2H), 8.32–8.12 (m,2H), 7.92–7.61 (m, 4H), 7.41–7.13 (m, 12H), 5.51 (d, 2H), 4.39 (m, 2H), 3.84 (m, 2H), 3.55 (s, 6H), 3.52 (m, 2H), 3.19 (m, 2H), 2.05–1.81 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.7, 168.5, 156.2, 139.8, 137.1, 135.9, 131.3, 128.6, 128.1, 127.9, 123.0, 122.3, 118.1, 60.8, 56.7, 51.7, 47.0, 29.3, 24.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{42}H_{42}Br_2N_6O_8$: 917.1504; found 917.1521.

*5.1.1.26. 9,9-Difluoro-2,7-dinitro-9H-fluorene (**30**).*
2,7-Nitro-9*H*-fluorene (100 mg, 0.39 mmol) and *N*-fluorobenzenesulfonimide (NFSI) (369 mg, 1.17 mmol) were dissolved in DMF and cooled to −20 °C. LiHMDS (1.0 M in THF, 1.17 mL, 1.17 mmol) was added dropwise over 5 min. After an additional 2 h at 0 °C, TLC indicated the completion of the reaction, and the excess base was quenched by addition of MeOH. The suspension was filtered over Celite and concentrated in vacuo. A silica gel mesh was prepared from the residue and submitted to flash chromatography (silica gel: CH_2Cl_2/hexane as eluent) to provide **30** as a yellow solid (98 mg, 86%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 8.63 (d, 2H), 8.53 (d, 1H), 8.56 (d, 1H), 8.36 (s, 1H), 8.34 (s, 1H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 149.1, 142.5, 138.5, 129.2, 144.3, 120.8, 119.6. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −110.3.

*5.1.1.27. 9,9-Dimethyl-2,7-dinitro-9H-fluorene (**31**).* A mixture of 2,7-nitro-9*H*-fluorene (100 mg, 0.39 mmol) and NaO*t*-Bu (75 mg, 0.78 mmol) were dissolved in DMF at ice bath under N_2. Then, CH_3I (49 μL, 0.78 mmol) was slowly added to the mixture, and stirred for 2 h. The solution was poured into water and a precipitate was formed. The product was filtered, washed with water, and air-dried. Without any purification, **31** (89 mg, 80%) was obtained as a yellow solid. ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 8.59 (d, 2H), 8.33 (m, 4H), 1.60 (s, 6H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 156.2, 148.1, 142.7, 123.6, 122.9, 118.7, 47.9, 25.6.

*5.1.1.28. 9,9-Difluoro-9H-fluorene-2,7-diamine (**32**).* A magnetic stirrer bar, Fe_3O_4 (purchased from Aldrich, 4 mg, 0.015 mmol), and DMF (0.5 mL) were added to an oven-dried Schlenk tube, and the mixture was sonicated in an ultrasound bath for 1 min under argon. Compound **31** (22 mg, 0.075 mmol) and hydrazine monohydrate (29 μL, 0.60 mmol) were then added to the mixture. The reaction mixture was stirred at 80 °C under an argon atmosphere until the reaction was completed. After magnetic separation of the catalyst, the organic layer was concentrated in vacuo. The residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Without any purification, **32** (17 mg, 98%) was obtained as a yellow solid. ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 7.19 (s, 1H), 7.17 (s, 1H), 6.8 (d, 2H), 6.61 (d, 1H), 6.59 (d, 1H), 5.3 (s, 4H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 148.0, 137.3, 137.1, 127.7, 123.7, 119.8, 116.45, 109.1. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −106.7. HRMS: Anal. calcd. for $[M+H]^+$ $C_{13}H_{10}F_2N_2$: 233.0885; found 233.0885.

*5.1.1.29. 9,9-Dimethyl-9H-fluorene-2,7-diamine (**33**).* Yield of 69 mg (98%) was obtained as a yellow solid. ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 7.21 (s, 1H), 7.19 (s, 1H), 6.6 (d, 2H), 6.47 (d, 1H), 6.45 (d, 1H), 4.9 (s, 4H), 1.3 (s, 6H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 153.5, 146.7, 128.4, 118.6, 112.6, 108.5, 45.6, 27.7. HRMS: Anal. calcd. for $[M+H]^+$ $C_{15}H_{16}N_2$: 225.1386; found 225.1383.

*5.1.1.30. 2,7-Diamino-9H-fluoren-9-one (**35**).* A mixture of 9H-fluorene-2,7-diamine (294 mg, 1.5 mmol) and Cs_2CO_3 (1.5 g, 4.5 mmol) in DMSO (7 mL) was stirred under an atmosphere of air. When TLC showed that no starting material remained, the solution was poured into water and a precipitate was formed. The product was

filtered, washed with water, and air-dried. Without any purification, **35** (239 mg, 76%) was obtained as a solid. 1H NMR (DMSO-d6, δ = 2.5 ppm, 400 MHz): 7.10 (s, 1H), 7.08 (s, 1H), 6.70 (d, 2H), 6.57 (d, 1H), 6.57 (d, 1H) 5.30 (s, 4H). 13C NMR (DMSO-d6, δ = 39.52 ppm, 100 MHz): 194.9, 148.2, 134.6, 133.3, 119.9, 118.6, 109.7. HRMS: Anal. calcd. for [M+H]+ $C_{13}H_{10}N_2O$: 211.0866; found 211.0867.

*5.1.1.31. (2S,2′S)-Di-tert-butyl 2,2′-(((9H-fluorene-2,7-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**36**).* A mixture of *N*-Boc-L-proline (323 mg, 1.5 mmol), EDCI (312 mg, 1.63 mmol), and 2,7-diaminofluorene (123 mg, 0.63 mmol) in CH_2Cl_2 (2 mL) was stirred at ambient temperature for 2 h. The resulting residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with 1.0 N aq HCl solution and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Without any purification, **36** was obtained as a solid (359 mg, 97%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.0 (d, 2H), 7.69 (d, 2H), 7.72 (d, 2H), 7.57-7.51 (m, 2H), 4.31–4.21 (m, 2H), 3.88 (s, 2H), 3.45–3.37 (m, 4H), 2.23 (m, 2H), 1.94–1.80 (m, 6H), 1.41 (app br s, 9H), 1.29 (app br s, 9H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 171.4, 153.2, 143.5, 137.6, 136.3, 119.5, 118.0, 116.1, 78.6, 78.5, 60.4, 46.6, 31.1, 28.2, 28.0, 24.0, 23.4. HRMS: Anal. calcd. for $[M+H]^+$ $C_{33}H_{42}N_4O_6$: 591.3177; found 591.3168.

*5.1.1.32. (2S,2′S)-di-tert-butyl 2,2′-(((9-oxo-9H-fluorene-2,7-diyl) bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**37**).* Yield 131 mg (91%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.24 (s, 2H), 7.91 (m, 2H), 7.91 (m, 2H), 7.62 (m, 2H), 4.26-4.16 (m, 2H), 3.46–3.40 (m, 2H), 3.37–3.31 (m, 2H), 2.24–2.16 (m, 2H), 1.94–1.76 (m, 6H), 1.40 (app br s, 9H), 1.27 (app br s, 9H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 192.9, 171.9, 171.4, 153.6, 153.1, 139.6, 138.8, 134.2, 125.0, 121.1, 115.0, 78.8, 78.6, 60.5, 60.1, 46.6, 46.1, 31.0, 30.2, 28.2, 28.0, 24.0, 23.4. HRMS: Anal. calcd. for $[M+H]^+$ $C_{33}H_{40}N_4O_7$: 605.2970; found 605.2980.

*5.1.1.33. (2S,2′S)-di-tert-butyl 2,2′-(((9,9-dimethyl-9H-fluorene-2,7-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**38**).* Yield 201 mg (97%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.10 (app br s, 2H), 7.84 (s, 1H), 7.79 (s, 1H), 7.66 (d, 2H), 7.51 (t, 2H), 4.31-4.28 (m, 1H), 4.24–4.21 (m, 1H), 3.44–3.40 (m, 2H), 3.37–3.31 (m, 2H), 2.21–2.16 (m, 2H), 1.95–1.80 (m, 6H), 1.40 (app br s, 9H), 1.34 (s, 6H), 1.28 (app br s, 9H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 174.3, 173.9, 171.4, 171.0, 153.6, 153.2, 138.1, 138.0, 133.7, 133.6, 119.7, 118.4, 118.2, 113.8, 113.6, 78.6, 78.5, 60.4, 60.0, 46.6, 46.1, 42.2, 42.1, 31.0, 30.3, 28.2, 28.1, 27.9, 27.1, 24.0, 23.4. HRMS: Anal. calcd. for $[M+H]^+$ $C_{35}H_{42}N_4O_6$: 619.3490; found 619.3496.

*5.1.1.34. (2S,2′S)-Di-tert-butyl 2,2′-(((9,9-difluoro-9H-fluorene-2,7-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-1-carboxylate) (**39**).* Yield 28 mg (95%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.29 (app br s, 2H), 8.06 (s, 1H), 8.02 (s, 1H), 7.69-7.66 (m, 4H), 4.30–4.25 (m, 1H), 4.21–4.18 (m, 1H), 3.45–3.41 (m, 2H), 3.37–3.34 (m, 2H), 2.23–2.16 (m, 2H), 1.92–1.80 (m, 6H), 1.40 (app br s, 9H), 1.27 (app br s, 9H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 174.3, 173.9, 171.9, 171.5, 153.6, 153.1, 139.4, 133.56, 133.49, 122.8, 122.7, 121.2, 114.5, 114.4, 78.8, 78.6, 60.5, 59.3, 58.6, 46.6, 46.2, 46.1, 31.0, 30.3, 28.2, 28.0, 27.9, 27.7, 24.0, 23.4. ^{19}F NMR (DMSO-d_6, 377 MHz): δ −108.9, -109.0. HRMS: Anal. calcd. for $[M+H]^+$ $C_{33}H_{40}F_2N_4O_6$: 627.2989; found 627.2997.

*5.1.1.35. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((9H-fluorene-2,7-diyl) bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**40**).* A mixture of biphenyl **36** (300 mg, 0.51 mmol) in CF_3CO_2H (2 mL) and CH_2Cl_2 (2 mL) was stirred at room temperature for 5 h. The volatile component was removed in vacuo. EDCI (253 mg, 1.3 mmol) and **9** (255 mg, 1.3 mmol) were added in batches over 4 min to a solution of *i*-Pr_2NEt (441 μL, 2.5 mmol) in CH_2Cl_2 (2 mL), and the reaction mixture was stirred at room temperature for 75 min. The residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. A silica gel mesh was prepared from the residue and submitted to flash chromatography (silica gel: EtOAc/hexane as eluent) to provide **40** as a white solid (198 mg, 50%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.92 (s, 2H), 7.91 (s, 2H), 7.75-7.69 (m, 4H), 7.56 (d, 2H), 7.44–7.13 (m, 10H), 5.52 (d, 2H), 4.43 (m, 2H), 3.88–3.83 (m, 2H), 3.55 (s, 6H), 3.21 (m, 2H), 2.04–1.78 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.2, 168.5, 156.2, 143.6, 137.5, 137.2, 136.4, 128.7, 128.1, 127.9, 119.6, 118.1, 116.2, 60.8, 56.8, 51.7, 47.0, 36.7, 29.4, 24.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{43}H_{44}N_6O_8$: 773.3293; found 773.3296.

*5.1.1.36. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((9-oxo-9H-fluorene-2,7-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl))bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**41**).* Yield 12 mg (66%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.10 (s, 2H), 7.91 (s, 2H), 7.76 (t, 4H), 7.64 (d, 2H), 7.43-7.10 (m, 10H), 5.51 (d, 2H), 4.39 (m, 2H), 3.85 (m, 2H), 3.55 (s, 6H), 3.20 (m, 2H), 2.05–1.79 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 192.8, 170.6, 168.5, 156.2, 139.5, 138.8, 137.1, 134.2, 128.6, 128.1, 127.9, 125.0, 121.1, 115.0, 60.8, 56.7, 51.7, 47.0, 29.3, 24.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{43}H_{42}N_6O_9$: 787.3086; found 787.3089.

*5.1.1.37. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((9,9-dimethyl-9H-fluorene-2,7-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl)) bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**42**).* Yield 135 mg (53%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 9.96 (s, 2H), 7.86 (s, 2H), 7.74 (d, 2H), 7.69 (d, 2H), 7.51 (dd, 2H), 7.43-7.10 (m, 10H), 5.52 (d, 2H), 4.43 (m, 2H), 3.83 (m, 2H), 3.55 (s, 6H), 3.20 (m, 2H), 2.05–1.78 (m, 8H), 1.41 (s, 6H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.1, 168.3, 156.1, 153.7, 138.0, 137.2, 133.7, 128.6, 128.1, 127.8, 119.8, 118.3, 113.7, 60.7, 56.7, 51.6, 47.0, 46.5, 29.3, 27.2, 24.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{45}H_{48}N_6O_8$: 801.3606; found 801.3611.

*5.1.1.38. Dimethyl ((1R,1′R)-((2S,2′S)-2,2′-(((9,9-difluoro-9H-fluorene-2,7-diyl)bis(azanediyl))bis(carbonyl))bis(pyrrolidine-2,1-diyl)) bis(2-oxo-1-phenylethane-2,1-diyl))dicarbamate (**43**).* Yield 18 mg (52%). ^{1}H NMR (DMSO-d_6, δ = 2.5 ppm, 400 MHz): 10.14 (s, 2H), 8.05 (s, 2H), 7.77 (d, 2H), 7.71 (s, 4H), 7.43-7.10 (m, 10H), 5.51 (d, 2H), 4.39 (m, 2H), 3.85 (m, 2H), 3.55 (s, 6H), 3.21 (m, 2H), 2.06–1.79 (m, 8H). ^{13}C NMR (DMSO-d_6, δ = 39.52 ppm, 100 MHz): 170.6, 168.5, 156.2, 139.3, 137.3, 137.0, 128.6, 128.4, 128.1, 127.9, 127.6, 122.8, 121.2, 114.5, 60.8, 56.7, 51.6, 47.0, 29.3, 24.3. HRMS: Anal. calcd. for $[M+H]^+$ $C_{43}H_{42}F_2N_6O_8$: 809.3105; found 809.3109.

5.2. Biological studies

5.2.1. Measurement of the anti-HCV activities of compounds using HCVcc

- **Cell line and cell culture**

Huh 7.5.1 cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with antibiotics (100 U/mL penicillin, 10 μg/mL streptomycin) and 10% heat-inactivated fetal bovine serum (ΔFBS; Thermo) at 37 °C in a humidified 6.0% CO_2 incubator.

- **Virus production**

In vitro transcription and RNA electroporation were performed as described in the previous report [122]. *In vitro* transcribed RNA of JFH5a-Rluc-ad34, which is a derivative of JFH1 with adaptive mutations in the E2 and p7 regions [82], was transfected into Huh7.5.1 cells by electroporation. JFH5a-Rluc-ad34 virus contains a reporter *Renilla* luciferase (Rluc) for convenient assay of virus proliferation [82]. Cell culture media containing HCV were collected 3~5 days after electroporation and then filtered through a 0.45 μM pore size filter.

- **Antiviral activity test with infectious HCV particles**

Huh 7.5.1 cells were inoculated with a JFH5a-Rluc-ad34 virus stock and cultivated for 3 h. At 3 h after the virus inoculation, HCV-infected Huh7.5.1 cells were cultivated with media containing serially diluted compounds. At 3 days after chemical treatment, the cells were harvested and luciferase activities in the cells were measured using a *Renilla* luciferase assay system (Promega) according to the manufacturer's direction. Finally the luciferase activities were normalized to those obtained from mock-treated cells.

5.2.2. Measurement of anti-HCV activities of compounds using HCV replicon

- **Cell line and cell culture**

Huh 7.5.1 cells containing a bicistronic HCV replicon NK/R2AZ (genotype 1b) were used to test the anti-HCV activities of the compounds (Fig. 5). The first and second open reading frames (ORFs) of the replicon contain the *Renilla* luciferase gene fused with the neomycin phosphotransferase gene and the HCV nonstructural genes NS3-5, respectively. The replicon-containing cells were cultivated under the same conditions as described above with an additional antibiotic G418 (0.5 mg/mL, Calbiochem).

- **NK/R2AN**
- **Antiviral activity assay test with HCV replicon**

Huh 7.5.1 cells containing HCV replicon (NK/R2AN) were plated on a 12-well plate (5×10^4 cells per well). At 16 h after cultivation, NK/R2AN-containing cells were incubated with media containing serially diluted compounds for 3 days. After the chemical treatment, the cells were harvested and luciferase activities were measured. Luciferase activities were measured using the *Renilla* luciferase assay system (Promega) according to the manufacturer's direction, and then normalized to those obtained from mock-treated cells.

5.2.3. Observation of the effects of co-treatment of two compounds

Huh 7.5.1 cells containing HCV replicon (NK/R2AN) were plated on a 12-well plate (5×10^4 cells per well). At 16 h after cultivation, NK/R2AN cells were treated with serially diluted compounds in the presence of 123 nM of sofosbuvir (EC_{30}). At 3 days after treatment, luciferase activities were measured using the *Renilla* luciferase assay system (Promega) according to the manufacturer's direction.

5.2.4. Observation of the effects of compounds on drug-resistant HCV mutants

We selected HCV mutants showing resistance against BMK-20113 by continuous cultivation of Huh 7.5.1 cells containing HCV replicon NK/R2AN in the presence of BMK-20113. We found that the majority of mutants had mutations at the N-terminal region of NS5A protein (L31V and/or Y93H in NS5A) similarly to the daclatasvir resistant mutants described previously [117,123]. We used NK/R2AN derivatives containing single mutations (L31V or Y93H in NS5A) to investigate the drug resistance of lead compounds. Briefly, Huh 7.5.1 cells were electroporated with RNAs containing resistant mutations and cultivated on 12-well plates. At 4 h after transfection, media were replaced with DMEM containing serially diluted compounds and further cultivated. At 3 days after compound treatment, the cells were harvested and luciferase activities were measured. Luciferase activities were normalized to those obtained from mock-treated cells.

5.2.5. hERG ligand binding assay [99]

The hERG ligand binding assay was performed by using the hERG Fluorescence Polarization Assay Kit (Cat No. PV5365), containing predictor™ hERG tracer red, predictor™ hERG membrane, and predictor™ hERG FP assay buffer. Test compounds (10 μM) and positive control (Astemizole) were added to the 384-well plate (5 μL each; low-volume polystyrene plate, corning #3677). Then, 10 μL of membrane and 5 μL of tracer were added to each plate by using a multi-pipette (Multi 16 channel pipette type 5–50 μL, Thermo). The plate was then covered with aluminum foil and was kept for 2–3 h at room temperature. Lastly, the fluorescence polarization of the hERG red tracer was measured using a microplate reader (molecular devices, SpectraMax M5e, excitation (540 nm) emission (585 nm) filters).

5.2.6. Plasma stability measurement

Rat plasma (297 μL) was pre-incubated for 5 min at 37 °C in a water bath. The plasma and 100 μM test compounds (3 μL) were mixed using a vortex, and incubated for 37 °C in a water bath. Then the mixture was sampled multiple times (0, 15, 30, 60, and 120 min). A mixture of crude sample (50 μL) and acetonitrile (100 μL) was then treated by centrifugation at 10,000 rpm for 10 min at 25 °C. Finally, supernatant was isolated and analyzed using an LC-MS/MS.

5.2.7. Method to determine stability in rat and human liver microsomes [103]

The metabolic stability of the compounds was evaluated in pooled rat (BD Gentest® Cat No. 452501) or human livers (BD GentestCat. 452161) microsomal fractions with β-NADPH over 30 m using 1 μM of compounds. The compounds in the supernatants were analyzed by LC-MS/MS.

5.2.8. Method of CYP_{450} inhibition

The interaction of human cytochrome CYP450 with compounds was studied on a cytochrome panel consisting of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (P450-glo, promega kit) according

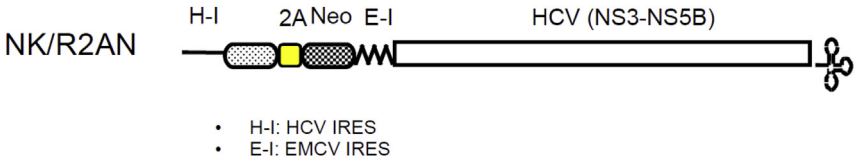


Fig. 5. The structure of replicon NKR2AN.

to the manufacturer's protocols. The inhibition effects were measured at 100, 50, 10, 5, 1, 0.1, and 0.01 μM of compounds using a luminometer (GENios, TECAN).

5.2.9. PK study

Pharmacokinetic studies were conducted in a parallel format with 2 male and 1 female Sprague–Dawley rats (supplied by Sippr-BK Lab Animal Ltd., Shanghai, China) for compounds per each dose routes. Intravenous (IV) dose was administrated via tail vein as a bolus injection by 5 mg/kg as a solution (5% DMSO, 10% solutol, 85% (2-hydroxypropyl)-β-cyclodextrin (HPBCD)). Blood samples were collected into polypropylene tubes containing K2-EDTA as anticoagulant at different time points and stored on wet ice. The samples were centrifuged at 8000 rpm for 6 min and the plasma samples were analyzed with LC/MS/MS. Non-compartmental pharmacokinetic analysis was performed with drug and statistics (DAS) 2.1.1 pharmacokinetic program.

5.2.10. Ames test [120]

The Ames test of compound was carried out with the Maron's method [120] using histidine-dependent strains of *S. typhimurium* (TA98, and TA100). Histidine requiring bacteria of each strain was treated with inhibitor compound **43** at 200 μg/plate and with positive (2-nitrofluorene, sodium azide, and benzo[a]pyrene) and negative controls. The number of colonies was counted three times for each medium to provide acceptable data for statistical analysis.

Acknowledgment

We thank Prof. Nakcheol Jung of Korea Basic Science Institute (KBSI) for the chiral resolution of racemic compounds through the use of SFC. This study was supported by the Bio R&D Program (No. 2012M3A9A9054974) through the National Research Foundation funded by the MEST, Republic of Korea.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.06.033.

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