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Structure-activity relationship (SAR) studies on the mutagenic properties of 2,7-diaminofluorene and 2,7-diaminocarbazole derivatives

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ABSTRACT

We discovered that 2,7-diaminofluorene or 2,7-diaminocarbazole moiety can be employed as a core structure of highly effective NS5A inhibitors that are connected through amide bonds to proline-valine-carbamate motifs. Amide bonds can be easily cleaved via various metabolic pathways upon administration into the body, and metabolites containing 2,7-diaminofluorene and 2,7-diaminocarbazole core structures have been known to be strong mutagens. To avoid the mutagenesis issue of these core structures, we examined various functional groups at the C9 or N9 position of 2,7-diaminofluorene or 2,7-diaminocarbazole, respectively, through the Ames test in TA98 and TA100 mutants of *Salmonella typhimurium* LT-2. We discovered that, through proper alkyl substitution at the C9 or N9 position, 2,7-diaminofluorene and 2,7-diaminocarbazole moieties can be successfully employed in drug discovery without necessarily causing mutagenicity problems.

9*H*-Fluorene (or fluorene) is a polycyclic compound, which has a violet fluorescence. As indicated in its name, fluorene has been used for the preparation of various organic dyes.[1] 9*H*-Carbazole (or carbazole) has similar structure as fluorene except for the nitrogen atom at the 9 position. Like fluorene, carbazole has also been utilized for many applications including dyes,[2] drugs,[3,4] and ligands.[5] Among various derivatives, 2,7-diaminofluorene and 2,7-diaminocarbazole have received most attention because they can be utilized as an excellent scaffold for symmetric or pseudo-symmetric molecules. Chemical applications of 2,7-diaminofluorene and 2,7-diaminocarbazole derivatives are various including chemical adsorbent,[6] nanoparticle for photodynamic therapy,[7] ATP sensing probe,[8] and covalent organic frameworks (COFs) for CO_2 capture,[9] etc. Among various usage of 2,7-diaminofluorene or 2,7-diaminocarbazole derivatives, we focused on their potential as key scaffolds for the construction of physiologically active molecules.

Daclatasvir (Fig. 1), which had been reported by Bristol-Myers Squibb in 2010, was approved in 2015 by the US Food and Drug Administration as an effective hepatitis C virus (HCV) non-structural protein 5A (NS5A) inhibitor.[10] Despite its extremely high efficacy, daclatasvir loses its inhibitory activity over mutated NS5A proteins.[11] To circumvent this problem, many pharmaceutical companies and laboratories worldwide have searched for and reported a number of new NS5A inhibitors based on the structure of daclatasvir.[12–20]

We have reported a new class of HCV NS5A inhibitors equipped with benzidine[21] and biaryl sulfate[22] as core structures, maintaining the proline-valine-carbamate motif of daclatasvir. In our continued research, we were curious if 2,7-diaminofluorene and 2,7-diaminocarbazole could be utilized as an important bridging moiety of a drug candidates, particularly in relation with HCV NS5A inhibitors. Compounds embedding these structures showed extremely potent inhibitory activities toward a variety of HCV genotype[23] (Fig. 2).

Although inhibitors can readily be constructed from 2,7-diaminofluorene or 2,7-diaminocarbazole through formation of a series of amide bonds and have extremely potent inhibitory activities, it is susceptible to cleavage by proteolysis once administered into the body, and the resulting metabolites may be potential mutagens.[24–26] The mutagenicity of 2,7-diaminofluorene and 2,7-diaminocarbazole core structures must be inspected for use in the discovery of new NS5A inhibitors. Therefore, we decided to explore the structure–activity-relationships (SAR) on the mutagenic properties of the 9-substituted 2,7-diaminofluorene and 2,7-diaminocarbazole. In this study, through the Ames test,[27] we revealed our systematic approach to circumvent the mutagenicity problems encountered in the use of 2,7-diaminofluorene and 2,7-diaminocarbazole derivatives, which warranted them as suitable

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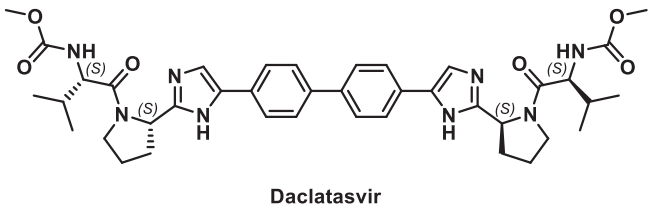


Fig. 1. Chemical structure of daclatasvir.

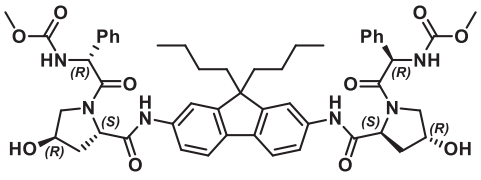


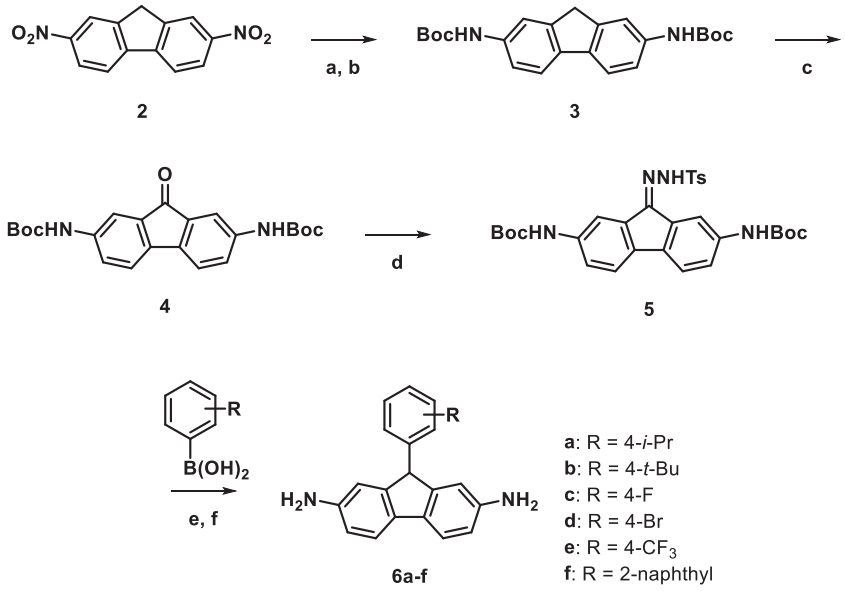
Fig. 2. Chemical structure and EC_{50} values of compound **1.**

new core structures of NS5A inhibitors.

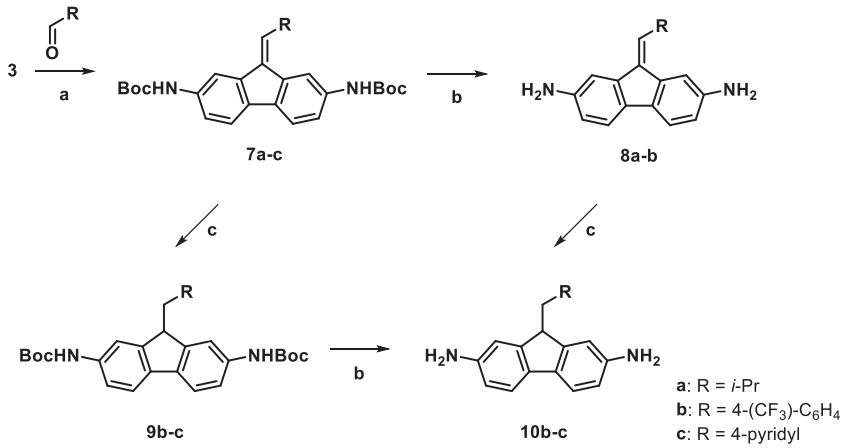
All diamine compounds used in the Ames test[27] were synthesized via the following synthetic schemes (Schemes 1–8) (See details in the Supplementary Material).

We describe the synthesis of 9-aryl substituted fluorene compounds in Scheme 1. Compound **2** was reduced to 2,7-diaminofluorene with the aid of iron oxide nanoparticles and hydrazine as reported in our previous report[28] and Boc protection of the resulting diamine afforded compound **3**. After oxidation at the 9 position, the resulting ketone **4** was converted to hydrazone **5** from treatment with *p*-$TsNHNH_2$. Metal-free reductive coupling[29] of **5** with the corresponding arylboronic acid was performed followed by Boc deprotection to give compounds **6a–f**. Synthesis of 2,7-diaminofluorene derivatives containing 9-alkyl or alkylidene substituents is described in Scheme 2. Compounds **7a–c** were synthesized from compound **3** via Knoevenagel condensation with various aldehydes. Compounds **7a–c** were converted to diamine through Boc deprotection (**8a–b**) or dehydrogenation followed by Boc deprotection (**10b–c**). Synthesis of 2,7-diaminofluorene derivatives containing 9,9-dialkyl substituents is described in Scheme 3. We introduced two alkyl groups through S_N2 reaction of compound **2** to obtain compounds **11a–d**. Reduction of the nitro groups of **11a–d** gave compounds **12a–d**. Scheme 4 describes the preparation of 9-alkyl substituted carbazole derivatives. In the synthesis of 2,7-diaminocarbazole derivatives, 2,7-dibromocarbazole (compound **13**) was used as starting material. Compound **13** was converted to *N*-alkylated carbazole (**14a–d**) from S_N2 reaction with proper alkyl bromides. Buchwald-Hartwig amination of aromatic bromide (**14a–d**) with the use of diphenylmethanimine followed by imine hydrolysis afforded **16a–d**. Transformation from aryl bromide to diamine via Buchwald-Hartwig amination and imine hydrolysis was repetitive in following Schemes (vide infra). Synthesis of 9-(10-morpholinodecyl)-9*H*-carbazole-2,7-diamine (**20**) is described in Scheme 5. Compound **17** was prepared via S_N2 reaction of compound **13** with 1,10-dibromodecane. Additional S_N2 reaction between compound **17** and morpholine gave compound **18** albeit in a moderate yield. Following the synthetic procedure mentioned in Scheme 4, we converted compound **18** to compound **20**. Scheme 6 describes the synthetic procedure for compounds **23a** and **23b**, which were prepared from *para*-alkoxy-substituted benzyl chlorides. S_N2 reaction between compound **13** and *para*-substituted benzyl chloride (**21a** and **21b**) afforded **22a** and **22b**, respectively. These compounds were converted to diamine (**23a** and **23b**) through above-mentioned methods in Scheme 4. Scheme 7 describes the preparation of 2,7-diaminocarbazole derivatives (**27a** and **27b**) containing *meta*-substituted benzyl group at the 9 position. *N*-Alkylation of compound **13** with 1-(bromomethyl)-3-methoxybenzene afforded compound **24** and demethylation of the resulting **24** furnished compound **25**. After proper *O*-alkylation (**26a** and **26b**), compounds **27a** and **27b** were synthesized via the same procedure described in Scheme 4. Compounds **30a–e** have similar structure as dialkylated fluorene derivatives (**12a–d**). Scheme 8 describes the preparation of compounds **30a–e**. These compounds (**30a–e**) were also prepared via the same way as described in Scheme 4: i) alkylation, ii) Buchwald-Hartwig amination, and iii) imine hydrolysis.

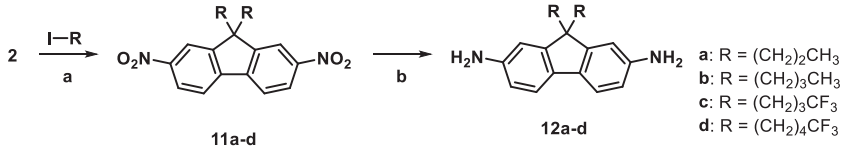
The mutagenicity of several 2,7-diaminofluorene derivatives and 2,7-diaminocarbazole derivatives was examined using the Ames test (see details in the Supplementary Material) in strains TA98 and TA100, both with and without S9 mix treatment. All compounds were tested at six different concentrations from 4 to 5000 μM. The results are presented in Tables 1 and 2, respectively, and the detailed mutagenic property of each examined compound is collected in the Supplementary Materials.



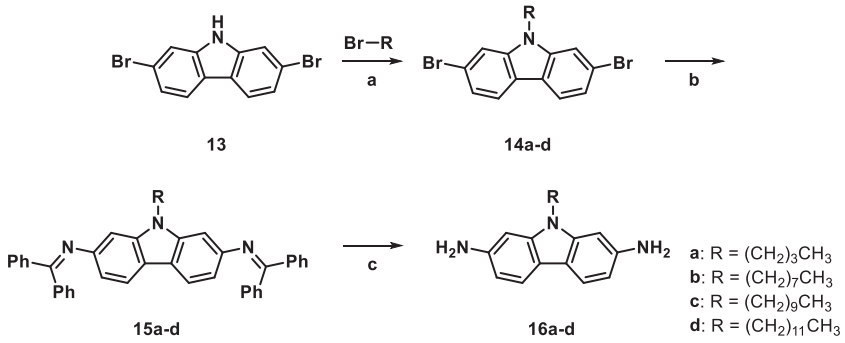
Scheme 1. Reagents and conditions: (a) Fe_3O_4, hydrazine monohydrate, EtOH, 75 °C, 2 h, 96%; (b) Boc_2O, NaOH, 1,4-dioxane, H_2O, 23 °C, 24 h, 84%; (c) Cs_2CO_3, DMSO, 23 °C, 18 h, 63%; (d) *p*-$TsNHNH_2$, MeOH, 60 °C, 4 h, 95%; (e) K_2CO_3, 1,4-dioxane, reflux, 2 h, 58–78%; (f) TFA, DCM, 23 °C, 2 h, 35–62%.



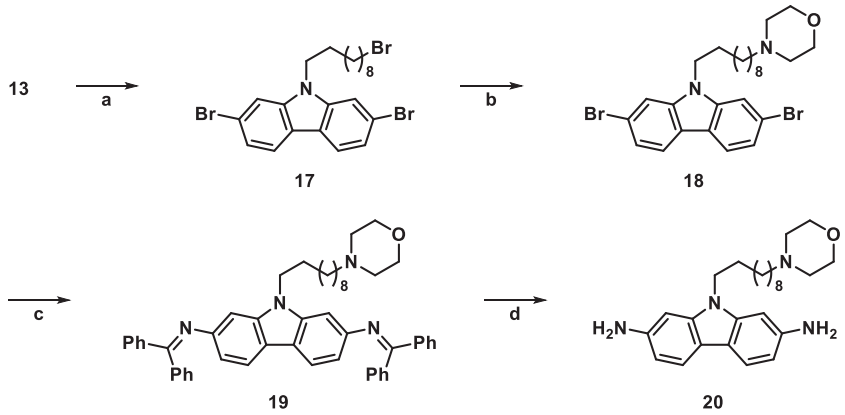
Scheme 2. Reagents and conditions: (a) *t*-BuOK, xylene, reflux, 1 h or KOH, DME, reflux, 4 h, 54–66%; (b) TFA, DCM, 23 °C, 2 h, 41–49%; (c) Pd/C, H_2, MeOH, 23 °C, 12 h, 51–58%.



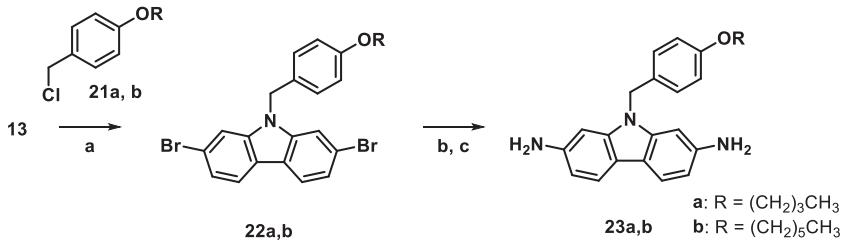
Scheme 3. Reagents and conditions: (a) Cs_2CO_3, DMF, 0 °C to 23 °C, 18 h, 40–64%; (b) Fe_3O_4, hydrazine monohydrate, EtOH, 75 °C, 2 h, 17–39%.



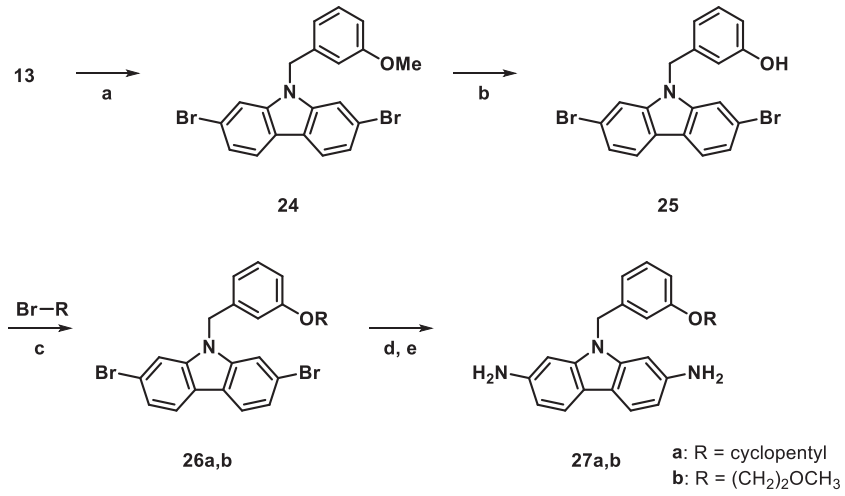
Scheme 4. Reagents and conditions: (a) NaH, DMF, 23 °C, 12 h, 69–98%; (b) $Pd_2(dba)_3 \cdot CHCl_3$, *t*-BuXPhos, *t*-BuONa, diphenylmethanimine, toluene, 100 °C, 16 h, 35–77%; (c) 4.0 M HCl/MeOH, 23 °C, 1 h, 86–93%.



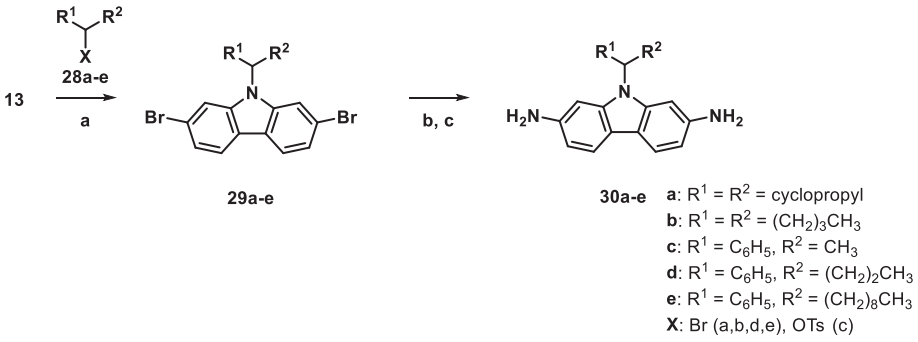
Scheme 5. Reagents and conditions: (a) NaH, 1,10-dibromodecane, DMF, 0 °C to 23 °C, 16 h, 99%; (b) K_2CO_3, morpholine, MeCN, reflux, 10 h, 47%; (c) $Pd_2(dba)_3 \cdot CHCl_3$, *t*-BuXPhos, *t*-BuONa, diphenylmethanimine, toluene, 100 °C, 16 h, 73%; (d) 4.0 M HCl/MeOH, 23 °C, 4 h, 69%.



Scheme 6. Reagents and conditions: (a) NaH, DMF, 0 °C to 23 °C, 12 h, 99%; (b) $Pd_2(dba)_3 \cdot CHCl_3$, *t*-BuXPhos, *t*-BuONa, diphenylmethanimine, toluene, 100 °C, 16 h, 69–79%; (c) 4.0 M HCl/MeOH, 23 °C, 1 h, 86–91%.



Scheme 7. Reagents and conditions: (a) NaH, 1-(bromomethyl)-3-methoxybenzene, DMF, 0 °C to 23 °C, 12 h, 99%; (b) NaI, TMSCl, MeCN, reflux, 3 h, 56%; (c) K_2CO_3, DMF, 90 °C, 5 h, 37–92%; (d) $Pd_2(dba)_3 \cdot CHCl_3$, *t*-BuXPhos, *t*-BuONa, diphenylmethanimine, toluene, 100 °C, 16 h, 37–72%; (e) 4.0 M HCl/MeOH, 23 °C, 2 h, 71–82%.



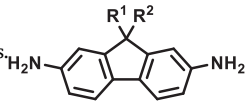
Scheme 8. Reagents and conditions: (a) NaH, DMF, 0 °C to 23 °C, 12 h, 38–91%; (b) $Pd_2(dba)_3 \cdot CHCl_3$, *t*-BuXPhos, *t*-BuONa, diphenylmethanimine, toluene, 100 °C, 16 h, 36–95%; (c) 4.0 M HCl/MeOH, 23 °C, 1 h, 48–69%.

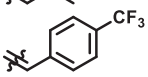
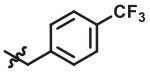
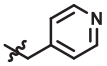
Compounds **6a**–**f**, equipped with various aromatic rings at the C9 position of fluorene, were found to be non-mutagenic in TA98 and TA100 strains in the absence of S9 mix. However, in the presence of S9 mix, these compounds turned to be mutagenic in TA98 strain. Compounds containing a double bond at the C9 position (**8a** and **8b**) were found to be mutagenic regardless of treatment with S9 mix. Compounds with a reduced double bond (**10b** and **10c**) were mutagenic in TA 98 strain only in the presence of S9 mix. However, compounds possessing dialkyl substituents at the C9 position of fluorene (**12a**–**d**) exhibited varying degrees of mutagenicity depending on the length of the alkyl groups. With dipropyl substituents, compound **12a** showed mutagenic activity in TA98 strain treated with S9 mix. In the cases of dibutyl, bis (trifluoromethylpropyl), and bis(trifluoromethylbutyl) substituted compounds **12b**, **12c**, and **12d**, respectively, they were found to be slightly mutagenic in TA98 strain treated with S9 mix only when the concentration was higher than 5 mM. Therefore, it can be concluded that the longer the length of alkyl chain is, the lower the probability of mutation.

In the case of carbazole derivatives, compounds equipped with monoalkyl substituents at the N9 position of carbazole (**16a**–**d** and **20**) showed a similar mutagenic pattern as in the fluorene derivatives; the mutagenicity was dependent on the length and bulkiness of the alkyl substituent. When the alkyl chains were longer than *n*-decyl, the corresponding carbazole derivatives were non-mutagenic. *N*-Benzyl derivatives with an alkoxy substitution, such as compounds **23a**, **23b**, **27a**, and **27b**, were all found to be mutagenic in TA98 strain treated with S9 mix, regardless of the position and type of the alkoxy groups attached to the benzene ring. Bis(cyclopropyl)methyl-substituted derivative (**30a**)

Table 1

Ames test results of 2,7-diaminofluorene derivatives.

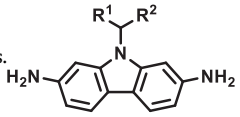


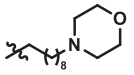
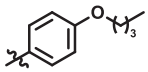
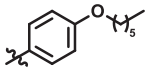
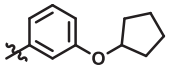
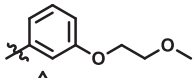
Entry	R^1	R^2	Ames result[a,b]
6a	H	4-(*i*-Pr)-C_6H_4	−,+,−,−
6b	H	4-(*t*-Bu)-C_6H_4	−,+,−,−
6c	H	4-F-C_6H_4	−,+,−,−
6d	H	4-Br-C_6H_4	−,+,−,-
6e	H	4-CF_3-C_6H_4	−,+,−,−
6f	H	2-naphthyl	−,+,−,−
8a			+,+,+,+
8b			+,+,−,−
10b	H		+,+,−,−
10c	H		−,+,−,+
12a	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	−,+,−,−
12b	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	−,+,−,−
12c	$(CH_2)_3CF_3$	$(CH_2)_3CF_3$	−,+,−,−
12d	$(CH_2)_4CF_3$	$(CH_2)_4CF_3$	−,−,−,−

[a] Ames results means the test result of TA98 −S9, TA98 + S9, TA100 −S9, and TA100 + S9, respectively.
[b] +: positive, −: negative.

Table 2

Ames test results of 2,7-diaminocarbazole derivatives.



Entry	R^1	R^2	Ames result[a,b]
16a	H	$(CH_2)_2CH_3$	+,+,+,+
16b	H	$(CH_2)_6CH_3$	−,+,−,−
16c	H	$(CH_2)_8CH_3$	−,+,−,−
16d	H	$(CH_2)_{10}CH_3$	−,−,−,−
20	H		−,−,−,−
23a	H		−,+,−,−
23b	H		−,+,−,−
27a	H		−,+,−,−
27b	H		−,+,−,−
30a			−,+,−,+
30b	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	−,−,−,−
30c	C_6H_5	CH_3	−,+,−,+
30d	C_6H_5	$(CH_2)_2CH_3$	−,+,−,−
30e	C_6H_5	$(CH_2)_8CH_3$	−,−,−,−

[a] Ames results means the test result of TA98 –S9, TA98 + S9, TA100 –S9, and TA100 + S9, respectively.
[b] +: positive, − negative.

also showed mutagenic activity in both TA98 and TA100 strains treated with S9 mix. A compound with a longer substitution such as a 5-nonyl group (**30b**) showed no mutagenicity in TA98 and TA100 strains treated with and without S9 mix. In compounds with (1-phenyl)alkyl substitution (**30c–e**), the alkyl chain length played a critical role in determining their mutagenicity, regardless of the phenyl group.

According to the precedent research, 2-aminofluorene (2-AF) or *N*-acetyl-2-aminofluorene (2-AAF) is transformed to *N*-hydroxy-2-AF or *N*-hydroxy-2-AAF via CYP_{450} monooxygenase oxidation.[25] *N*-Hydroxy-2-AF or *N*-hydroxy-2-AAF can be metabolized to electrophilic species, such as *N*-SO_4-2-AF, *N*-acetoxyl-2-AF, and *N*-SO_4-2-AAF.[25] These electrophiles can form DNA adducts through a reaction with the guanine base, which we considered as a major mutation pathway in the aminofluorene case.

Compounds **6a–f**, equipped with various aromatic ring substitutions at the C9 position of 2,7-diaminofluorene, were found to be mutagenic in TA98 strain treated with S9 mix. The fact that these compounds did not act as mutagens in TA98 without S9 treatment indicated that the formation of metabolites through DNA adduct can be a cause of mutation. In addition, the negative result for mutation in TA100 strain treated with S9 mixture indicated that the DNA adduct caused a frame-shift mutation rather than a base-pair substitution.[30] In the case of fluorene derivatives substituted with dialkyl groups at the C9 position (compounds **12a–d**), shorter alkyl chain substituents tended to cause mutagenicity. From the results of previous[25] and current study, we hypothesized that the mutation of 2,7-diaminofluorene can be prevented by introducing sterically bulky dialkyl groups at the C9 position, presumably because they prevent the guanine base from approaching electrophilic metabolites.

In the case of 2,7-diaminofluorene, introduction of dibutyl-, bis(trifluoromethylpropyl)-, or bis(trifluoromethylbutyl)- chains, as in compounds **12b**, **12c**, and **12d**, respectively, diminished mutation propensity. However, the carbazole moiety needed to be substituted with a longer *N*-alkyl chain than an *n*-decyl group (as in **16c**, **16d** and **20**) to avoid mutagenicity issues. These results agree well with our hypothesis because carbazole needed to be substituted with a longer chain monoalkyl group than fluorene with longer-than-propyl dialkyl groups at the C9 position. Therefore, to block the formation of DNA adduct, carbazole has to be substituted with a longer alkyl chain than that of fluorene. In the case of carbazoles substituted with a symmetric secondary alkyl group (**30a** and **30b**), which mimics the dialkyl group of fluorene derivatives, compounds substituted with a long branched alkyl chain, such as (1-butyl)pentyl substitution (mimicking 9,9-dipentyl groups at the fluorene) (**30b**), may avoid mutagenic issues, but not those with a short chain (**30a**). The importance of the alkyl chain length in determining the mutagenicity of compounds could be explained by the (1-phenyl)alkyl substitution cases (**30c–e**). Regardless of the phenyl group, mutagenicity pattern was consistent with the length of the alkyl chain. With methyl (**30c**) or propyl (**30d**) substitution, the compounds were found to be mutagenic, but compounds with longer substituent (e.g. nonyl group, **30e**) were non-mutagenic. Our hypothesis was also confirmed by the results of *N*-benzyl derivatives with an alkoxy substitution (**23a, 23b**, **27a** and **27b**). In these cases, any changes in the alkoxy group did not affect the Ames results of the four compounds because those variations did not affect the bulkiness of the compounds.

In this study, using the Ames test, we investigated the important factors affecting the mutagenicity of the aniline derivatives 2,7-diaminofluorene and 2,7-diaminocarbazole, which are often employed in drug discovery. Ames test results showed that mutagenicity problems with derivatives of 2,7-diaminofluorene and 2,7-diaminocarbazole can be solved by equipping these derivatives with a long alkyl chain at a proper location. The results imply that 2,7-diaminofluorene and 2,7-diaminocarbazole can successfully be employed in drug discovery as long as they are equipped with proper substituents. In addition, several of the examined compounds showed extremely high antiviral activities,[23] proving that fluorene and carbazole can be used as effective core structures of HCV NS5A inhibitors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127662.

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