



Synthesis, activity, and pharmacokinetic properties of a series of conformationally-restricted thiourea analogs as novel hepatitis C virus inhibitors

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ABSTRACT

A series of novel conformationally-restricted thiourea analogs were designed, synthesized, and evaluated for their anti-HCV activity. Herein we report the synthesis, structure–activity relationships (SARs), and pharmacokinetic properties of this new class of thiourea compounds that showed potent inhibitory activities against HCV in the cell-based subgenomic HCV replicon assay. Among compounds tested, the fluorene compound **4b** was found to possess the most potent activity ($EC_{50} = 0.3 \mu\text{M}$), lower cytotoxicity ($CC_{50} > 50 \mu\text{M}$), and significantly better pharmacokinetic properties compared to its corresponding fluorenone compound **4c**.

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1. Introduction

Hepatitis C is a liver disease caused by the hepatitis C virus (HCV) that was first discovered in 1989.¹ HCV, a single-stranded positive RNA virus in the family Flaviviridae, has six genotypes and several subtypes with varying geographic distribution.² An estimated more than 170 million people worldwide are chronically infected with this virus.³ HCV infection is associated with severe liver disease, including cirrhosis, liver cancer, and liver failure, a leading indication for liver transplantation.⁴ Currently, no vaccine is available to prevent HCV infection and treatments for chronic hepatitis C are limited.⁵ Interferon- α (IFN- α), alone or in combination with ribavirin, is the currently approved treatment for chronic hepatitis C.⁶ Unfortunately, response rates to this treatment are significantly lower in patients infected with HCV genotype 1, the most common type in the United States and Europe.⁷ In addition, this treatment is frequently associated with significant side effects that can lead to discontinuation of therapy in approximately 20% of patients.⁷ Thus there is an urgent need for the development of more efficacious anti-HCV agents with fewer limitations and less side effects.

According to the literature review, many emerging antiviral agents are targeted against specific HCV enzymes, such as NS3 serine protease and NS5B RNA-dependent RNA polymerase.⁸ The

development of the HCV replicon provides a cell-based assay system for the evaluation of antiviral agents targeted to viral and host proteins involved in HCV replication.⁹ Moreover, the use of combinations of anti-HCV agents with different mechanisms of action seems to be an important strategy to prevent viral resistance.⁸

Recently, the arylthiourea compound **1**¹⁰ (Fig. 1) has been reported to exert strong anti-HCV activity ($EC_{50} = 0.49 \mu\text{M}$) in a cell-based subgenomic HCV replicon assay.¹¹ By maintaining the arylthiourea moiety of **1**, the initial structure–activity relationships of this class of compounds was explored. Though the mechanism of action of this class of compounds is not yet fully understood,¹² some arylthiourea derivatives were found to possess potent activity with nanomolar range in a cell-based HCV replicon assay. Unfortunately, these compounds showed significant cytotoxicity and poor pharmacokinetic properties which have high clearance, low drug exposure and poor oral bioavailabilities in rats from our previous studies. In continuing efforts to discover new HCV

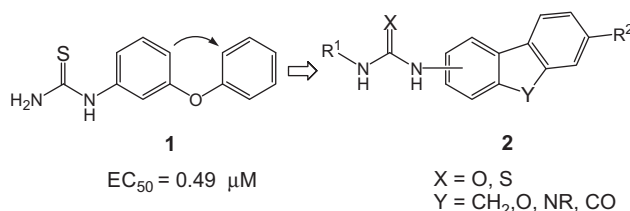


Figure 1. Arylthiourea compound **1** and its tricyclic analog **2**.

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inhibitors with high potency, low cytotoxicity and suitable pharmacokinetic properties, we elected to extend our optimization strategy to conformationally-restricted scaffold **2**, such as fluorene, fluorenone, dibenzofuran, and carbazole (Fig. 1). In this letter, we would like to report the synthesis, activity, and pharmacokinetic properties of these new scaffolds as potential HCV inhibitors. Details of this investigation will be described herein.

2. Results and discussion

2.1. Chemistry

A series of conformationally-restricted thiourea analogs **4a–k** and urea **5a**, including tricyclic and bicyclic scaffolds, have been synthesized according to the procedure outlined in Scheme 1. In general, the starting material of amine **3** was obtained from commercial sources or prepared by reduction of the corresponding nitro compounds with tin (II) chloride.¹⁰ The amine **3** was subsequently reacted with 1,1'-thiocarbonyldiimidazole (TCDI) or 1,1'-carbonyldiimidazole (CDI) in dichloromethane at room temperature followed by reaction with 25% ammonia solution to give the desired compounds **4a–k** and **5a**.

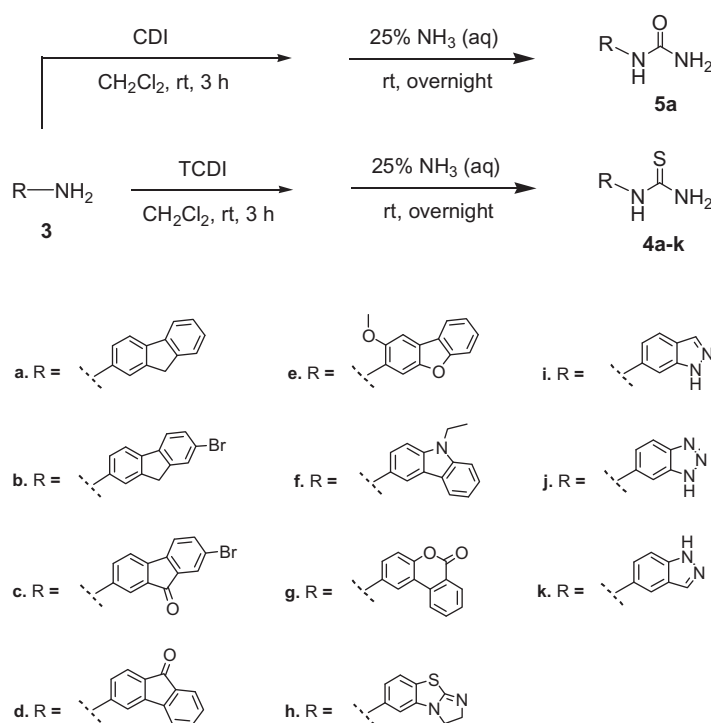
As shown in Scheme 2, several 7-bromofluorene analogs **6–12** were prepared from 2-amino-7-bromofluorene in one step by

coupling reaction with commercially available isothiocyanate in dichloromethane.

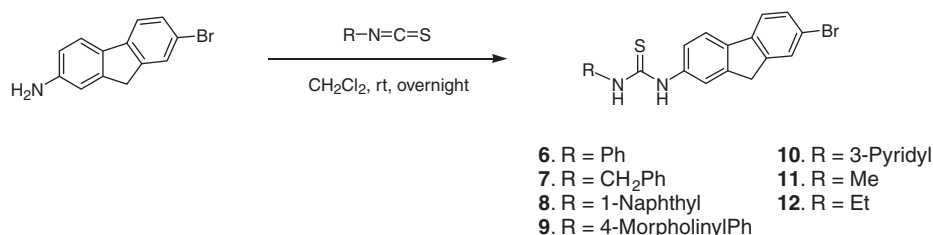
In addition, a variety of 7-aminofluorene, 7-alkylaminofluorene, and 7-dialkylaminofluorene analogs **15–23** were prepared according to a general synthetic method shown in Scheme 3 starting from commercially available 2,7-diaminofluorene. N-protection with di-*tert*-butyl dicarbonate in the presence of sodium carbonate produced the *N*-Boc derivative **13**, which was reacted with TCDI and then treated with 25% ammonia solution, followed by deprotection with trifluoroacetic acid (TFA) to give (7-amino-9*H*-fluoren-2-yl)-thiourea **15**. On the other hand, N-alkylation of **13** with a variety of alkyl iodides (*n*-Pr, *n*-Bu, and CH₂Ph) in the presence of potassium carbonate in acetonitrile gave a mixture of the *N*-alkylated and *N,N*-dialkylated derivatives, which were reacted with TFA to give the corresponding amines **14**. It is worth to mention that the *N*-alkylation of **13** with highly reactive alkylating agents such as methyl iodide and ethyl iodide provided only *N,N*-dialkylated derivatives under the same reaction condition in good yields. Subsequent treatment of **14** with TCDI followed by reaction with 25% ammonia solution gave the desired thiourea compounds **16–23**.

2.2. Bioactivity

All compounds prepared above were tested for anti-HCV activity using an in vitro assay system that is suitable for monitoring



Scheme 1. Synthesis of compounds **4a–k** and **5a**.



Scheme 2. Synthesis of compounds **6–12**.

On the basis of these biological results, the fluorene compound **4b** and its corresponding fluorenone compound **4c** were selected for further pharmacokinetic evaluation since they appeared to demonstrate potent inhibitory activity against HCV. To determine the pharmacokinetic behavior in vivo, compounds **4b** and **4c** were administered intravenously and orally to rats ($n=3$) at 5 and 25 mg/kg, respectively. The plasma was analyzed by LC/MS/MS for the concentration of **4b** and **4c**, and the calculated pharmacokinetic parameters are summarized in Tables 4 and 5. Compound **4c** is cleared rapidly following intravenous administration and has a relatively shorter half-life ($t_{1/2}$) when compared with **4b** (Table 4). The apparent volume of distribution is large relative to the

Table 1
Anti-HCV activity and cytotoxicity for compounds **4a–k**

Compound	Structure	1b EC ₅₀ ^a (μM)	CC ₅₀ ^a (μM)
1		0.49	>50
4a		2.09	>50
4b		0.3	>50
4c		0.57	>50
4d		20.97	>50
4e		5.51	>50
4f		7.55	>50
4g		>50	>50
4h		>50	>50
4i		>50	>50
4j		>50	>50
4k		>50	>50

1b, there are several genotypes in HCV, our assay employed genotype 1b subgenomic replicon.

^a Mean of triplicate well values. All experiments were performed at least twice.

Table 2
Anti-HCV activity and cytotoxicity for compounds **5a** and **6–12**

Compound	X	R	1b EC ₅₀ ^a (μM)	CC ₅₀ ^a (μM)
1	—	—	0.49	>50
4b	S	H	0.3	>50
5a	O	H	>50	>50
6	S	Ph	42.84	46.22
7	S	CH ₂ Ph	>50	>50
8	S	1-Naphthyl	15.38	>50
9	S	4-MorpholinylPh	34.14	18.10
10	S	3-Pyridyl	>50	19.71
11	S	Me	>50	>50
12	S	Et	>50	>50

1b, there are several genotypes in HCV, our assay employed genotype 1b subgenomic replicon.

^a Mean of triplicate well values. All experiments were performed at least twice.

plasma volume of the rat, indicating extensive distribution into tissues which is consistent with the lipophilic properties of both com-

Table 3
Anti-HCV activity and cytotoxicity for compounds **15–23**

Compound	R ³	R ⁴	1b EC ₅₀ ^a (μM)	CC ₅₀ ^a (μM)
1	—	—	0.49	>50
4b	—	—	0.3	>50
15	H	H	>50	>50
16	Me	Me	1.11	>50
17	Et	Et	0.83	>50
18	<i>n</i> -Pr	<i>n</i> -Pr	0.44	19.49
19	<i>n</i> -Bu	<i>n</i> -Bu	0.72	40.69
20	CH ₂ Ph	CH ₂ Ph	0.39	35.57
21	H	<i>n</i> -Pr	0.42	>50
22	H	<i>n</i> -Bu	0.38	>50
23	H	CH ₂ Ph	0.49	>50

1b, there are several genotypes in HCV, our assay employed genotype 1b subgenomic replicon.

^a Mean of triplicate well values. All experiments were performed at least twice.

Table 4
Pharmacokinetic parameters of **4b** and **4c** following iv administration^a to rats^b

Parameter	Compound	
	4b	4c
CL (mL/min/kg)	17.0 ± 0.9	127.4 ± 10.7
V _{ss} (L/kg)	2.8 ± 0.2	2.0 ± 0.6
<i>t</i> _{1/2} (h)	2.6 ± 0.2	1.4 ± 0.2
AUC (ng/mL h)	4937 ± 255	655 ± 51

^a Compound was formulated as a solution in DMA/propyl glycol (20:80, v/v) and administered at 5 mg/kg.

^b *n* = 3.

Table 5
Pharmacokinetic parameters of **4b** and **4c** following oral administration^a to rats^b

Parameter	Compound	
	4b	4c
C _{max} (ng/mL)	106.5 ± 22.5	65.3 ± 39.5
T _{max} (h)	0.7 ± 0.3	0.25 ± 0.0
<i>t</i> _{1/2} (h)	3.8 ± 3.1	1.7 ± 0.4
AUC (ng/mL h)	294 ± 73	43 ± 16
Bioavailability (%)	1.2	1.3

^a Compound was formulated as a solution in DMA/propyl glycol (20:80, v/v) and administered at 25 mg/kg.

^b *n* = 3.

pounds **4b** and **4c**. Additionally, compound **4b** exhibited a much higher AUC than **4c** in rats (AUC = 4937 vs 655 ng/mL h for **4b** and **4c**, respectively). These results indicated that the fluorine compound **4b** is metabolically more stable than the corresponding fluorone compound **4c**.

Following oral administration (25 mg/kg) of **4b** and **4c** to rats, the maximum concentration (C_{max}) was 106.5 and 65.3 ng/mL, and the time to reach the maximum concentration (T_{max}) was 0.7 and 0.25 h, respectively (Table 5). The oral plasma half-lives (*t*_{1/2}) of **4b** and **4c** were 3.8 and 1.7 h, respectively. Although compound **4b** showed approximately seven-fold higher oral exposure than that achieved by **4c** (AUC = 294 vs 43 ng/mL h for **4b** and **4c**, respectively), both compounds **4b** and **4c** showed poor oral bioavailability (*F* < 2%) and therefore are not suitable for oral use. The underlying cause of this effect is not fully understood and worthy of further study.

3. Conclusion

In summary, we have discovered a series of conformationally-restricted thiourea analogs as novel HCV inhibitors. According to our SAR investigation, introduction of a bromo group at the 7-position of the fluorene ring can significantly enhance the anti-HCV activity. The presence of a free thiourea group at the 2-position of the fluorene ring was essential for their anti-HCV activity. Replacement of the bromo group at the 7-position of the fluorene ring with a variety of alkylamino substituents resulted in an interesting pattern of activity. The pharmacokinetic properties of the fluorene compound **4b** and its corresponding fluorenone compound **4c** after iv and po dosing to rats were dramatically different. Among compounds synthesized, compound **4b** was found to exhibit promising in vitro activity in a cell-based subgenomic HCV replicon assay. In addition, this compound showed lower cytotoxicity and better pharmacokinetic properties compared to the arylthiourea derivatives from our previous studies following iv dosing, although its poor oral bioavailability required further optimization. Further SAR studies as well as mechanistic and pharmacokinetic studies on this class of compounds are currently under active investigation and will be reported in due course.

4. Experimental

4.1. Chemistry

All commercial chemicals and solvents are reagent grade and were used without further treatment unless otherwise noted. ¹H NMR spectra were obtained with a Varian Mercury-300 or a Varian Mercury-400 spectrometer. Chemical shifts were recorded in parts per million (ppm, δ) and were reported relative to the solvent peak or TMS. LC/MS data were measured on an Agilent MSD-1100 ESI-MS/MS System. Flash column chromatography was done using silica gel (Merck Kieselgel 60, No. 9385, 230–400 mesh ASTM). Reactions were monitored by TLC using Merck 60 F₂₅₄ silica gel glass backed plates; zones were detected visually under ultraviolet irradiation (254 nm) or by spraying with phosphomolybdic acid reagent (Aldrich) followed by heating at 80 °C. Melting points were determined on an Electrothermal IA9000 Series Digital Melting Point Apparatus.

4.1.1. General procedure for the preparation of compounds 4a–k

1,1'-Thiocarbonyldiimidazole (TCDI, 1.2 mmol) was added to a stirred solution of amine **3** (1.0 mmol) in CH₂Cl₂ (10 mL) at room temperature. The reaction mixture was treated with excess 25% ammonia solution about 3 h later and continually stirred overnight at room temperature. The precipitate was filtered in vacuo and washed with methanol and water to give the corresponding products **4a–k**.

4.1.1.1. (9H-Fluoren-2-yl)-thiourea (4a). Compound **4a** was prepared from corresponding amine **3** in 68% yield as a white solid, mp 213–214 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 3.89 (s, 2H), 7.24–7.38 (m, 3H), 7.55 (d, *J* = 7.5 Hz, 1H), 7.63 (s, 1H), 7.82 (d, *J* = 8.1 Hz, 1H), 7.83 (d, *J* = 7.5 Hz, 1H), 9.75 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 36.49, 119.73, 120.05, 120.12, 122.07, 125.09, 126.39, 126.80, 137.62, 137.82, 140.78, 142.98, 143.65, 180.88. MS (ESI⁺) *m/z* calcd for C₁₄H₁₂N₂S: 240.07; found: 241.0 (M+H), 263.0 (M+Na).

4.1.1.2. (7-Bromo-9H-fluoren-2-yl)-thiourea (4b). Compound **4b** was prepared from corresponding amine **3** in 93% yield as a white solid, mp 218–219 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 3.91 (s, 2H),

7.34 (dd, *J* = 1.8, 8.4 Hz, 1H), 7.53 (dd, *J* = 2.0, 8.0 Hz, 1H), 7.65 (s, 1H), 7.75–7.85 (m, 3H), 9.78 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 36.44, 119.32, 119.88, 120.44, 121.48, 122.11, 128.09, 129.67, 136.42, 138.31, 140.12, 143.55, 145.55, 180.90. MS (ESI⁺) *m/z* calcd for C₁₄H₁₁BrN₂S: 319.98; found: 320.0 (M+H), 342.0 (M+Na).

4.1.1.3. (7-Bromo-9-oxo-9H-fluoren-2-yl)-thiourea (4c). Compound **4c** was prepared from corresponding amine **3** in 65% yield as a orange solid, mp 220–221 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.57 (dd, *J* = 1.2, 8.0 Hz, 1H), 7.68–7.80 (m, 4H), 7.86 (s, 1H), 9.98 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 118.30, 121.60, 121.76, 122.79, 126.65, 128.63, 133.29, 135.46, 137.64, 138.31, 141.01, 142.93, 181.20, 191.42. MS (ESI⁺) *m/z* calcd for C₁₄H₉BrN₂OS: 333.96; found: 334.9 (M+H), 356.9 (M+Na).

4.1.1.4. (9-Oxo-9H-fluoren-3-yl)-thiourea (4d). Compound **4d** was prepared from corresponding amine **3** in 41% yield as a yellow solid, mp 211–212 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.35–7.43 (m, 2H), 7.54–7.61 (m, 3H), 7.71 (d, *J* = 7.2 Hz, 1H), 7.96 (d, *J* = 1.8 Hz, 1H), 10.11 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 114.13, 120.99, 121.56, 123.56, 124.84, 128.28, 129.59, 134.07, 134.96, 143.06, 144.88, 145.91, 181.12, 191.81. MS (ESI⁺) *m/z* calcd for C₁₄H₁₀N₂O₂S: 254.05; found: 255.0 (M+H), 277.0 (M+Na).

4.1.1.5. (2-Methoxy-dibenzofuran-3-yl)-thiourea (4e). Compound **4e** was prepared from corresponding amine **3** in 35% yield as a white solid, mp 191–192 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 3.97 (s, 3H), 7.37 (dd, *J* = 7.2, 8.1 Hz, 1H), 7.46 (dd, *J* = 7.2, 8.0 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.80 (s, 1H), 8.09 (d, *J* = 8.1 Hz, 1H), 8.54 (s, 1H), 9.27 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 56.41, 102.66, 107.14, 111.50, 119.28, 120.68, 122.84, 124.08, 126.72, 128.09, 147.89, 148.93, 155.98, 181.23. MS (ESI⁺) *m/z* calcd for C₁₄H₁₂N₂O₂S: 272.06; found: 273.1 (M+H), 295.0 (M+Na).

4.1.1.6. (9-Ethyl-9H-carbazol-3-yl)-thiourea (4f). Compound **4f** was prepared from corresponding amine **3** in 83% yield as a white solid, mp 199–200 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.30 (t, *J* = 7.2 Hz, 3H), 4.44 (q, *J* = 7.2 Hz, 2H), 7.19 (dd, *J* = 7.2, 7.5 Hz, 1H), 7.32 (d, *J* = 8.7 Hz, 1H), 7.45 (dd, *J* = 7.5, 7.8 Hz, 1H), 7.57–7.61 (m, 2H), 8.04 (s, 1H), 8.15 (d, *J* = 7.8 Hz, 1H), 9.64 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 13.74, 37.01, 109.17, 117.00, 118.69, 120.60, 122.02, 122.22, 123.64, 125.90, 130.14, 137.46, 139.97, 181.31. MS (ESI⁺) *m/z* calcd for C₁₅H₁₅N₃S: 269.10; found: 270.2 (M+H), 292.1 (M+Na).

4.1.1.7. (6-Oxo-6H-benzo[c]chromen-2-yl)-thiourea (4g). Compound **4g** was prepared from corresponding amine **3** in 54% yield as a white solid, mp 209–210 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.40 (d, *J* = 8.7 Hz, 1H), 7.50 (dd, *J* = 2.4, 8.7 Hz, 1H), 7.69 (dd, *J* = 7.6, 8.0 Hz, 1H), 7.97 (ddd, *J* = 1.4, 7.6, 8.0 Hz, 1H), 8.26 (dd, *J* = 1.4, 8.0 Hz, 1H), 8.32 (d, *J* = 8.0 Hz, 1H), 8.40 (d, *J* = 2.4 Hz, 1H), 9.80 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 117.53, 117.59, 118.32, 120.49, 122.64, 126.79, 129.48, 129.82, 134.06, 135.47, 135.84, 147.66, 160.22, 181.47. MS (ESI⁺) *m/z* calcd for C₁₄H₁₀N₂O₂S: 270.05; found: 271.0 (M+H).

4.1.1.8. (2,3-Dihydro-benzo[d]imidazo[2,1-*b*]thiazol-6-yl)-thiourea (4h). Compound **4h** was prepared from corresponding amine **3** in 86% yield as a yellow solid, mp 250–251 °C. MS (ESI⁺) *m/z* calcd for C₁₀H₁₀N₄S₂: 250.03; found: 251.1 (M+H).

4.1.1.9. (1H-Indazol-6-yl)-thiourea (4i). Compound **4i** was prepared from corresponding amine **3** in 84% yield as a white solid, mp 194–195 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 6.96 (dd, *J* = 1.5, 8.4 Hz, 1H), 7.52 (br s, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 7.80 (s, 1H),

7.99 (s, 1H), 9.80 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 103.14, 117.30, 119.97, 120.64, 133.34, 137.13, 140.05, 180.99. MS (ESI $^+$) m/z calcd for $\text{C}_8\text{H}_8\text{N}_4\text{S}$: 192.05; found: 193.1 (M+H), 215.1 (M+Na).

4.1.1.10. (3H-Benzotriazol-5-yl)-thiourea (4j). Compound **4j** was prepared from corresponding amine **3** in 81% yield as a brown solid, mp 203–204 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 7.29 (dd, J = 1.5, 9.0 Hz, 1H), 7.58 (br s, 1H), 7.87 (d, J = 9.0 Hz, 1H), 8.08 (s, 1H), 9.90 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 106.44, 116.35, 121.37, 122.33, 137.21, 181.38. MS (ESI $^+$) m/z calcd for $\text{C}_7\text{H}_7\text{N}_5\text{S}$: 193.04; found: 194.3 (M+H), 216.2 (M+Na).

4.1.1.11. (1H-Indazol-5-yl)-thiourea (4k). Compound **4k** was prepared from corresponding amine **3** in 85% yield as a violet solid, mp 192–193 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 7.22 (dd, J = 1.5, 8.7 Hz, 1H), 7.35 (br s, 1H), 7.49 (d, J = 8.7 Hz, 1H), 7.67 (s, 1H), 8.04 (s, 1H), 9.62 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 110.31, 115.59, 122.83, 124.51, 131.57, 133.65, 137.88, 181.35. MS (ESI $^+$) m/z calcd for $\text{C}_8\text{H}_8\text{N}_4\text{S}$: 192.05; found: 193.3 (M+H), 215.3 (M+Na).

4.1.2. Preparation of (7-bromo-9H-fluoren-2-yl)-urea (5a)

1,1'-Carbonyldiimidazole (CDI, 1.2 mmol) was added to a stirred solution of 2-amino-7-bromofluorene (1.0 mmol) in CH_2Cl_2 (10 mL) at room temperature. The reaction mixture was treated with excess 25% ammonia solution about 3 h later and continually stirred overnight at room temperature. The precipitate was filtered in vacuo and washed with methanol and water to give product **5a** in 90% yield as a white solid, mp 274–275 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 3.87 (s, 2H), 5.88 (s, 2H), 7.30 (d, J = 8.7 Hz, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.69–7.75 (m, 4H), 8.65 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 36.41, 114.33, 116.64, 118.38, 120.41, 120.77, 127.87, 129.47, 133.23, 140.22, 140.66, 143.78, 145.07, 155.96. MS (ESI $^+$) m/z calcd for $\text{C}_{14}\text{H}_{11}\text{BrN}_2\text{O}$: 302.01; found: 303.0 (M+H).

4.1.3. General procedure for the preparation of compounds 6–10 and 12

Commercially available isothiocyanate (1.2 mmol) was added to a stirred solution of 2-amino-7-bromofluorene (1.0 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was stirred overnight at room temperature. The precipitate was filtered in vacuo and washed with ether/ CH_2Cl_2 (10:1) to give the corresponding products **6–10** and **12**.

4.1.3.1. 1-(7-Bromo-9H-fluoren-2-yl)-3-phenyl-thiourea (6). Compound **6** was prepared from phenyl isothiocyanate in 77% yield as a white solid, mp 189–190 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 3.93 (s, 2H), 7.09–7.14 (m, 1H), 7.30–7.35 (m, 2H), 7.43–7.50 (m, 3H), 7.54 (dd, J = 1.2, 8.1 Hz, 1H), 7.74 (s, 1H), 7.75 (s, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.85 (d, J = 8.1 Hz, 1H), 9.81 (s, 1H), 9.90 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 36.40, 119.29, 120.18, 120.38, 121.46, 122.68, 123.70, 124.42, 128.08, 128.43, 129.66, 136.44, 138.74, 139.49, 140.15, 143.24, 145.56, 179.51. MS (ESI $^+$) m/z calcd for $\text{C}_{20}\text{H}_{15}\text{BrN}_2\text{S}$: 394.01; found: 394.9 (M+H), 416.9 (M+Na).

4.1.3.2. 1-Benzyl-3-(7-bromo-9H-fluoren-2-yl)-thiourea (7). Compound **7** was prepared from benzyl isothiocyanate in 43% yield as a white solid, mp 183–184 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 3.92 (s, 2H), 4.74 (d, J = 5.7 Hz, 2H), 7.22–7.29 (m, 1H), 7.33 (s, 2H), 7.35 (s, 2H), 7.38 (dd, J = 1.2, 8.4 Hz, 1H), 7.54 (dd, J = 0.9, 8.1 Hz, 1H), 7.68 (s, 1H), 7.75 (s, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.85 (d, J = 8.1 Hz, 1H), 8.21 (br t, 1H), 9.71 (br s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 36.42, 47.24, 119.28, 120.22, 120.39, 121.45, 122.47, 126.87, 127.43, 128.08, 128.27, 129.67, 136.38, 138.41, 139.04, 140.14, 143.47, 145.52, 180.74. MS (ESI $^+$) m/z calcd for $\text{C}_{21}\text{H}_{17}\text{BrN}_2\text{S}$: 408.03; found: 409.0 (M+H), 430.9 (M+Na).

4.1.3.3. 1-(7-Bromo-9H-fluoren-2-yl)-3-naphthalen-1-yl-thiourea (8). Compound **8** was prepared from 1-naphthalenyl isothiocyanate in 45% yield as a white solid, mp 206–207 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 3.92 (s, 2H), 7.47–7.60 (m, 6H), 7.75–7.86 (m, 5H), 7.94–8.00 (m, 2H), 9.87 (s, 1H), 9.89 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 36.38, 119.29, 120.08, 120.86, 121.48, 123.10, 123.14, 125.37, 125.64, 126.08, 126.16, 126.72, 128.08, 129.65, 129.97, 133.91, 135.16, 136.53, 138.86, 140.15, 143.13, 145.57, 181.20. MS (ESI $^+$) m/z calcd for $\text{C}_{24}\text{H}_{17}\text{BrN}_2\text{S}$: 444.03; found: 467.0 (M+Na).

4.1.3.4. 1-(7-Bromo-9H-fluoren-2-yl)-3-(4-morpholin-4-yl-phenyl)-thiourea (9). Compound **9** was prepared from 4-morpholinyl-phenyl isothiocyanate in 25% yield as a yellow solid, mp 200–201 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 3.07 (br t, J = 4.7 Hz, 4H), 3.72 (br t, J = 4.7 Hz, 4H), 3.92 (s, 2H), 6.91 (d, J = 8.8 Hz, 2H), 7.28 (d, J = 8.8 Hz, 2H), 7.43 (d, J = 8.4 Hz, 1H), 7.54 (d, J = 8.1 Hz, 1H), 7.73 (s, 1H), 7.75 (s, 1H), 7.79 (d, J = 8.4 Hz, 1H), 7.83 (d, J = 8.1 Hz, 1H), 9.60 (s, 1H), 9.68 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 36.40, 48.65, 66.10, 115.01, 119.22, 120.10, 120.34, 121.42, 122.64, 125.34, 128.06, 129.65, 131.02, 136.24, 138.95, 140.20, 143.16, 145.53, 148.43, 179.50. MS (ESI $^+$) m/z calcd for $\text{C}_{24}\text{H}_{22}\text{BrN}_3\text{OS}$: 479.07; found: 480.0 (M+H).

4.1.3.5. 1-(7-Bromo-9H-fluoren-2-yl)-3-pyridin-3-yl-thiourea (10). Compound **10** was prepared from 3-pyridinyl isothiocyanate in 90% yield as a yellow solid, mp 170–171 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 3.94 (s, 2H), 7.36 (dd, J = 5.1, 8.1 Hz, 1H), 7.45 (dd, J = 1.2, 8.3 Hz, 1H), 7.55 (dd, J = 1.4, 8.4 Hz, 1H), 7.73 (s, 1H), 7.76 (s, 1H), 7.82 (d, J = 8.1 Hz, 1H), 7.88 (d, J = 8.3 Hz, 1H), 7.94 (br d, J = 8.4 Hz, 1H), 8.31 (d, J = 5.1 Hz, 1H), 8.61 (d, J = 2.7 Hz, 1H), 9.87 (s, 1H), 10.13 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 36.43, 119.41, 120.34, 120.58, 121.54, 122.87, 123.11, 128.11, 129.69, 131.48, 136.39, 136.79, 138.33, 140.07, 143.39, 145.26, 145.51, 145.59, 180.15. MS (ESI $^+$) m/z calcd for $\text{C}_{19}\text{H}_{14}\text{BrN}_3\text{S}$: 395.01; found: 395.9 (M+H), 417.9 (M+Na).

4.1.3.6. 1-(7-Bromo-9H-fluoren-2-yl)-3-ethyl-thiourea (12). Compound **12** was prepared from ethyl isothiocyanate in 90% yield as a white solid, mp 209–210 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 1.1 (t, J = 7.2 Hz, 3H), 3.36 (br s, 1H), 3.44–3.53 (m, 2H), 3.91 (s, 2H), 7.34 (dd, J = 1.8, 8.4 Hz, 1H), 7.54 (dd, J = 2.0, 8.1 Hz, 1H), 7.64 (s, 1H), 7.75 (s, 1H), 7.79 (d, J = 8.4 Hz, 1H), 7.84 (d, J = 8.1 Hz, 1H), 9.53 (br s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 14.22, 36.41, 38.74, 119.21, 119.99, 120.35, 121.40, 122.25, 128.06, 129.65, 136.15, 138.52, 140.17, 143.44, 145.49, 179.98. MS (ESI $^+$) m/z calcd for $\text{C}_{16}\text{H}_{15}\text{BrN}_2\text{S}$: 346.01; found: 346.9 (M+H), 368.9 (M+Na).

4.1.4. Preparation of 1-(7-bromo-9H-fluoren-2-yl)-3-methyl-thiourea (11)

1,1'-Thiocarbonyldiimidazole (TCDI, 1.2 mmol) was added to a stirred solution of 2-amino-7-bromofluorene (1.0 mmol) in CH_2Cl_2 (10 mL). The reaction mixture was treated with excess 40% methyl amine aqueous solution about 3 h later and continually stirred overnight at room temperature. After concentrating in vacuo, the crude product was recrystallized with methanol and CH_2Cl_2 (1:5) to give pure product **11** in 28% yield as a white solid, mp 186–187 °C. ^1H NMR (300 MHz, DMSO- d_6) δ 2.36 (q, J = 5.6 Hz, 1H), 2.92 (d, J = 5.6 Hz, 3H), 3.91 (s, 2H), 7.34 (d, J = 8.3 Hz, 1H), 7.54 (dd, J = 1.5, 8.1 Hz, 1H), 7.63 (s, 1H), 7.75 (d, J = 1.5 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.84 (d, J = 8.1 Hz, 1H), 9.69 (br s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 31.23, 36.41, 119.22, 119.92, 120.39, 121.42, 122.11, 128.08, 129.65, 136.20, 138.51, 140.17, 143.47, 145.50, 181.01. MS (ESI $^+$) m/z calcd for $\text{C}_{15}\text{H}_{13}\text{BrN}_2\text{S}$: 332.00; found: 333.0 (M+H), 354.9 (M+Na).

4.1.5. Preparation of (7-amino-9H-fluoren-2-yl)-carbamic acid tert-butyl ester (**13**)

2,7-Diaminofluorene (5.0 mmol) and sodium carbonate (10.0 mmol) were suspended in a solution of 1,4-dioxane (20 mL) and H₂O (10 mL). Di-*tert*-butyl dicarbonate (6.0 mmol) was added dropwise to the suspension solution at ice-bath. The reaction mixture was warmed to room temperature and stirred for 6 h. The mixture was diluted with CH₂Cl₂ and washed sequentially with saturated ammonium chloride. The organic layer was dried over MgSO₄, concentrated in vacuo and purified by column chromatography using EtOAc/hexane (1:3) as an eluant to give desired product **13** in 41% yield as a off-white solid, mp 172–173 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.47 (s, 9H), 3.67 (s, 2H), 5.09 (s, 2H), 6.53 (dd, *J* = 1.8, 8.1 Hz, 1H), 6.72 (d, *J* = 1.8 Hz, 1H), 7.29 (dd, *J* = 1.8, 8.4 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 7.60 (s, 1H), 9.25 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 28.21, 36.31, 78.79, 110.55, 112.72, 115.03, 116.87, 117.85, 119.79, 129.77, 136.44, 136.65, 142.23, 144.18, 147.65, 152.91. MS (ESI⁺) *m/z* calcd for C₁₈H₂₀N₂O₂: 296.15; found: 297.1 (M+H).

4.1.6. Preparation of (7-amino-9H-fluoren-2-yl)-thiourea (**15**)

1,1'-Thiocarbonyldiimidazole (TCDI, 1.2 mmol) was added to a stirred solution of compound **13** (1.0 mmol) in CH₂Cl₂ (10 mL). The reaction mixture was treated with excess 25% ammonia solution about 3 h later and continually stirred overnight at room temperature. After filtering and washing with methanol, the precipitate was dissolved in CH₂Cl₂ (2 mL) and introduced by TFA (2 mL) at ice-bath. The reaction mixture was warmed to room temperature and stirred for 1 h. The mixture was diluted with CH₂Cl₂ and neutralized by saturated sodium bicarbonate. The organic layer was washed by brine, dried over MgSO₄, and concentrated in vacuo. The precipitate was washed by methanol to give the pure product **15** in 54% yield as a off-white solid, mp 250–251 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.71 (s, 2H), 5.17 (s, 2H), 6.56 (dd, *J* = 2.0, 8.2 Hz, 1H), 6.73 (s, 1H), 7.18 (br d, *J* = 8.0 Hz, 1H), 7.43 (br s, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.53 (d, *J* = 8.2 Hz, 1H), 9.74 (br s, 1H). ¹³C NMR (400 MHz, DMSO-*d*₆) δ 36.27, 110.43, 112.83, 117.87, 120.29, 120.34, 122.24, 129.30, 135.48, 139.18, 142.17, 144.65, 148.14, 180.83. MS (ESI⁺) *m/z* calcd for C₁₄H₁₃N₃S: 255.08; found: 256.1 (M+H).

4.1.7. General Procedure for the preparation of compounds **16**–**23**

Alkyl iodide (3.0 mmol) was added to the mixture of compound **13** (1.0 mmol) and potassium carbonate (1.2 mmol) in acetonitrile (5 mL). The reaction mixture was stirred at 60 °C for 3 h and then extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄ and concentrated in vacuo to give the crude product which was used without further purification. The crude product in CH₂Cl₂ (2 mL) was deprotected with TFA (2 mL) at ice-bath and followed by treating with TCDI (1.2 mmol) and excess 25% ammonia solution. The final product was purified by column chromatography using EtOAc/hexane (1:3) as an eluant to give the desired products **16**–**23**.

4.1.7.1. (7-Dimethylamino-9H-fluoren-2-yl)-thiourea (16**).** Compound **16** was prepared from compound **13** for three steps in 32% overall yield as a pink solid, mp 196–197 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 2.94 (s, 6H), 3.79 (s, 2H), 6.73 (dd, *J* = 1.8, 8.4 Hz, 1H), 6.93 (s, 1H), 7.19 (d, *J* = 8.1 Hz, 1H), 7.47 (s, 1H), 7.60 (d, *J* = 8.1 Hz, 1H), 7.61 (d, *J* = 8.4 Hz, 1H), 9.65 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 36.60, 40.49, 108.99, 111.40, 118.38, 120.23, 120.34, 122.29, 129.60, 135.70, 138.85, 142.53, 144.64, 149.90, 180.74. MS (ESI⁺) *m/z* calcd for C₁₆H₁₇N₃S: 283.11; found: 284.1 (M+H), 306.1 (M+Na).

4.1.7.2. (7-Diethylamino-9H-fluoren-2-yl)-thiourea (17**).** Compound **17** was prepared from compound **13** for three steps in 21% overall yield as a white solid, mp 172–173 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.10 (t, *J* = 7.0 Hz, 6H), 3.34 (q, *J* = 7.0 Hz, 4H), 3.77 (s, 2H), 6.66 (dd, *J* = 2.2, 8.4 Hz, 1H), 6.85 (s, 1H), 7.18 (d, *J* = 8.0 Hz, 1H), 7.44 (s, 1H), 7.56 (d, *J* = 8.0 Hz, 1H), 7.57 (d, *J* = 8.4 Hz, 1H), 9.66 (s, 1H). ¹³C NMR (400 MHz, DMSO-*d*₆) δ 12.56, 36.63, 43.97, 108.21, 110.66, 118.06, 120.30, 120.50, 122.26, 128.63, 135.51, 139.00, 142.35, 144.98, 147.00, 180.81. MS (ESI⁺) *m/z* calcd for C₁₈H₂₁N₃S: 311.15; found: 312.1 (M+H), 334.1 (M+Na).

4.1.7.3. (7-Dipropylamino-9H-fluoren-2-yl)-thiourea (18**).** Compound **18** was prepared from compound **13** for three steps in 51% overall yield as a yellow solid, mp 166–167 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 0.89 (t, *J* = 7.4 Hz, 6H), 1.52–1.57 (m, 4H), 3.24–3.31 (m, 4H), 3.77 (s, 2H), 6.63 (dd, *J* = 2.2, 8.8 Hz, 1H), 6.82 (s, 1H), 7.16 (d, *J* = 8.0 Hz, 1H), 7.43 (s, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 7.56 (d, *J* = 8.8 Hz, 1H), 9.66 (s, 1H). ¹³C NMR (400 MHz, DMSO-*d*₆) δ 11.31, 20.12, 36.67, 52.32, 108.07, 110.56, 118.08, 120.32, 120.47, 122.28, 128.51, 135.48, 139.03, 142.39, 144.96, 147.42, 180.78. MS (ESI⁺) *m/z* calcd for C₂₀H₂₅N₃S: 339.18; found: 340.2 (M+H), 362.2 (M+Na).

4.1.7.4. (7-Dibutylamino-9H-fluoren-2-yl)-thiourea (19**).** Compound **19** was prepared from compound **13** for three steps in 15% overall yield as a white solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.91 (t, *J* = 7.4 Hz, 6H), 1.28–1.36 (m, 4H), 1.46–1.54 (m, 4H), 3.29 (t, *J* = 7.5 Hz, 4H), 3.77 (s, 2H), 6.63 (dd, *J* = 2.4, 8.7 Hz, 1H), 6.82 (s, 1H), 7.17 (dd, *J* = 1.5, 8.1 Hz, 1H), 7.44 (s, 1H), 7.55 (d, *J* = 8.1 Hz, 1H), 7.56 (d, *J* = 8.7 Hz, 1H), 9.63 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 13.91, 19.75, 29.11, 36.64, 50.25, 108.13, 110.63, 118.04, 120.30, 120.44, 122.26, 128.51, 135.44, 139.01, 142.36, 144.92, 147.41, 180.78. MS (ESI⁺) *m/z* calcd for C₂₂H₂₉N₃S: 367.21; found: 368.1 (M+H), 390.1 (M+Na).

4.1.7.5. (7-Dibenzylamino-9H-fluoren-2-yl)-thiourea (20**).** Compound **20** was prepared from compound **13** for three steps in 8% overall yield as a white solid, mp 181–182 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 3.70 (s, 2H), 4.73 (s, 4H), 6.68 (dd, *J* = 2.0, 8.4 Hz, 1H), 6.91 (d, *J* = 1.5 Hz, 1H), 7.16–7.35 (m, 11H), 7.43 (s, 1H), 7.52 (d, *J* = 8.4 Hz, 1H), 7.54 (d, *J* = 8.1 Hz, 1H), 9.68 (s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 36.58, 54.34, 109.01, 111.53, 118.28, 120.14, 120.29, 122.15, 126.66, 126.73, 128.50, 129.77, 135.76, 138.58, 138.98, 142.44, 144.64, 147.80, 180.72. MS (ESI⁺) *m/z* calcd for C₂₈H₂₅N₃S: 435.18; found: 436.3 (M+H).

4.1.7.6. (7-Propylamino-9H-fluoren-2-yl)-thiourea (21**).** Compound **21** was prepared from compound **13** for three steps in 11% overall yield as a brown solid, mp 193–194 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.94 (t, *J* = 7.4 Hz, 3H), 1.54–1.61 (m, 2H), 3.00 (dt, *J* = 5.7, 6.9 Hz, 2H), 3.73 (s, 2H), 5.70 (t, *J* = 5.7 Hz, 1H), 6.57 (dd, *J* = 1.8, 8.3 Hz, 1H), 6.74 (s, 1H), 7.16 (d, *J* = 8.1 Hz, 1H), 7.42 (s, 1H), 7.50 (d, *J* = 8.1 Hz, 1H), 7.53 (d, *J* = 8.3 Hz, 1H), 9.65 (br s, 1H). ¹³C NMR (300 MHz, DMSO-*d*₆) δ 11.70, 21.96, 36.39, 44.91, 108.05, 111.18, 117.87, 120.30, 120.35, 122.28, 128.93, 135.28, 139.26, 142.18, 144.72, 148.62, 180.77. MS (ESI⁺) *m/z* calcd for C₁₇H₁₉N₃S: 297.13; found: 298.1 (M+H), 320.1 (M+Na).

4.1.7.7. (7-Butylamino-9H-fluoren-2-yl)-thiourea (22**).** Compound **22** was prepared from compound **13** for three steps in 14% overall yield as a off-white solid, mp 195–196 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 0.91 (t, *J* = 7.4 Hz, 3H), 1.35–1.43 (m, 2H), 1.50–1.57 (m, 2H), 3.03 (dt, *J* = 5.4, 6.9 Hz, 2H), 3.73 (s, 2H), 5.67 (t, *J* = 5.4 Hz, 1H), 6.56 (dd, *J* = 2.0, 8.4 Hz, 1H), 6.73 (d, *J* = 1.2 Hz, 1H), 7.16 (d, *J* = 8.1 Hz, 1H), 7.42 (s, 1H), 7.50 (d, *J* = 8.1 Hz, 1H),

7.53 (d, $J = 8.4$ Hz, 1H), 9.63 (s, 1H). ^{13}C NMR (300 MHz, DMSO- d_6) δ 13.85, 19.88, 30.92, 36.42, 42.74, 108.06, 111.19, 117.89, 120.32, 120.35, 122.29, 128.95, 135.30, 139.27, 142.20, 144.73, 148.66, 180.78. MS (ESI $^+$) m/z calcd for $\text{C}_{18}\text{H}_{21}\text{N}_3\text{S}$: 311.15; found: 312.1 (M+H), 334.1 (M+Na).

4.1.7.8. (7-Benzylamino-9H-fluoren-2-yl)-thiourea (23). Compound **23** was prepared from compound **13** for three steps in 15% overall yield as a yellow solid, mp 197–198 °C. ^1H NMR (400 MHz, DMSO- d_6) δ 3.70 (s, 2H), 4.30 (d, $J = 6.0$ Hz, 2H), 6.37 (t, $J = 6.0$ Hz, 1H), 6.59 (dd, $J = 2.0, 8.1$ Hz, 1H), 6.77 (s, 1H), 7.15–7.41 (m, 7H), 7.48 (d, $J = 8.1$ Hz, 1H), 7.52 (d, $J = 8.1$ Hz, 1H), 9.65 (s, 1H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 37.07, 47.33, 109.35, 112.15, 118.69, 120.98, 121.01, 122.97, 127.33, 127.89, 128.98, 130.11, 136.11, 139.79, 140.96, 142.93, 145.31, 148.91, 181.46. MS (ESI $^+$) m/z calcd for $\text{C}_{21}\text{H}_{19}\text{N}_3\text{S}$: 345.13; found: 346.3 (M+H).

4.2. Biology

Huh-7 cells containing HCV subgenomic replicons (Ava5) were provided by Apath, LLC (St. Louis, MO). The reporter-based HCV subgenomic replicon, Ava5-EG(D4AB)SEAP, has previously been described.¹¹ Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD). Cell viability was determined by the MTS assay that was essentially as described.

4.2.1. Subgenomic HCV inhibitory assay

In 96-well plates, Ava5-EG(D4AB)SEAP cells were seeded at a density of 7×10^3 cells per well. After incubation at 37 °C for 1 day, cells were treated with various drugs at final 10 μM . Two days later, culture medium was replaced with fresh phenol red-free DMEM/10% FBS containing the same concentration of drugs and cells were incubated for one more day. Culture supernatants were collected from each well and SEAP activities were measured using Phospha-Light assay kit (Tropix, Foster City, CA), according to the manufacturer's instruction.

4.3. Pharmacokinetic study

4.3.1. Pharmacokinetic analysis of 4b and 4c in Sprague–Dawley rats

The SD rats for the pharmacokinetic study were obtained from BioLASCO Taiwan Co., Ltd (Ilan, Taiwan, ROC), and housed in the animal facility at the National Health Research Institutes, Taiwan, ROC. The animal studies were performed according to committee approved procedures. Male rats, each weighing 330–380 g (9–10 weeks old), were quarantined for 1 week before use. The animals were surgically implanted with a jugular-vein cannula 1 day before treatment, and were fasted before treatment. The compound was given to the rats ($n = 4$) as an intravenous (5 mg/kg) or oral (20 mg/kg) dose prepared in a mixture of dosing vehicles. The volume of the dosing solution given was adjusted according to the body weight recorded before the drug was administered. At 0 (immediately before dosing), 2, 5 (intravenous only), 15 and

30 min and 1, 2, 4, 6, 8, and 24 h after dosing, a blood sample (~150 mL) was taken from each animal via the jugular-vein cannula and stored in ice (0–4 °C). The processing of the plasma and analysis by high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was carried out as described.¹³ The plasma concentration data were analyzed with a standard non-compartmental method.

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