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Original article

Discovery of novel *N*-substituted carbazoles as neuroprotective agents with potent anti-oxidative activity

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ABSTRACT

Carbazole moiety is an important scaffold with a variety of biological applications, for example, anti-oxidative stress. Our previous synthesized carbazoles were screened for their neuroprotective properties against two individual oxidative stresses. Some of the new carbazole derivatives were observed with modest to good neuroprotective effects on neuronal cells HT22 against cell injury induced by glutamate or homocysteic acid (HCA). Substituents introduced to the carbazole ring system play crucial roles in their biological activities. In particular, a bulky group favors the neuroprotective activity of the compounds. One of the new compounds, **6**, showed the best neuroprotective effects, which might result from its anti-oxidative activity with a GSH-independent mechanism. These findings might provide an alternative strategy for the development of novel carbazole derivatives for the treatment of CNS diseases such as Alzheimer's disease.

1. Introduction

Increased oxidative stress has been recognized as a common culprit of many neurological disorders including Alzheimer's disease (AD), Parkinson's disease (PD), and stroke [1–3]. Oxidative stress is often the result of unregulated production of reactive oxygen species (ROS) including hydrogen peroxide, nitric oxide, superoxide, and reactive hydroxyl radicals [2]. Cells have developed a highly elaborate mechanism to regulate cellular level of oxidant species including ROS using exogenous and endogenous antioxidants [2]. Endogenous antioxidants such as glutathione peroxidase and superoxide dismutase are essential to detoxify or scavenge oxidant species. In some circumstances, however, the production of oxidant species can exceed the scavenging ability of the endogenous antioxidants. The consequent oxidative imbalance can lead to cellular oxidative stress, cellular functions' alteration, and even cell death. Central nervous system (CNS) is particularly susceptible to oxidative stress because, comparing to other organs, it is rich in highly oxygen-consuming polyunsaturated fatty acids, and possesses a relatively low level of antioxidant and low regenerative capacity [2,3].

Naturally occurring carbazoles have attracted interests of chemists and biologists due to their structural features and intriguing pharmacological activities. A large array of natural carbazoles and synthetic derivatives are endowed with profound biological activities. These unique compounds include anti-inflammatory caprofen [4], antitumor ellipticine [5], antibiotic carbazomycin B [6], anti-oxidative carvedilol [7], neuronal cell-protecting agent carbazomadurin A and P7C3 [8], and anti-prion tetrahydrocarbazoles [9] (Fig. 1).

Anti-oxidative activity of carbazoles might contribute to their neuroprotective properties although the exact mechanism still remains to be explored [10]. Substituted carbazoles were often reported to be neuroprotective, for example, carvedilol and P7C3 [7,8]. These findings indicated that a high possibility to move substitutes around the carbazole core and to change their chemical properties could still keep their neuroprotective activity. Therefore, it is of high interest to search for a library of substituted carbazoles

Abbreviations: CNS, central nerve system; AD, Alzheimer's disease; ROS, reactive oxygen species; *m*CPBA, *meta*-chloroperoxybenzoic acid.

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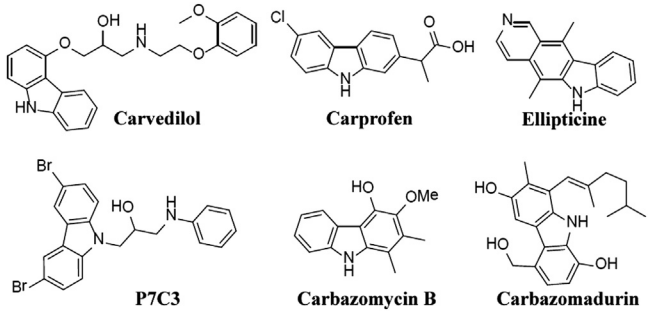


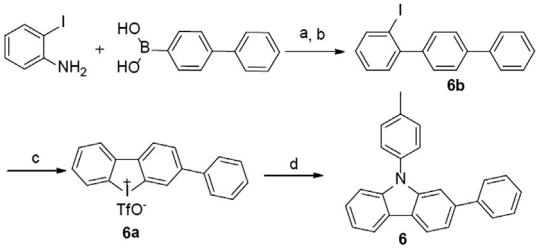
Fig. 1. Chemical structures of known carbazoles.

in order to identify a potent lead compound that will be further developed into a drug for AD.

2. Chemistry

Recently, we have developed a novel strategy to prepare diversified carbazoles from cyclic dipenyleneiodoniums with a broad range of amines including aromatic and aliphatic amines and aryl sulfonamides (Scheme 1) [11]. Specifically, our method has employed an inexpensive catalyst copper (II) acetate ($Cu(OAc)_2$) and a base Na_2CO_3. The reaction of diphenyleniodoniums with a variety of amines in a refluxing isopropanol/ethylene glycol (9/1) proceeded smoothly in modest to good yields. In the end, a small chemical library of structurally diversified carbazoles has been generated.

The general procedure for the preparation of these carbazoles was exemplified by the synthesis of **6** (Scheme 2). Firstly, Suzuki reaction of 2-iodoaniline with [1,1′-biphenyl]-4-ylboronic acid and subsequent Sandmeyer reaction afforded iodo-terphenyl **6b**. Then, **6b** was oxidized and subsequently cyclized to form **6a** using *m*CPBA (85%) in anhydrous dichloromethane with trifluoromethanesulfonic acid. Finally, **6** was prepared in a good yield using the method discussed above.

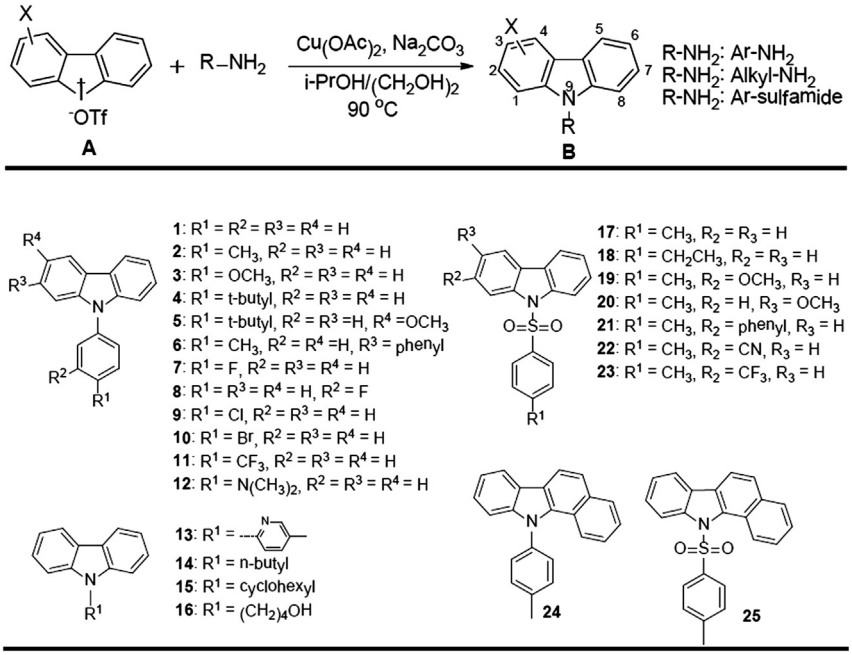


Scheme 2. Synthesis of **6**. Reaction conditions: (A) $Pd(PPh_3)_4$, K_3PO_4, EtOH, reflux, 6 h, 85%. (B) i. $NaNO_2$, 0 °C THF, 4M HCl, ii. KI, r.t., 81%. (C) *m*CPBA (85%), TfOH, CH_2Cl_2, r.t., 2 h, 100%. (D) *p*-toluidine, Na_2CO_3, $Cu(OAc)_2$, *i*PrOH/$(CH_2OH)_2$, 90 °C, 16 h, 71%.

3. Results and discussions

The serendipitously obtained carbazoles with a structural diversity resemble the neuroprotective agents, and they could unexpectedly provide a good lead to elaborate drug candidates to target AD. So the synthetic compounds were taken for a biological screening against the death of neuronal HT22 cells induced by neurotoxins including glutamate and HCA. Our results indicated that several of the new carbazoles were significantly neuroprotective at the concentration of 30 μM (Fig. 2).

A substituent at the N9 position of the carbazole is essential for neuroprotective ability while unsubstituted carbazole did not prevent HT22 from the death induced by glutamate or HCA at the concentration of 30 μM (Table 1). This was further confirmed by structurally similar compounds, for example, dibenzofuran (DBF) and dibenzothiophene (DBT) that showed no neuroprotective activity at the concentration of 30 μM. The spatial properties of the substituents play important roles in their biological activities. The neuroprotective activity was significantly increased when a bulky group, such as methoxylphenyl (**3**), *t*-butylphenyl (**4**), trifluorophenyl (**11**), and *N*,*N*-dimethylphenyl (**12**) was introduced to the N9 position of the carbazole. So it is not surprising that compound **6** with methylphenyl and phenyl attached to N9 position and C2 position had displayed the best neuroprotective ability, resulting



Scheme 1. Synthetic carbazoles from diphenyleneiodoniums.

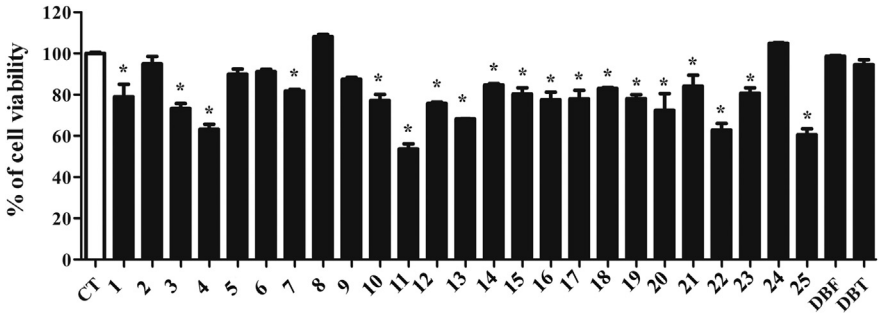


Fig. 2. The effect of carbazoles on the viability of mice hippocampal cell line HT22 cells. Cells were pretreated ahead with carbazoles alone at 30 μM for 24 h. After that, the cell viability was tested by MTT assay ($n = 6$). $^*P < 0.05$ compared with glutamate-treated group.

from cumulative spatial effects. The electronic property of the substituents on the introduced benzene ring did not make any significant difference (**11** and **12**). Substituents attached to C3 position of carbazoles play a negative role (**5**). Carbazoles with an *N*-alkyl substituent showed none or low neuroprotective capacity (**14** or **15**, **16**). With *N*-arylsulfonyl, an electron-rich methoxy group on the side benzene ring of carbazoles was detrimental (**19**, **20**). With 2-subsituted phenyl and trifluoromethyl, or none substitute, the *N*-arylsulfonyl carbazoles showed modest neuroprotective ability (**21**, **23**, **17**, **18**). In general, carbazole with *N*-alkyl and benzenesulfonyl substitutions are less potent compared to the ones with aryl substitutions. However, one exception is compound **25**, which showed neuroprotection while the counterpart **24** did not (see Table 1). In addition, the neuroprotective effects of compound **6**, in glutamate and HCA model, are in "bell-shape" manners. Further study demonstrated that compound **6** inhibits the proliferation but not induces cell toxicity at 30 μM using LDH assay (cell toxicity assay) (Data not shown). Further studies are under way to uncover the exact mechanism.

It is interesting that compound **6** dramatically prevented HT22 cells from the cell death induced by glutamate or HCA at the concentration as low as 3 μM (see Table 1, Fig. 3 and Supplemental Figure 1). Both glutamate and HCA were reported to induce ROS through depleting the intracellular GSH [12–14]. Previous study demonstrated that glutamate-induced ROS is associated with the cell damage in HT22 cells [15]. To investigate whether the novel carbazole **6** could decrease the glutamate-induced ROS, an ROS predictor DHE was used. We found that the cells changed to red color under fluorescence microscopy when exposed to glutamate (see Fig. 4B). Compound **6** significantly inhibited the changes in a concentration-dependent manner (see Fig. 4C–E), suggesting that **6** decreased glutamate-induced ROS level in a concentration-dependent manner. However, **6** did not reverse the glutamate-induced intracellular GSH depletion (see Fig. 5), indicating that it might quench ROS through a GSH-independent way. Previous report showed that melatonin, a potent natural antioxidant also did not restore cytosolic GSH depletion although it potently prevented mitochondrial ROS production [15]. These data suggest that compound **6** might target mitochondria in a similar way as melatonin. Further study is under way to uncover the exact mechanism(s) of neuroprotective effects of **6**.

Table 1
Neuroprotective effects of the carbazoles on HT22 cells against cell injury induced by neurotoxins, glutamate and HCA.

Chemicals	Inhibitory rate of cell injury induced by glutamate (%)			Inhibitory rate of cell injury induced by HCA (%)		
	3 μM	10 μM	30 μM	3 μM	10 μM	30 μM
1	0	7	6	0	0	5
2	0	0	0	0	0	0
3	**3**	**25**	**59**	**0**	**32**	**71**
4	**0**	**35**	**70**	**2**	**30**	**63**
5	0	0	0	0	0	0
6	**63**	**69**	**50**	**64**	**77**	**44**
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	20	0	0	40
10	0	0	0	0	0	0
11	**0**	**14**	**68**	**0**	**10**	**80**
12	**20**	**40**	**77**	**0**	**30**	**81**
13	0	0	5	0	5	12
14	0	0	0	0	0	0
15	3	8	20	7	8	10
16	0	1	4	0	1	12
17	**0**	**10**	**47**	**4**	**13**	**34**
18	**0**	**3**	**28**	**0**	**6**	**30**
19	0.8	2	4	0	0	0
20	0	3	5.2	0	0	0
21	**12**	**19**	**30**	**11**	**18**	**30**
22	3	2	7	0	0	0
23	**0**	**10**	**42**	**0**	**6**	**38**
24	0	0	0	0	0	0
25	**4**	**16**	**26**	**18**	**30**	**30**

Bold values highlight that these compounds are effectively neuroprotective.

4. Conclusion

In summary, we have screened a series of substituted carbazoles for their neuroprotective activities. The results of the anti-oxidation screening have revealed that the compounds **3**, **4**, **6**, **9**, **11**, **12**, **17**, and **21** showed moderate to excellent neuroprotective capacity. The most potent compound, **6** prevented cell death through a GSH-

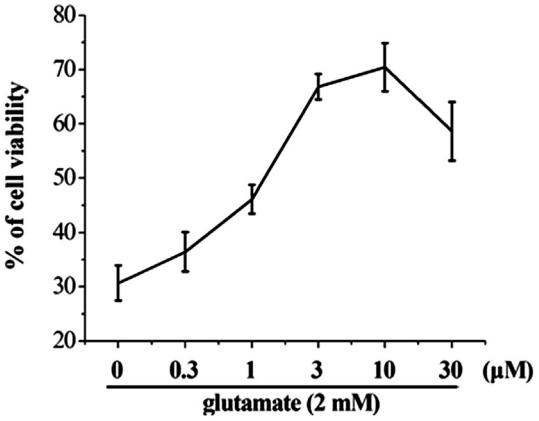


Fig. 3. Compound **6** dramatically attenuates the cell death induced by glutamate in a dose-dependent manner in mice hippocampal cell line HT-22 cells. Cells were pretreated ahead with compound **6** at different concentrations for 30 min and then exposed to glutamate (2 mM) for 24 h. After that, the cell viability was tested by MTT assay ($n = 6$).

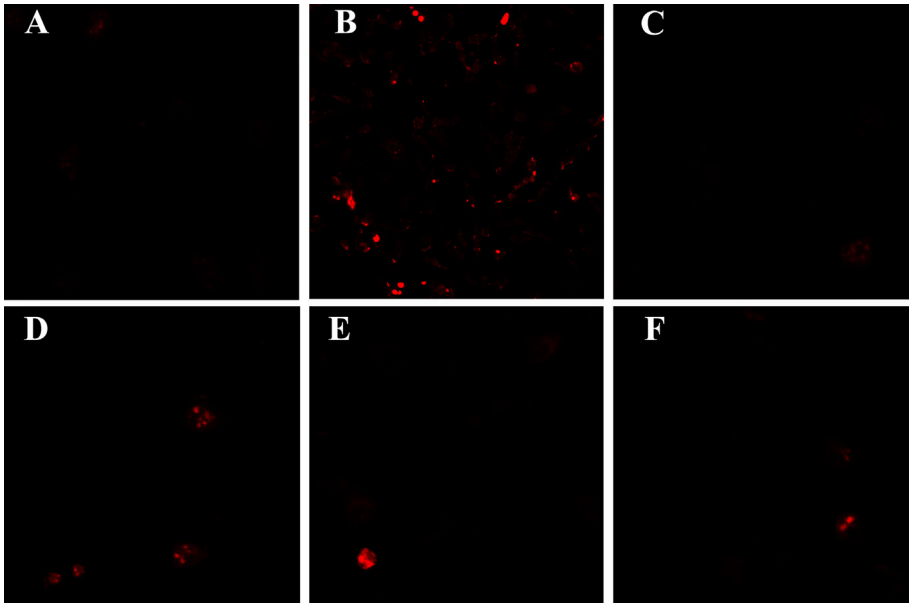


Fig. 4. Compound **6** dramatically attenuates the ROS induced by glutamate in mice hippocampal cell line HT22 cells. HT22 cells were treated with Compound **6** for 30 min and further exposed to glutamate (2 mM) for 10 h. DHE staining was applied for ROS detection. (A) Vehicle; (B) Glutamate; (C) Compound **6** (30 μM); (D) Compound **6** (3 μM) + glutamate; (E) Compound **6** (10 μM) + glutamate and (F) Compound **6** (30 μM) + glutamate group, respectively. The data showed here are representative results of three independent experiments.

independent way. With low toxicity and potent neuroprotective effects, **6** could serve as a potential lead compound to further develop into novel agents with potent neuroprotection against oxidative stress-associated CNS diseases such as AD.

5. Experiments

5.1. Chemistry

5.1.1. General information

All solvents were commercially available and were used without a further purification unless stated. The chemicals used were either purchased from commercial sources or prepared according to literature procedures. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance spectrometer 400 at 400 MHz and 100 MHz respectively. Chemical shifts are given in ppm (δ) referenced to *$CDCl_3$* with 7.26 for ^{1}H and 77.10 for ^{13}C, and to *d_6-DMSO* with 2.50 for ^{1}H and 39.50 for ^{13}C. In the case of multiplet, the signals are reported as intervals. Signals are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are expressed in Hertz. The progress of the reactions was monitored by thin-layer chromatography on a glass plate coated with silica gel with fluorescent indicator (GF254). Column chromatography was performed on silica gel (200–300 mesh). Purity of all final compounds was determined by Agilent 1100 HPLC system using an Eclipse XDB-C18 column. In HPLC conditions, flow rate was set at 1 mL/min, and gradient elution was run for 20 min, from 85% MeOH/H_2O to 95% MeOH/H_2O in 15 min and 95% MeOH/H_2O in 5 min.

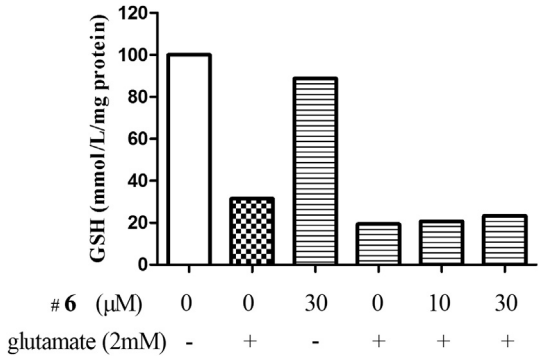


Fig. 5. Compound **6** does not reverse the GSH depletion induced by glutamate in mice hippocampal cell line HT22 cells. HT22 cells were treated with Compound **6** for 30 min and further exposed to glutamate (2 mM) for 10 h. GSH levels were analyzed by the GSH detection kit. The data showed here are representative results of three independent experiments.

5.1.2. Synthesis and characterization of the carbazole derivatives

General procedure for the synthesis of carbazoles: To a stirred solution of cyclic diphenyliodonium trifluoromethanesulfonate (100 mg, 234 μmol, 1 eq) in *i*PrOH (1.8 mL) and ethylene glycol (0.2 mL), was added amine (4 eq), sodium carbonate (3 eq), $Cu(OAc)_2$ (0.2 eq). The reaction proceeded at a reflux for 16 h under argon atmosphere before *i*PrOH was removed by a rotary evaporation. The remained mixture was partitioned between water and EtOAc, and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with H_2O and brine, dried over anhydrous Na_2SO_4, evaporated in a vacuo. The residue was purified by column chromatography on a silica gel (PE/EtOAc) to provide carbazole derivatives.

*5.1.2.1. 2-Iodo-1,1′:4′,1″-terphenyl (**6b**).* To a stirred solution of [1,1′-biphenyl]-4-ylboronic acid (542.5 mg, 2.74 mmol) in EtOH (10 mL) was added 2-iodoaniline (500 mg, 2.28 mmol), K_3PO_4 (1.21 g, 5.71 mmol), $Pd(PPh_3)_4$ (131.9 mg, 0.11 mmol). The reaction proceeded at a reflux for 6 h under argon atmosphere before EtOH was removed by rotary evaporation. The remained mixture was partitioned between water and EtOAc, and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with H_2O and brine, dried over anhydrous Na_2SO_4, and evaporated in a vacuo. The residue was purified by column chromatography on a silica gel (PE/EtOAc = 20/1–10/1) to provide coupled aminoterphenyl (476 mg, 85% yield) as a yellow liquid. To a stirred

solution of amino-terphenyl (278 mg, 1.13 mmol) in THF (5 mL) was added 4 M aqueous HCl (2.8 mL), and the solution was cooled in an ice water bath. A solution of $NaNO_2$ (93.8 mg, 1.36 mmol) in H_2O (3 mL) was added dropwise. After 20 min, a solution of KI (470.3 mg, 2.83 mmol) in H_2O (5 mL) was added, and stirred for 10 min in the ice water bath. Then the solution was slowly warmed up to r.t. and stirred for 1 h before 1 M aqueous $Na_2S_2O_3$ was added until the color of the mixture didn't change. The phases were separated, and the aqueous phase extracted with EtOAc (15 mL × 3). The combined organic layers were washed with H_2O (5 mL × 2) and brine (5 mL × 1), dried over anhydrous Na_2SO_4, concentrated in a vacuo. The residue was purified by column chromatography on silica gel (PE/EtOAc = 20/1–10/1) to provide **6b** (326 mg, 81% yield) as a yellow liquid.

*5.1.2.2. 3-phenyldibenzo[b,d]iodol-5-ium trifluoromethanesulfonate (**6a**).* To a stirred solution of **6b** (150 mg, 421.1 μmol) in anhydrous CH_2Cl_2 (5 mL) was added *m*CPBA (75%, 145.4 mg, 631.7 μmol), TfOH (111.5 μL, 1.26 mmol). The solution was stirred for 1 h at r.t. before CH_2Cl_2 was removed by rotary evaporation. Et_2O (5 mL) was added to the remained solid. The mixture was stirred for 20 min, and then filtered. The obtained solid was washed with Et_2O three times, dried in high vacuo to provide **6a** (213 mg, 100% yield) as a black solid.

*5.1.2.3. 2-phenyl-9-(p-tolyl)-9H-carbazole (**6**).* Following the general procedure for the synthesis of carbazoles, **6** was obtained as a yellow solid at 71% yield. HPLC t_R = 9.074 min, 95.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 7.9 Hz, 1H), 7.64–7.56 (m, 2H), 7.50 (d, *J* = 1.5 Hz, 1H), 7.47–7.36 (m, 4H), 7.32 (t, *J* = 7.3 Hz, 1H), 7.21–7.12 (m, 2H), 7.07 (m, 5H), 2.30 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.8, 141.9, 139.8, 131.2, 130.0, 129.5, 128.9, 128.8, 127.5, 127.3, 120.5, 118.6, 114.0, 101.2, 20.9 ppm. IR υ 3048, 2949, 1709, 1589, 1423, 1333, 1252, 1120, 1056, 969, 780, 650, 589 cm^{-1}. M.P. 107.2–108.1 °C. LRMS (ESI, *m*/*z*): 334.2 $[M + H]^+$. HRMS calcd for $C_{25}H_{20}N$ $[M + H]^+$: 334.1596, found: 334.1602.

*5.1.2.4. 9-phenyl-9H-carbazole (**1**).* A white solid. HPLC t_R = 8.079 min, 100%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.7 Hz, 2H), 7.65–7.54 (m, 4H), 7.47 (t, *J* = 6.9 Hz, 1H), 7.41 (d, *J* = 3.7 Hz, 4H), 7.33–7.27 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.9, 137.8, 129.9, 127.5, 127.2, 125.9, 123.4, 120.3, 119.9, 109.8 ppm. IR υ 3047, 2926, 1675, 1589, 1446, 1321, 1226, 1169, 1011, 922, 746 cm^{-1}. LRMS (ESI, *m*/*z*): 244.2 $[M + H]^+$.

*5.1.2.5. 9-(p-tolyl)-9H-carbazole (**2**).* A white solid. HPLC t_R = 9.764 min, 99.21%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.8 Hz, 2H), 7.47–7.35 (m, 9H), 7.29 (d, *J* = 1.9 Hz, 1H), 2.49 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.1, 137.4, 135.0, 130.5, 127.0, 125.8, 123.2, 120.2, 119.7, 109.8, 21.2 ppm. IR υ 3039, 2933, 1601, 1589, 1456, 1331, 1222, 1169, 1011, 931, 735 cm^{-1}. M.P. 100.7–101.0 °C. LRMS (ESI, *m*/*z*): 258.2 $[M + H]^+$. HRMS calcd for $C_{19}H_{16}N$ $[M + H]^+$: 258.1277, found: 258.1285.

*5.1.2.6. 9-(4-methoxyphenyl)-9H-carbazole (**3**).* A white solid. HPLC t_R = 7.452 min, 97.99%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.7 Hz, 2H), 7.49–7.37 (m, 4H), 7.36–7.26 (m, 4H), 7.12 (d, *J* = 8.5 Hz, 2H), 3.93 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.8, 140.3, 129.3, 127.5, 124.8, 122.1, 119.2, 118.6, 114.0, 108.6, 54.5 ppm. IR υ 3047, 2939, 2837, 1597, 1511, 1453, 1317, 1242, 1172, 1109, 1026, 829, 749, 583 cm^{-1}. LRMS (ESI, *m*/*z*): 274.2 $[M + H]^+$. HRMS calcd for $C_{19}H_{16}NO$ $[M + H]^+$: 274.1226, found: 274.1240.

*5.1.2.7. 9-(4-(tert-butyl)phenyl)-9H-carbazole (**4**).* A yellow solid. HPLC t_R = 15.021 min, 99.21%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (m, 2H), 7.60 (m, 2H), 7.48 (m, 2H), 7.41 (m, 4H), 7.26 (m, 2H), 1.43 (d, *J* = 3.6 Hz, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.5, 141.1, 135.0, 126.7, 126.6, 125.8, 123.3, 120.2, 119.7, 34.8, 31.4 ppm. IR υ 3129, 3048, 2953, 1913, 1781, 1670, 1597, 1515, 1451, 1317, 1230, 1182, 1110, 1013, 920, 834, 742, 625, 551 cm^{-1}. LRMS (ESI, *m*/*z*): 300.2 $[M + H]^+$.

*5.1.2.8. 9-(4-(tert-butyl)phenyl)-3-methoxy-9H-carbazole (**5**).* A white solid. HPLC t_R = 9.517 min, 98.76%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13–8.05 (m, 1H), 7.65–7.55 (m, 3H), 7.52–7.46 (m, 2H), 7.43–7.31 (m, 3H), 7.25 (s, 1H), 7.07–7.00 (m, 1H), 3.94 (d, *J* = 5.5 Hz, 3H), 1.42 (d, *J* = 5.5 Hz, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.2, 150.2, 141.4, 136.0, 135.2, 126.7, 126.4, 125.8, 123.6, 123.1, 120.2, 119.3, 114.9, 110.7, 110.0, 103.1, 56.1, 34.8, 31.4 ppm. IR υ 3052, 2945, 1598, 1464, 1270, 1187, 1023, 840, 741, 620 cm^{-1}. M.P. 104.9–106.4 °C. LRMS (ESI, *m*/*z*): 330.2 $[M + H]^+$. HRMS calcd for $C_{23}H_{23}NO$ $[M + H]^+$: 330.1852, found: 330.1867.

*5.1.2.9. 9-(4-fluorophenyl)-9H-carbazole (**7**).* A yellow solid. HPLC t_R = 7.206 min, 96.54%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.7 Hz, 2H), 7.56–7.50 (m, 2H), 7.45–7.39 (m, 2H), 7.35–7.28 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.6 (d, ^1J = 247.4 Hz), 140.0, 132.6, 128.0 (d, ^3J = 8.6 Hz), 126.7, 125.0, 123.7 (d, ^3J = 8.8 Hz), 122.2, 119.3, 118.9, 115.8 (d, ^2J = 22.6 Hz), 115.0 (d, ^2J = 22.9 Hz), 108.5 ppm. IR υ 3054, 2946, 1897, 1599, 1505, 1448, 1328, 1224, 1093, 1025, 916, 823, 739, 568 cm^{-1}. LRMS (ESI, *m*/*z*): 262.2 $[M + H]^+$.

*5.1.2.10. 9-(3-fluorophenyl)-9H-carbazole (**8**).* A yellow solid. HPLC t_R = 8.188 min, 98.44%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.7 Hz, 2H), 7.58 (q, *J* = 8.0 Hz, 1H), 7.50–7.36 (m, 5H), 7.35–7.27 (m, 3H), 7.18 (td, *J* = 8.4, 2.5 Hz, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.5, 131.0 (d, ^3J = 9.3 Hz), 126.1, 123.5, 122.7 (d, ^4J = 3.1 Hz), 120.4, 120.3, 114.5 (d, ^3J = 6.9 Hz), 114.3 (d, ^3J = 8.7 Hz), 109.7 ppm. IR υ 3041, 2958, 1895, 1597, 1500, 1450, 1319, 1223, 1083, 1015, 913, 822, 746, 570 cm^{-1}. LRMS (ESI, *m*/*z*): 262.2 $[M + H]^+$.

*5.1.2.11. 9-(4-chlorophenyl)-9H-carbazole (**9**).* A purple solid. HPLC t_R = 10.229 min, 98.45%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.6 Hz, 2H), 7.55 (q, *J* = 8.0 Hz, 4H), 7.46–7.34 (m, 4H), 7.30 (t, *J* = 6.8 Hz, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.7, 136.3, 133.1, 130.1, 128.4, 126.1, 123.5, 120.4, 120.2, 109.6 ppm. IR υ 3032, 2949, 1895, 1569, 1436, 1358, 1238, 1080, 1032, 836, 759, 650, 533 cm^{-1}. LRMS (ESI, *m*/*z*): 278.2 $[M + H]^+$.

*5.1.2.12. 9-(4-bromophenyl)-9H-carbazole (**10**).* A yellow solid. HPLC t_R = 10.997 min, 98.46%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.6 Hz, 2H), 7.83–7.61 (m, 3H), 7.50–7.35 (m, 5H), 7.30 (t, *J* = 6.8 Hz, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.6, 138.9, 136.8, 133.1, 132.4, 129.9, 129.4, 128.7, 128.0, 126.1, 124.4, 123.5, 120.9, 120.4, 120.2, 109.6 ppm. IR υ 3052, 2928, 1902, 1576, 1474, 1328, 1227, 1063, 1003, 828, 747, 615, 529 cm^{-1}. LRMS (ESI, *m*/*z*): 322.2, 324.2 $[M + H]^+$.

*5.1.2.13. 9-(4-(trifluoromethyl)phenyl)-9H-carbazole (**11**).* A brown solid. HPLC t_R = 8.929 min, 96.83%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.7 Hz, 2H), 7.88 (d, *J* = 7.2 Hz, 2H), 7.73 (d, *J* = 8.1 Hz, 2H), 7.47–7.39 (m, 4H), 7.38–7.28 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl3) δ 139.3, 137.9, 137.7, 133.9, 130.1, 129.6, 128.9, 128.3, 128.1, 127.4, 127.0, 126.5, 126.1 (d, ^3J = 6.1 Hz), 125.2, 122.7, 119.5, 119.4 (d, ^2J = 31.8 Hz), 113.6, 108.5 ppm. IR υ 3042, 2949, 1623, 1559, 1424, 1376, 1170, 1031, 950, 750 cm^{-1}. LRMS (ESI, *m*/*z*): 312.3 $[M + H]^+$.

*5.1.2.14. 4-(9H-carbazol-9-yl)-N,N-dimethylaniline (**12**).* A white solid. HPLC t_R = 9.701 min, 96.32%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.8 Hz, 2H), 7.45–7.30 (m, 7H), 7.29–7.22 (m, 1H), 6.91 (d, *J* = 7.2 Hz, 2H), 3.07 (d, *J* = 1.5 Hz, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$)

δ 148.8, 140.6, 127.2, 125.2, 124.6, 121.9, 119.1, 118.3, 112.0, 108.8, 39.6 ppm. IR υ 3049, 2931, 1731, 1608, 1526, 1453, 1345, 1237, 1104, 944, 811, 741, 565 cm^{-1}. M.P. 210.4–211.5 °C. LRMS (ESI, *m/z*): 287.2 $[M + H]^+$. HRMS calcd for $C_{20}H_{19}N_2$ $[M + H]^+$: 287.1543, found: 287.1556.

*5.1.2.15. 9-(5-methylpyridin-2-yl)-9H-carbazole (**13**).* A yellow solid. HPLC t_R = 4.486 min, 98.46%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.55 (s, 1H), 8.12 (d, *J* = 7.7 Hz, 2H), 7.81–7.70 (m, 3H), 7.53 (d, *J* = 8.2 Hz, 1H), 7.43 (t, *J* = 7.7 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 2.46 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.8, 149.5, 139.8, 139.0, 131.0, 126.1, 124.1, 120.6, 120.2, 118.8, 111.0, 18.1 ppm. IR υ 3046, 2982, 1623, 1557, 1423, 1386, 1196, 1125, 945, 749, 683 cm^{-1}. M.P. 104.4–105.1 °C. LRMS (ESI, *m/z*): 259.2 $[M + H]^+$. HRMS calcd for $C_{18}H_{15}N_2$ $[M + H]^+$: 259.1230, found: 259.1233.

*5.1.2.16. 9-butyl-9H-carbazole (**14**).* A white solid. HPLC t_R = 7.786 min, 98.96%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, *J* = 7.8 Hz, 2H), 7.52–7.38 (m, 4H), 7.26–7.20 (m, 2H), 4.32 (t, *J* = 7.2 Hz, 2H), 1.95–1.80 (m, 2H), 1.42 (dq, *J* = 14.8, 7.4 Hz, 2H), 0.96 (t, *J* = 7.4 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.4, 125.6, 122.8, 120.3, 118.7, 108.6, 42.8, 31.1, 20.6, 13.9 ppm. IR υ 3049, 2938, 1740, 1593, 1460, 1336, 1224, 1142, 1017, 740 cm^{-1}. LRMS (ESI, *m/z*): 224.2 $[M + H]^+$. HRMS calcd for $C_{16}H_{18}N$ $[M + H]^+$: 224.1434, found: 224.1445.

*5.1.2.17. 9-cyclohexyl-9H-carbazole (**15**).* A yellow solid. HPLC t_R = 9.948 min, 96.99%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, *J* = 7.4 Hz, 2H), 7.57 (d, *J* = 8.0 Hz, 2H), 7.43 (t, *J* = 7.7 Hz, 2H), 7.21 (t, *J* = 7.4 Hz, 2H), 4.59–4.40 (m, 1H), 2.49–2.32 (m, 2H), 2.00 (t, *J* = 14.5 Hz, 4H), 1.63–1.48 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 125.2, 120.3, 118.5, 55.4, 30.7, 26.5, 25.7 ppm. IR υ 3054, 2926, 2855, 1595, 1450, 1326, 1215, 1134, 814, 743 cm^{-1}. LRMS (ESI, *m/z*): 250.2 $[M + H]^+$.

*5.1.2.18. 4-(9H-carbazol-9-yl)butan-1-ol (**16**).* A white solid. HPLC t_R = 2.966 min, 98.66%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.7 Hz, 2H), 7.50–7.38 (m, 4H), 7.25–7.20 (m, 2H), 4.45–4.29 (m, 2H), 3.72–3.57 (m, 2H), 2.06–1.92 (m, 2H), 1.42 (brs, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.4, 125.6, 122.9, 120.4, 118.8, 108.6, 62.5, 42.8, 30.3, 25.5 ppm. IR υ 3445, 3036, 2975, 1689, 1573, 1439, 1336, 1247, 1156, 1055, 760 cm^{-1}. LRMS (ESI, *m/z*): 240.2 $[M + H]^+$.

*5.1.2.19. 9-tosyl-9H-carbazole (**17**).* A white solid. HPLC t_R = 5.013 min, 100%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, *J* = 8.3 Hz, 2H), 7.91 (d, *J* = 7.7 Hz, 2H), 7.70 (d, *J* = 8.2 Hz, 2H), 7.54–7.45 (m, 2H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.10 (d, *J* = 8.1 Hz, 2H), 2.26 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.8, 138.4, 135.0, 129.6, 127.4, 126.5, 126.4, 123.9, 120.0, 115.2, 21.5 ppm. IR υ 2931, 1691, 1589, 1441, 1357, 1146, 959, 843, 750, 669, 550 cm^{-1}. LRMS (ESI, *m/z*): 322.0 $[M + H]^+$. HRMS calcd for $C_{19}H_{15}NO_2SNa$ $[M + Na]^+$: 334.0716, found: 334.0728.

*5.1.2.20. 9-((4-ethylphenyl)sulfonyl)-9H-carbazole (**18**).* A white solid. HPLC t_R = 6.063 min, 100%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.34 (d, *J* = 8.4 Hz, 2H), 7.91 (d, *J* = 7.7 Hz, 2H), 7.74 (d, *J* = 6.6 Hz, 2H), 7.50 (t, *J* = 7.8 Hz, 2H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.13 (d, *J* = 7.0 Hz, 2H), 2.57 (q, *J* = 7.3 Hz, 2H), 1.13 (td, *J* = 7.6, 2.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.8, 138.4, 135.3, 128.5, 127.4, 126.6, 126.4, 123.9, 120.0, 115.1, 28.7, 14.7 ppm. IR υ 2933, 1919, 1726, 1595, 1439, 1358, 1168, 970, 827, 751, 653, 578 cm^{-1}. M.P. 122.1–123.0 °C. LRMS (ESI, *m/z*): 336.2 $[M + H]^+$. HRMS calcd for $C_{20}H_{16}NO_2S$ $[M - H]^-$: 334.0902, found: 334.0906.

*5.1.2.21. 2-methoxy-9-tosyl-9H-carbazole (**19**).* A white solid. HPLC t_R = 5.176 min, 96.66%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.26 (m, 1H), 7.88 (brs, 1H), 7.84–7.73 (m, 2H), 7.69 (m, 2H), 7.39 (m, 1H), 7.31 (m, 1H), 7.11 (m, 2H), 6.96 (m, 1H), 3.95 (d, *J* = 5.1 Hz, 3H), 2.27 (d, *J* = 4.6 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.8, 144.9, 139.8, 138.4, 135.0, 129.7, 126.5, 126.0, 123.9, 120.6, 119.1, 115.1, 112.1, 99.9, 55.8, 21.5 ppm. IR υ 3053, 2929, 1724, 1602, 1446, 1361, 1270, 1165, 986, 821, 668, 585 cm^{-1}. M.P. 125.3–126.1 °C. LRMS (ESI, *m/z*): 352.2 $[M + H]^+$. HRMS calcd for $C_{20}H_{16}NO_3S$ $[M - H]^-$: 350.0851, found 350.0845.

*5.1.2.22. 3-methoxy-9-tosyl-9H-carbazole (**20**).* A white solid. HPLC t_R = 4.803 min, 94.39%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.30 (d, *J* = 6.3 Hz, 1H), 8.22 (d, *J* = 7.3 Hz, 1H), 7.85 (s, 1H), 7.64 (d, *J* = 6.3 Hz, 2H), 7.48 (d, *J* = 6.1 Hz, 1H), 7.34 (s, 2H), 7.08 (s, 3H), 3.90 (d, *J* = 4.2 Hz, 3H), 2.26 (d, *J* = 4.4 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.8, 144.7, 139.1, 134.8, 132.7, 129.6, 127.6, 127.4, 126.6, 126.4, 123.8, 119.9, 116.3, 115.5, 115.3, 103.2, 100.0, 55.8, 21.5 ppm. IR υ 2931, 1602, 1451, 1360, 1174, 1029, 974, 812, 741, 675, 570 cm^{-1}. M.P. 127.5–128.3 °C. LRMS (ESI, *m/z*): 352.3 $[M + H]^+$. HRMS calcd for $C_{20}H_{16}NO_3S$ $[M - H]^-$: 350.0851, found: 350.0855.

*5.1.2.23. 2-phenyl-9-tosyl-9H-carbazole (**21**).* A yellow solid. HPLC t_R = 9.577 min, 97.28%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.34 (d, *J* = 8.4 Hz, 1H), 7.95 (d, *J* = 8.1 Hz, 1H), 7.92 (d, *J* = 7.8 Hz, 1H), 7.76–7.70 (m, 4H), 7.61 (d, *J* = 8.0 Hz, 1H), 7.50 (q, *J* = 7.6 Hz, 3H), 7.44–7.35 (m, *J* = 14.7, 7.1 Hz, 2H), 7.11 (d, *J* = 8.2 Hz, 2H), 2.27 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.9, 141.2, 140.8, 139.1, 138.8, 135.0, 129.7, 128.9, 127.6, 127.4, 126.5, 126.2, 125.5, 124.0, 123.4, 120.2, 120.0, 115.2, 113.6, 21.5 ppm. IR υ 3048, 2923, 1728, 1597, 1458, 1367, 1283, 1173, 1093, 974, 755, 674, 578 cm^{-1}. M.P. 107.5–108.8 °C. LRMS (ESI, *m/z*): 398.3 $[M + H]^+$. HRMS calcd for $C_{25}H_{13}NO_2SNa$ $[M + Na]^+$: 420.1029, found: 420.1029.

*5.1.2.24. 9-tosyl-9H-carbazole-2-carbonitrile (**22**).* A white solid. HPLC t_R = 4.004 min, 98.59%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.65 (s, 1H), 8.36 (d, *J* = 8.4 Hz, 1H), 8.04–7.92 (m, 2H), 7.72 (d, *J* = 7.5 Hz, 2H), 7.67–7.57 (m, 2H), 7.43 (t, *J* = 7.5 Hz, 1H), 7.16 (d, *J* = 8.0 Hz, 2H), 2.30 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.6, 139.3, 137.6, 134.5, 130.0, 129.8, 129.4, 127.2, 126.5, 124.8, 124.5, 120.9, 120.8, 119.3, 119.0, 115.2, 110.2, 21.6 ppm. IR υ 2948, 2245, 1576, 1413, 1340, 1155, 979, 815, 728, 670, 591 cm^{-1}. M.P. 174.8–176.1 °C. LRMS (ESI, *m/z*): 347.2 $[M + H]^+$. HRMS calcd for $C_{20}H_{13}N_2O_2S$ $[M - H]^-$: 345.0698, found: 345.0704.

*5.1.2.25. 9-tosyl-2-(trifluoromethyl)-9H-carbazole (**23**).* A yellow solid. HPLC t_R = 6.427 min, 100%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.63 (s, 1H), 8.36 (d, *J* = 8.4 Hz, 1H), 7.98 (dd, *J* = 16.9, 7.9 Hz, 2H), 7.71 (d, *J* = 8.3 Hz, 2H), 7.66–7.54 (m, 2H), 7.41 (t, *J* = 7.6 Hz, 1H), 7.14 (d, *J* = 8.4 Hz, 2H), 2.29 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.4, 139.2, 137.8, 134.7, 129.8, 129.1, 128.7, 126.5, 125.1, 124.3, 120.8–120.6 (m), 120.3, 115.2, 112.5 (d, ^3J = 4.3 Hz) ppm. IR υ 2932, 1596, 1433, 1325, 1169, 983, 814, 728, 660, 577 cm^{-1}. M.P. 153.1–153.9 °C. LRMS (ESI, *m/z*): 390.2 $[M + H]^+$. HRMS calcd for $C_{20}H_{13}F_3NO_2S$ $[M - H]^-$: 388.0619, found: 388.0620.

*5.1.2.26. 11-(p-tolyl)-11H-benzo[a]carbazole (**24**).* A white solid. HPLC t_R = 12.615 min, 98.93%. ^1H NMR (400 MHz, $CDCl_3$) δ 8.28–8.15 (m, 2H), 7.99 (d, *J* = 8.1 Hz, 1H), 7.72 (d, *J* = 8.4 Hz, 1H), 7.53–7.39 (m, 6H), 7.39–7.30 (m, 2H), 7.23 (s, 1H), 7.18 (d, *J* = 7.8 Hz, 1H), 2.57 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 142.2, 138.8, 137.4, 135.6, 133.5, 130.7, 129.1, 128.8, 124.9, 124.7, 123.3, 122.3, 122.1, 121.0, 120.2, 119.4, 119.3, 119.1, 110.4, 21.4 ppm. IR υ 3030, 2946, 1699, 1601, 1463, 1378, 1220, 1083, 1045, 920, 809, 750, 678, 556 cm^{-1}. LRMS (ESI, *m/z*): 308.1 $[M + H]^+$.

*5.1.2.27. 11-tosyl-11H-benzo[a]carbazole (**25**).* A yellow solid. HPLC t_R = 5.900 min, 95.28%. ^1H NMR (400 MHz, $CDCl_3$) δ 9.02 (d,

J = 8.4 Hz, 1H), 8.34 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.3 Hz, 1H), 7.84 (d, J = 8.6 Hz, 1H), 7.79–7.75 (m, 1H), 7.73–7.64 (m, 2H), 7.57 (t, J = 7.5 Hz, 1H), 7.45 (t, J = 7.8 Hz, 1H), 7.35 (t, J = 7.2 Hz, 1H), 6.83 (d, J = 6.8 Hz, 2H), 6.74 (d, J = 7.5 Hz, 2H), 2.14 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.2, 141.8, 136.7, 133.9, 131.5, 130.1, 128.5, 128.2, 127.8, 127.6, 127.0, 126.9, 126.6, 126.3, 126.1, 125.9, 125.6, 119.8, 119.4, 117.4, 21.4 ppm. IR υ 3050, 2926, 1725, 1589, 1450, 1360, 1172, 1092, 1029, 915, 815, 755, 669, 569 cm^{-1}. M.P. 144.9–145.7 °C. LRMS (ESI, m/z): 372.1 $[M + H]^+$.

5.2. Biological assay

5.2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibico-BRL (Grand Island, NY, USA). Dihydroethidium (DHE), 3-(3,4-dimehylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. Carbazoles were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C.

5.2.2. Cell culture and cell treatment

HT22 murine hippocampal neuronal cells (a gift from Prof. Jun Liu, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, China) [16] were maintained in DMEM supplemented with 10% (v/v) FBS and incubated at 37 °C under 5% CO_2. To study the neuroprotective effects of carbazoles, cells were seeded in 96-well plates at a density of 4000 cells per well. The solution of carbazoles (30 mM in DMSO) was diluted in DMEM supplemented with 10% (v/v) FBS before addition to each well at the desired final concentrations. After 30 min, cells were exposed to toxins, glutamate (2 mM) or HCA (500 μM). Cells in the control group were treated with vehicle alone. Cell morphology could be observed by an inverse phase contrast microscopy (Olympus, Japan).

5.2.3. MTT assay

For determining cell viability, the MTT assay was used. MTT (10 μl, at 5 mg/mL) was added to each well in 96-well plate at a final concentration of 500 μg/mL; the mixture was further incubated for 1 h at 37 °C; and the liquid in the wells was removed thereafter. DMSO (150 μl) was added to each well, and the absorbance was read with microplate reader (Bio-Tek) at 570 nm. Results were expressed as the percentage of MTT reduction and the absorbance of control cells was set as 100%.

5.2.4. Measurement of intracellular ROS accumulation

The intracellular reactive oxygen species (ROS) level was measured using fluorescent dyes, Dihydroethidium (DHE). Once internalized, the DHE reacts with superoxide production [16,17] and is dehydrogenated to ethidium, a red fluorescent product, which then intercalates into DNA [18]. A working stock of 10 mM DHE in anhydrous DMSO was freshly prepared immediately before use. In the presence of an oxidant, DHE, a dye, combines with ROS, especially hydrogen peroxide or peroxyl radicals to produce red fluorescence [16,17]. For assay, HT22 cells were cultured for overnight and then treated with glutamate in the presence or absence of carbazole for 12 h. Cells were washed twice with PBS (pH 7.4) and incubated with DHE (10 μM) in serum-free DMEM for 30 min. After washed twice with PBS, multiple viewing fields were photographed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) and the fluorescence intensity of DHE was measured by a fluorimetric plate reader Flex Station 3 (Molecular Devices, USA) at an excitation wavelength of 485 nm and an emission wavelength of 595 nm.

5.2.5. Estimation of intracellular glutathione

A glutathione assay kit (Jiancheng Biochemical, Nanjing, China) was used to measure intracellular glutathione (GSH) concentrations. Cells were collected and sonicated in 50 μl of ice-cold lysis buffer. After centrifuging at 10,000× g for 15 min at 4 °C, the supernatant was collected. 50 μl of TEAM reagent was added to 1 mL supernatant. 50 μl of sample or the standard solution provided in the kit was applied to each sample wells. 150 μl of the freshly prepared assay mixture was added to each of the wells containing standards and samples. The plate was incubated in the dark on an orbital shaker at room temperature. Absorbance was measured at 405 nm using a plate reader (Bio-Tek) after 25 min incubation. GSH concentrations were calculated according to the instructions provided by the supplier. All GSH values were normalized to per mg protein of each sample.

5.2.6. Statistical analysis

All quantitative data and experiments described in this study were repeated at least three times. Data were presented as mean ± S.D. of multiple independent experiments. Statistics were analyzed with one-way ANOVA followed by a least significant difference test (SPSS 17.0 software). Statistical difference was considered at $P < 0.05$.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.07.029.

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