Electronic Supplementary Information

Water-soluble Chiral Tetrazine Derivatives: Towards the Application of Circularly Polarized Luminescence from Upper-Excited States to Photodynamic Therapy

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The synthesis procedures of tetrazine derivatives[1]

(R)-6-chloro-N-(1-phenylethyl)-1,2,4,5-tetrazin-3-amine: 2,4,6-Collidine (0.44 mmol, 53.32 mg, 2.2 eq) is added to a solution of 3,6-dichlorotetrazine (0.2 mmol, 30.19 mg, 1.0 eq) and (R)-(+)-1-phenylethylamine (0.44 mmol, 53.24 mg, 2.2 eq) in dichloromethane (3 mL). The resulting solution is stirred overnight at room temperature then concentrated and purified by column chromatography (eluent: petroleum ether/dichloromethane at 40/1) to yield 35 mg of (R)-6-chloro-N-(1-phenylethyl)-1,2,4,5-tetrazin-3-amine as an orange-red liquid. **[α]20 D** = + 44.8 (c = 0.5, CH_3OH) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.40 – 7.25 (m, 5H, ArH), 6.54 (d, *J* = 5.3 Hz, 1H, NH), 5.25 (p, *J* = 7.0 Hz, 1H, CH), 1.68 (d, *J* = 6.9 Hz, 3H, CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 160.67, 160.61, 141.86, 128.84, 127.88, 126.13, 51.45, 22.28. **HRMS** (ESI) *m/z*: calcd for $C_{10}H_{11}ClN_5^+$ $[M + H]^+$: 236.0697, found: 236.0703.

(S)-6-chloro-N-(1-phenylethyl)-1,2,4,5-tetrazin-3-amine: The synthesis step refers to another enantiomer. **[α]20 D** = - 48.0 (c = 0.5, CH_3OH) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.38 – 7.23 (m, 5H, ArH), 6.62 (d, *J* = 6.9 Hz, 1H, NH), 5.23 (p, *J* = 7.0 Hz, 1H, CH), 1.66 (d, *J* = 6.9 Hz, 3H, CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 160.68, 160.60, 141.91, 128.86, 127.90, 126.17, 51.48, 22.33. **HRMS** (ESI) *m/z*: calcd for $C_{10}H_{11}ClN_5^+$ $[M + H]^+$: 236.0697, found: 236.0703.

(S)-6-chloro-N-(hexan-2-yl)-1,2,4,5-tetrazin-3-amine: 2,4,6-Collidine (0.44 mmol, 53.32 mg, 2.2 eq) is added to a solution of 3,6-dichlorotetrazine (0.2 mmol, 30.19 mg, 1.0 eq) and (S)-(+)-2-aminonhexane (0.44 mmol, 44.52 mg, 2.2 eq) in dichloromethane (3 mL). The resulting solution is stirred overnight at room temperature then concentrated and purified by column chromatography (eluent: petroleum ether/dichloromethane at 40/1) to yield 30 mg of (S)-6-chloro-N-(hexan-2-yl)-1,2,4,5-tetrazin-3-amine as an orange-red liquid. **[α]20 D** = - 10.0 (c = 0.5, CH_3OH) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.97 (s, 1H), 4.18 (dt, *J* = 13.5, 6.7 Hz, 1H, CH), 1.71 – 1.54 (m, 2H, CH_2), 1.35 (m, *J* = 2.0 Hz, 4H, CH_2CH_2), 1.31 (d, *J* = 6.5 Hz, 3H, CH_3), 0.89 (t, *J* = 6.9 Hz, 3H, CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 161.10, 160.12, 47.81, 36.15, 28.01, 22.45, 20.27, 13.90. **HRMS** (ESI) *m/z*: calcd for $C_8H_{15}ClN_5^+$ $[M + H]^+$: 216.1010, found: 216.1012.

(R)-6-chloro-N-(hexan-2-yl)-1,2,4,5-tetrazin-3-amine: The synthesis step refers to another enantiomer. **[α]20 D** = + 13.4 (c = 0.5, CH_3OH) **^{1}H NMR** (400 MHz,$CDCl_3$) δ 5.98 (s, 1H), 4.18 (dt, *J* = 14.8, 6.6 Hz, 1H, CH), 1.67 – 1.58 (m, 2H, CH_2), 1.34 (m, *J* = 2.0 Hz, 4H, CH_2CH_2), 1.31 (d, *J* = 6.5 Hz, 3H, CH_3), 0.89 (t, *J* = 7.0 Hz, 3H, CH_3). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 161.10, 160.12, 47.82, 36.15, 28.03, 22.46, 20.28, 13.93. **HRMS** (ESI) *m/z*: calcd for $C_8H_{15}ClN_5^+$ $[M + H]^+$: 216.1010, found: 216.1012.

The details of photodynamic therapy activity on cancer cells

Cell Culture: In growth media (DMEM supplemented with fetal bovine serum (10%), streptomycin (100.0 mg/L) and penicillin (100 IU/mL)), HeLa cells were cultured and maintained in a humidified atmosphere of 5% carbon dioxide at 37 °C for following use.

Cell imaging: Hela cells were seeded in glass-bottom dishes and grown till 70 ~ 80% confluency. Subsequently, cells were incubated with 1-R (20 μM) for 4 h, and then further incubated with 200 nM of the LysoTracker™ Red DND-99 (Thermo Fisher Scientific). Cells were then washed three times with PBS, then imaged with an inverting-typed confocal microscope (Zeiss LSM 880 meta NLO). Images of 1-R stained cells were collected at 420 ~ 480 nm by using a laser excitation wavelength at 405 nm. Images of LysoTracker™ Red stained cells were collected at 620 ~ 650 nm by using a laser excitation wavelength at 559 nm. Background signals of all images were verified to be nearly zero by imaging the same cells treated with a buffer control.

ROS imaging in HeLa cells. HeLa cells were seeded in glass-bottom dishes and grown till 70 ~ 80% confluences. Subsequently, cells were incubated with 1-R (20 μM) for 4 h. Then, according to manufacturer's instructions, ROS probe (DCFH-DA, Thermo Fisher Scientific) was added and incubated for 20 min. After that, cells were irradiated with a white light LED source for 0, 5 or 10 min. Images from DCFH-DA solution were recorded in 500-530 nm emission range with the excitation wavelength of 488 nm using confocal microscope (Zeiss LSM 880 meta NLO).

Confocal Imaging of Photoinduced Cell Death: After HeLa cells were incubated with 1-R (20 μM) for 4 h, these cells were further stained with Calcein-AM and PI and irradiated under white light LED source for 10 min. Then, the cells were inculcated another 4 h for apoptosis. Finally, confocal microscope (Zeiss LSM 880 meta NLO) was used to simultaneously acquire images of Calcein-AM emission (500-550 nm) and PI emission (600-650 nm). The excitation wavelength was at 488 nm.

Flow Cytometry: HeLa cells were seeded in the six-well plates for 24 h. Then 1-R (20 μM) was added for 4 h incubation. The medium was then replaced with fresh culture medium and irradiated by white light LED source for 10 min irradiation. Then, cells were incubated for another 4 hours to apoptosis. Afterward, these cells were stained with Annexin V–FITC/PI according to the manufacturer's instruction, trypsinized, harvested, rinsed with PBS, resuspended, and subjected to perform flow cytometric assay using BD FACSCanto II flow cytometry.

Cellular cytotoxicity: The cytotoxicity of materials is very important for bio-application. So, we use Hela cells to carry out MTT assays. In a 96-well, Hela cells was well plated for 24 h. Then, different concentrations of 1-R were added. After an occupation of 12 h, 20 μL MTT solution (20 μL, 5 mg mL^{-1}) was added to each well and incubated for another 4 h. After that, we remove supematant and add 200 μL dimethyl sulfoxide to every well. The absorbance intensity was recorded by a PowerWave XS/XS2 microplate spectrophotometer at 490 nm. The cellular viability relative to the untreated cells (control group) was calculated according to our previous paper.[2]

Statistical analysis: The statistical analysis of the samples was undertaken using a Student's t-test, and p-values < 0.05 were considered statistically significant. All data reported are means ± standard deviations, unless otherwise noted.

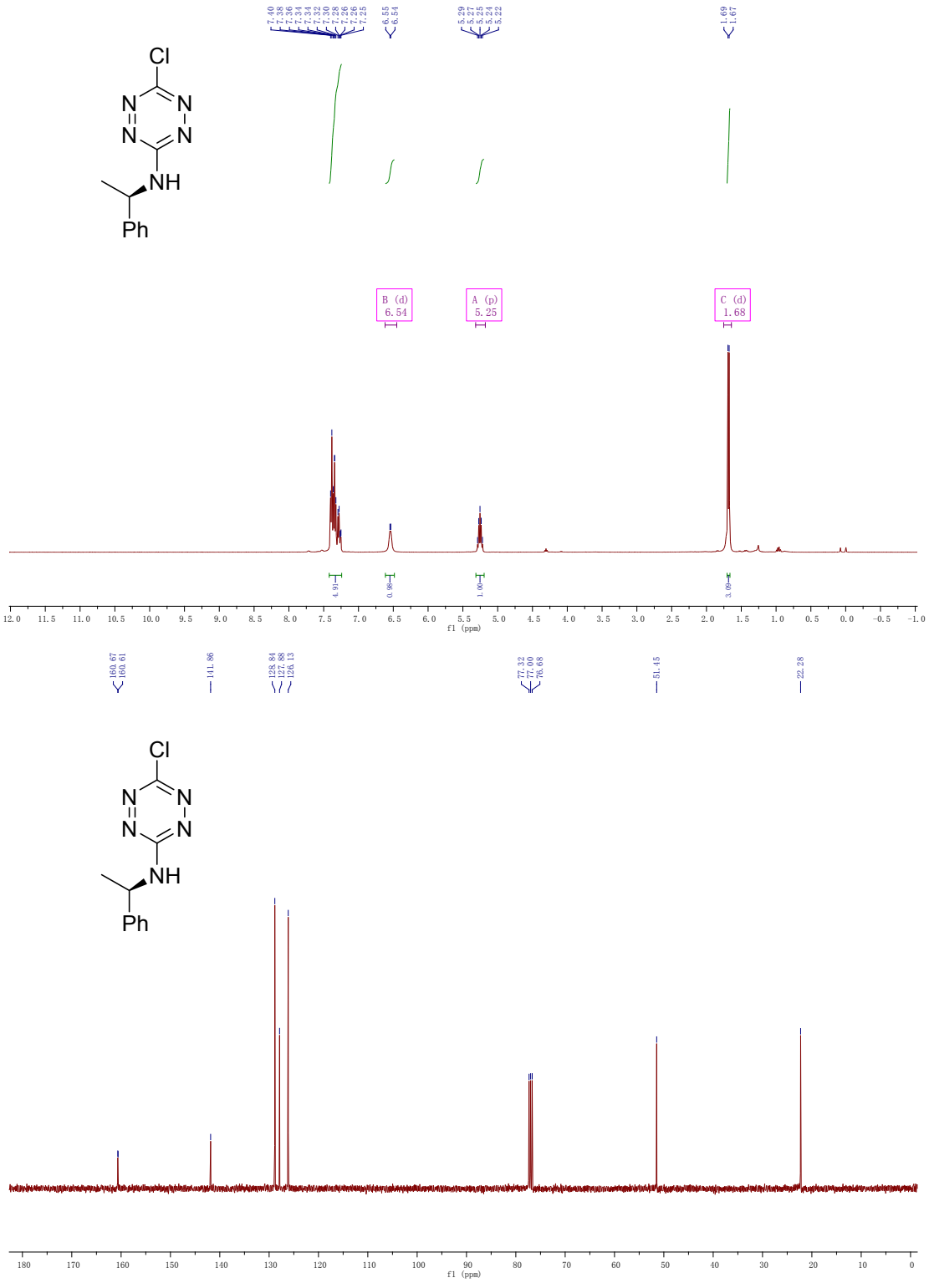


Fig. S1 ^{1}H and ^{13}C NMR spectra of (R)-6-chloro-N-(1-phenylethyl)-1,2,4,5-tetrazin-3-amine

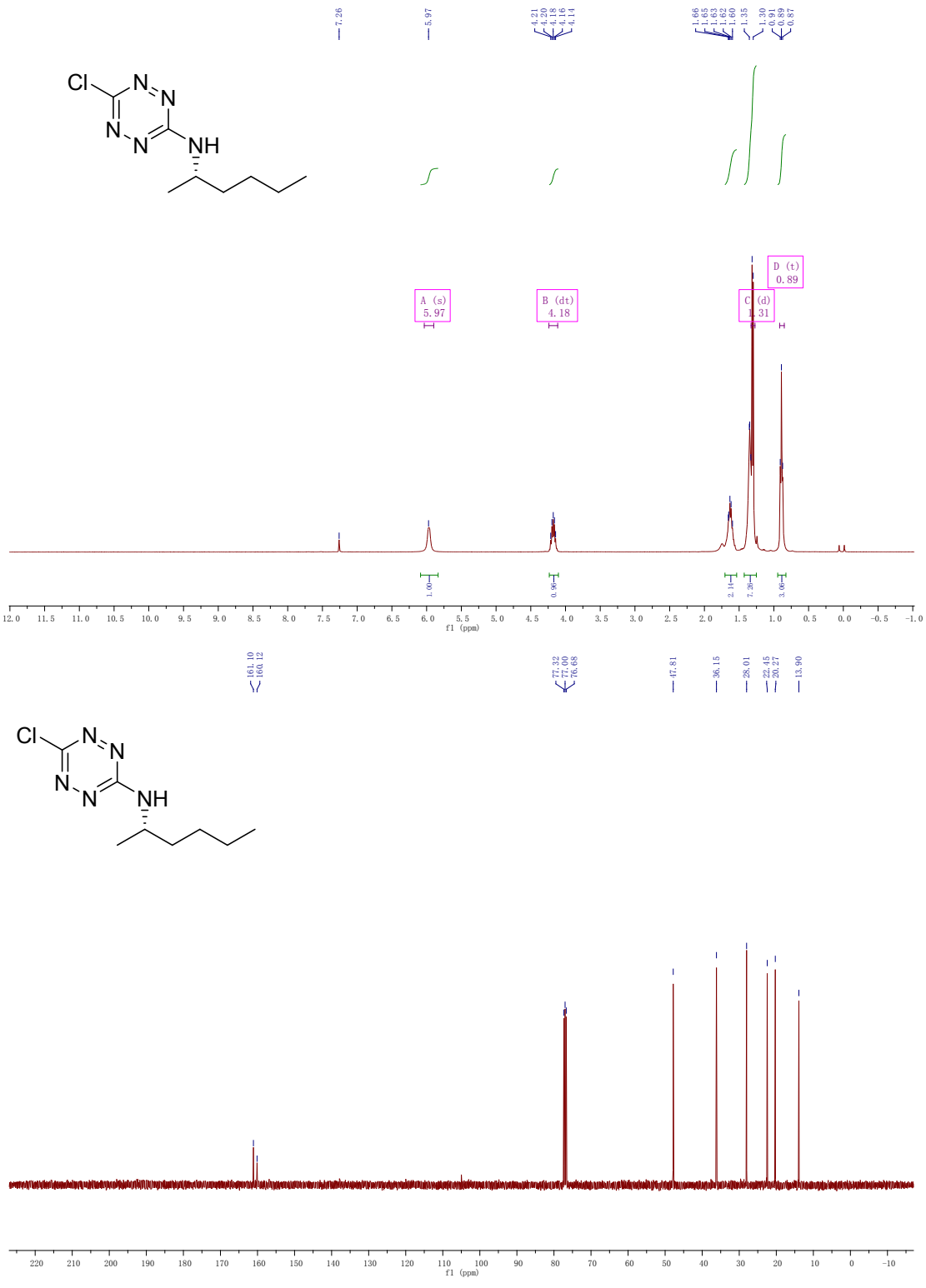


Fig. S2 ^{1}H and ^{13}C NMR spectra of (S)-6-chloro-N-(hexan-2-yl)-1,2,4,5-tetrazin-3-amine

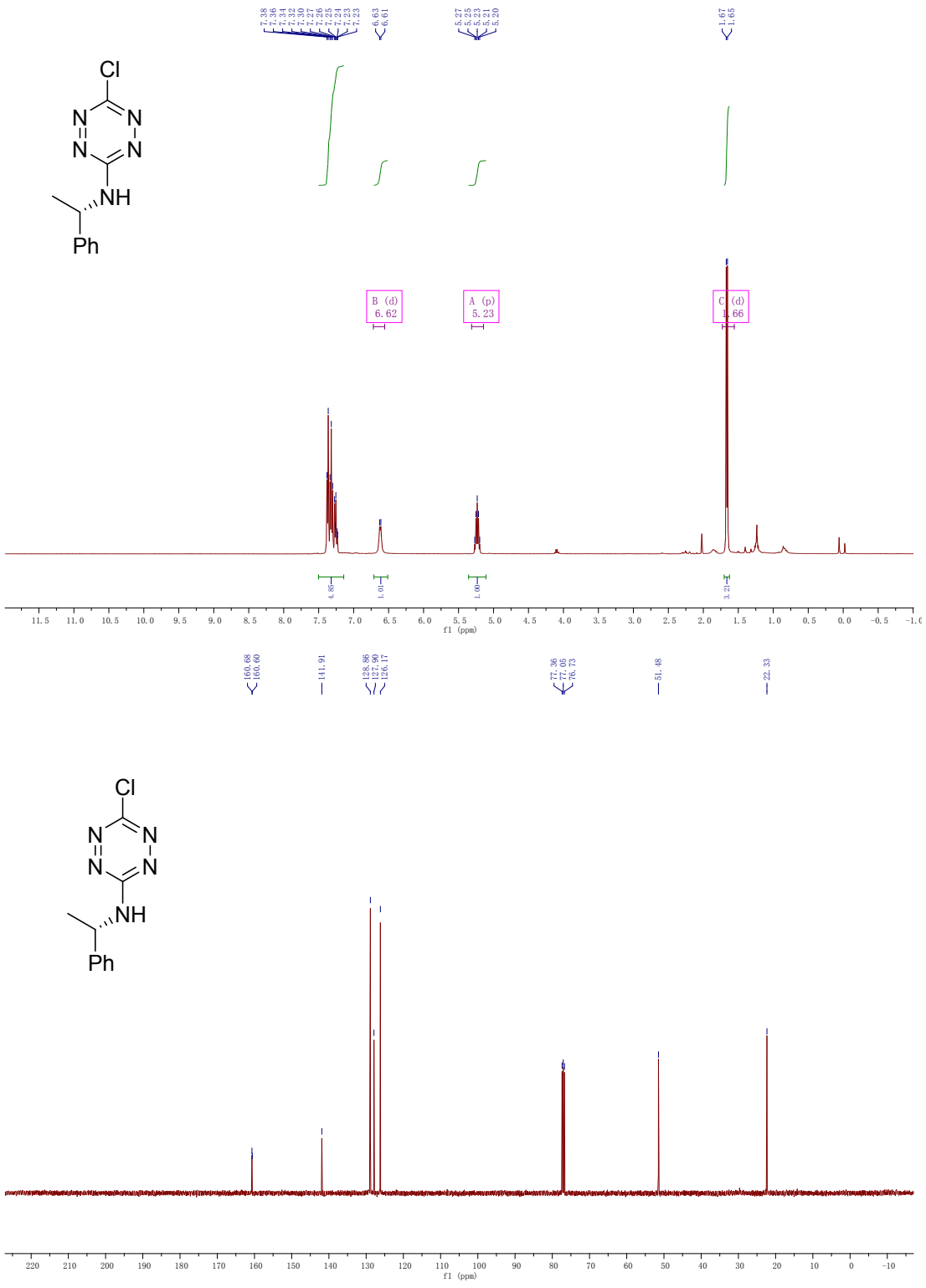


Fig. S3 ^{1}H and ^{13}C NMR spectra of (S)-6-chloro-N-(1-phenylethyl)-1,2,4,5-tetrazin-3-amine

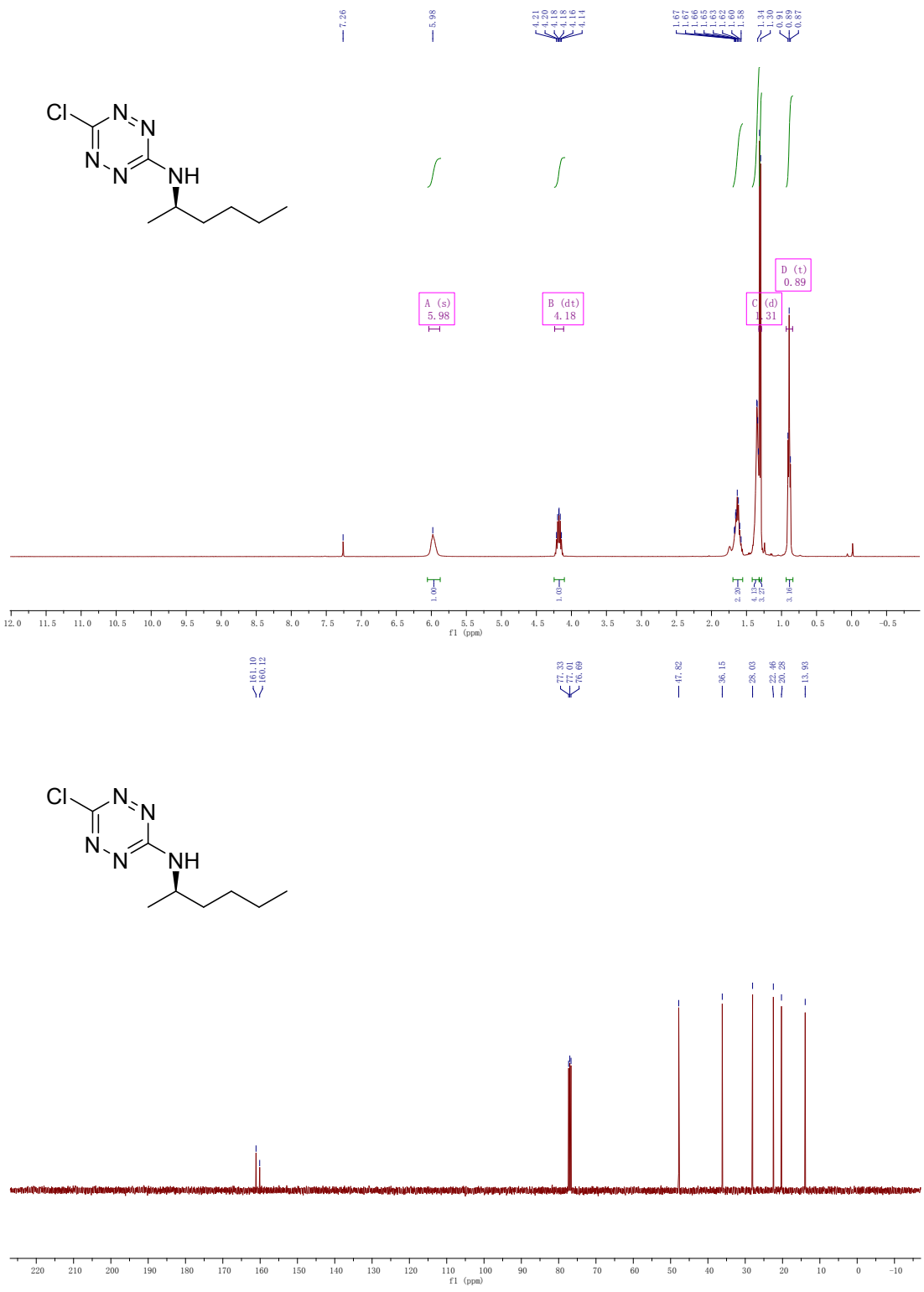


Fig. S4 ^{1}H and ^{13}C NMR spectra of (R)-6-chloro-N-(hexan-2-yl)-1,2,4,5-tetrazin-3-amine

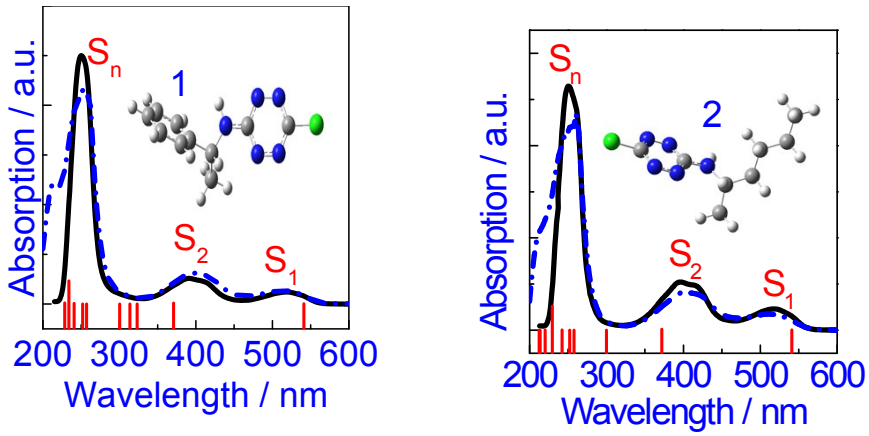


Fig. S5 Comparison of experimental absorption spectra (solid lines) and theoretically calculated spectra (dashed lines) at the B3LYP6-31g(d) level in vacuum (96 excitations) for **1** and **2**. the oscillator strength of each transition is represented by sticks. The inset: the twisted molecular geometry of **1** and **2**.

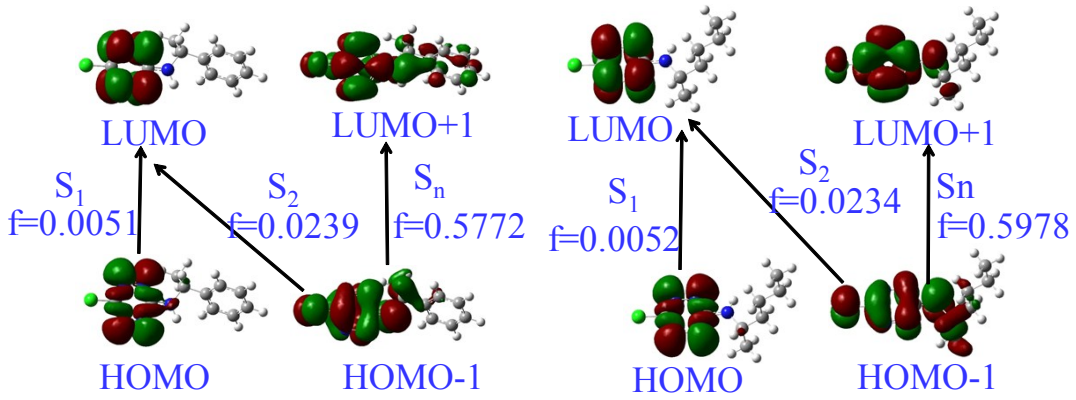
Table S1. TD-DFT calculated the first 10 transitions, corresponding excitation energies (vertical transition) and wavelength, and their oscillation strength (f) at the B3LYP-6-31g(d) level for **1** and **2**.

Molecule 1

Transition	Energy (eV)	Wavelength (nm)	Oscillation Strength
1	2.3240	533.51	0.0051
2	3.2139	385.77	0.0239
3	3.5511	349.14	0.0004
4	3.6456	340.10	0.0002
5	4.2213	293.71	0.0002
6	4.8505	255.61	0.0006
7	5.0253	246.72	0.0003
8	5.1640	240.09	0.1021
9	5.2194	237.54	0.5772
10	5.3538	231.58	0.0630

Molecule 2

Transition	Energy (eV)	Wavelength (nm)	Oscillation Strength
1	2.3209	534.20	0.0052
2	3.1957	387.97	0.0234
3	4.2240	293.52	0.0000
4	4.8280	256.80	0.0006
5	5.0255	246.71	0.0008
6	5.0732	244.39	0.0112
7	5.2731	235.13	0.5978
8	5.3817	230.38	0.0376
9	5.4811	226.21	0.0038
10	5.6531	219.32	0.0001



Chromophore 1 **Chromophore 2**

Fig. S6 Pictorial drawing of frontier orbitals, transitions and oscillation strength of **1** and **2**, calculated at the B3LYP-6-31g(d) level.

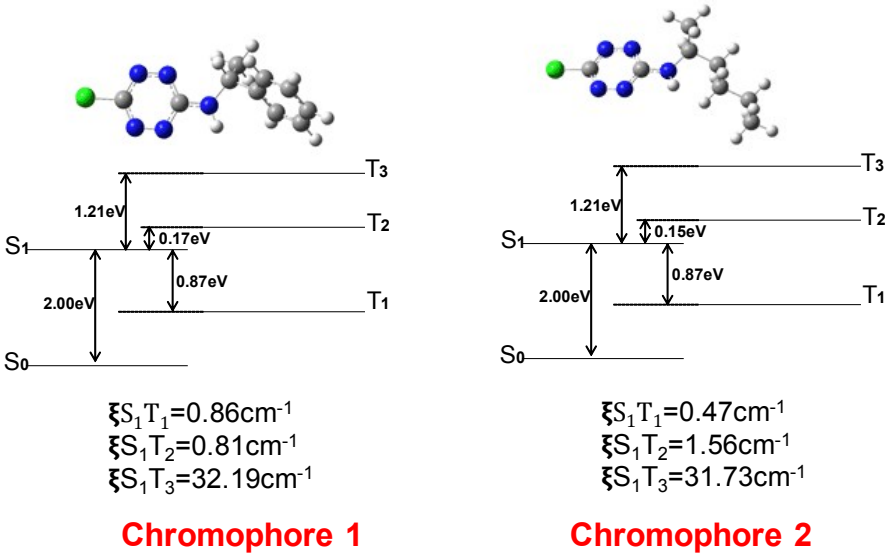


Fig. S7 Calculated vertical excitation energies of S_0, S_1, T_1, T_2 and T_3 states at fixed geometry and estimated average spin-orbit coupling (SOC) between these excited electronic states.

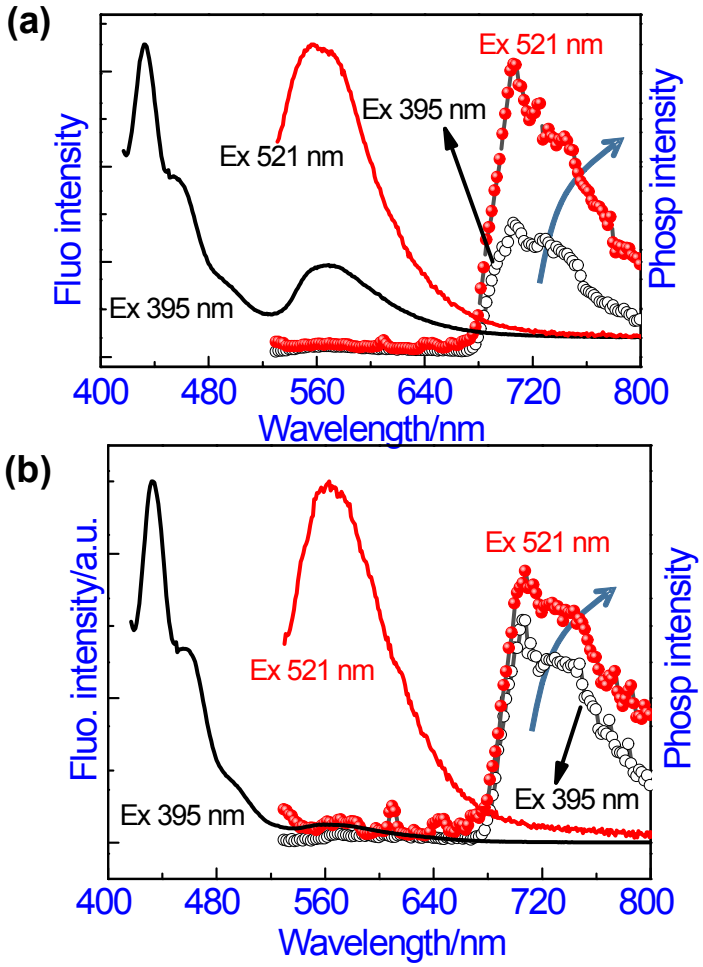


Fig. S8 fluorescence and phosphorescence spectra for **1** and **2**. The phosphorescence spectra were measured at 70 K.

References:

1. Y. -H. Gong, F. Miomandre, R. Méallet-Renault, S. Badré, L. Galmiche, J. Tang, P. Audebert, and G. Clavier, *Eur. J. Org. Chem.* 2009, **2009**, 6121-6128; C. Allain, J. Piard, A. Brosseau, M. Han, J. Paquier, T. Marchandier, M. Lequeux, C. Boissière, and P. Audebert, *ACS Appl. Mater. Interfaces* 2016, **8**, 19843-19846.
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4. W. Hu, Q. Wang, X. Miao, L. Bai, L. Li, G. S. He, J. Li, S. Yao, T. He, X. Lu, W. Huang, P. N. Prasad, Q. Fan, *J. Phys. Chem. C* 2018, **122**, 20945.