Supplemental Information

A Photolabile Curcumin-Diazirine Analogue Enables Phototherapy with Physically and Molecularly Produced Light for Alzheimer's Disease Treatment

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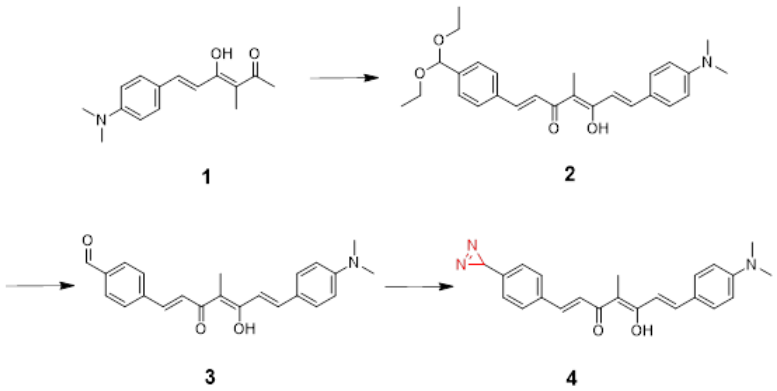
TABLE OF CONTENTS

Experimental Section

Materials and Measurements

All reagents were purchased from Sigma-Aldrich, of reagent grade quality, and were used as received unless stated otherwise. Column chromatography was performed on a glass column slurry-packed with silica gel (60 Å, 40–63 mm; SiliCycle Inc.). Recombinant Aβ peptide (1-40) was purchased from rPeptide. Aβ aggregates for *in vitro* studies were generated by a slow stirring of $Aβ_{40}$ in PBS buffer for 3 days at room temperature. ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz on JOEL spectrometers in $CDCl_3$ solution at room temperature. Liquid chromatography-mass spectrometry (LC-MS) was performed using an Agilent 1200 Series apparatus with an LC/MSD trap and Daly conversion dynode detector with UV detection at 254 nm. Fluorescence measurements were carried out using an F-7100 fluorescence spectrophotometer (Hitachi). The IVIS Spectrum imaging system (PerkinElmer) was used for *in vitro* and *in vivo* imaging.

Synthesis and characterization



Scheme S1. Synthesis of the CRANAD-147.

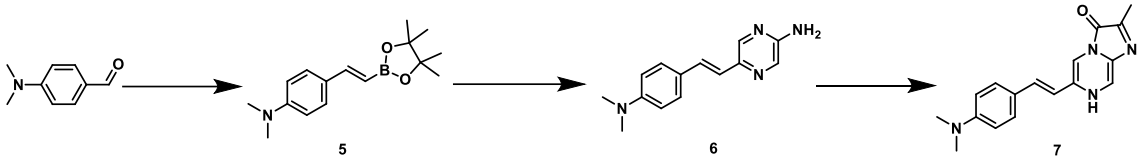
Synthesis of 6-(4-(dimethylamino)phenyl)-4-hydroxy-3-methylhexa-3,5-dien-2-one (1): B_2O_3 (0.7g, 10 mmol) was added into a solution of DMF (8 mL), and the resulting mixture was heated to 120 °C for 30 min until dissolved. To the above mixture, 3-methylpentane-2,4-dione (1.14g, 10 mmol) was added drop-wise. After that, the heating temperature for the mixture was decreased to 110 °C, which was followed by an addition of tributyl borate (4.6 g, 20 mmol). The resulting mixture was stirred for an additional 5 min at 110 °C, followed by an addition of 4-(dimethylamino)benzaldehyde (1.5 g, 10 mmol). The resulting solution was stirred for 5 min, followed by the addition of a solution of 1,2,3,4-tetrahydroquinoline (0.2 mL) and acetic acid (0.4 mL) in DMF (5 mL). The reaction mixture was stirred at 110 °C for 2 h. Upon completion, after cooling to room temperature, the mixture was poured into 400g ice. The resulting solution was filtered and washed with cold water. The precipitate was collected, and the crude product was further purified by flash silica chromatography (HEX: EA=2:1). **Compound 1** was obtained as an orange solid with a 25 % yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.60 (d, J = 15.4 Hz, 1H), 7.45 (d, J = 8.9 Hz, 2H), 6.72 (d, J = 15.4 Hz, 1H), 6.67 (d, J = 8.8 Hz, 2H), 3.01 (s, 6H), 2.21 (s, 3H), 1.99 (s, 3H).

Synthesis of 1-(4-(diethoxymethyl)phenyl)-7-(4-(dimethylamino)phenyl)-5-hydroxy-4-methylhepta-1,4,6-trien-3-one (2): Compound 1 (200 mg, 0.8 mmol) was dissolved in 10 mL butanol, followed by addition of 4-(diethoxymethyl)benzaldehyde (155 µL, 1 eq) and piperidine (120 µL, 1.5 eq). The resulting mixture was refluxed for 24 h. After cooling to room temperature, the solvent was removed under reduced pressure, and the residue was washed with water and extracted by ethyl acetate. After removing the solvent under reduced pressure, the crude products were obtained and used for the next step without further purification.

Synthesis of 4-(7-(4-(dimethylamino)phenyl)-5-hydroxy-4-methyl-3-oxohepta-1,4,6-trien-1-yl)benzaldehyde (3): Compound 2 was dissolved with 10 mL dd water and 10 mL CH_3OH. The resulting solution was refluxed for 2 h. After cooling to room temperature, the solvent was removed under the reduced pressure and the residue was extracted by ethyl acetate (20 mL*3). The organic phase was collected and then the solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography (HEX: EA=2:1). **Compound 3** was obtained as a red solid with a 20 % yield.

Synthesis of 1-(4-(3H-diazirin-3-yl)phenyl)-7-(4-(dimethylamino)phenyl)-5-hydroxy-4-methylhepta-1,4,6-trien-3-one (4) CRANAD-147: Compound 3 (23 mg, 0.06 mmol) was added in a round flask that was covered with foil paper, followed by adding of $NH_3{\cdot}H_2O$ (14N, 75 µL, 1.05 mmol, 17.5 eq) and CH_3OH (75 µL) at 0 °C. The flask was flushed with Argon gas, and the resulting mixture was stirred for 30 min at 0 °C. Then (diacetoxyiodo)benzene (50 mg, 0.15 mmol, 2.5 eq) was added into the flask that was under an inert atmosphere of Argon. The resulting solution was stirred at 0 °C for another 4 h, and the reaction mixture was slowly warmed to

room temperature overnight. Upon completion, the reaction mixture was concentrated under reduced pressure, and the residue was purified by flash silica chromatography (HEX: EA=2:1) under the dark. **Compound 4 (CRANAD-147)** was obtained as a red solid with a 28 % yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.67 (d, J = 15.4 Hz), 7.62 (d, J = 15.7 Hz), 7.48 (d, J = 8.7 Hz), 7.43 (d, J = 8.8 Hz), 6.91 (d, J = 15.3 Hz), 6.73 – 6.65 (m), 6.63 (dd, J = 12.1, 3.2 Hz), 4.09 (q, J = 6.9 Hz), 3.04 (s), 3.02 (s), 2.14 (s), 1.44 (d, J = 6.9 Hz). ^{13}C NMR (126 MHz, $CDCl_3$) δ 142.42, 138.34, 135.00, 131.74, 128.86, 128.66, 123.93, 121.01, 113.30, 45.84, 8.53. ESI-MS (m/z): 374.5 [M+H$^+$].



Scheme S2. Synthesis of the ADLumin-4.

Synthesis of (E)-6-(4-(dimethylamino)styryl)-2-methylimidazo[1,2-a]pyrazin-3(7H)-one (7) ADLumin-4: **ADLumin-4** was obtained as an orange solid using the same method as for **ADLumin-1** [1]. Compound 7 (ADLumin-4) was obtained as a red solid with a 62 % yield. ^{1}H NMR (500 MHz, CD_3OD): δ 9.07 (s, 1H), 8.50 (s, 1H), 7.86 (d, J = 8.1 Hz, 2H), 7.79 (d, J = 16.2 Hz, 1H), 7.73 (d, J = 7.8 Hz, 2H), 7.45 (d, J = 16.0 Hz, 1H), 3.31 (s, 6H), 2.53 (s, 1H). ^{13}C NMR (126 MHz, CD_3OD): δ 142.42, 138.34, 135.00, 131.74, 128.86, 128.66, 123.93, 121.01, 113.30, 45.84, 8.53. ESI-MS (m/z): 295.4 [M+H$^+$].

Binding affinity Kd measurement and binding kinetics: For binding affinity Kd: to a solution of CRANAD-147 (1.0 μM) in PBS, different concentrations of $A\beta_{40}$ aggregates were added, and the fluorescence intensity was recorded until the intensity was not changed. The recorded peak values were fitted by GraphPad with the one-site binding model to provide binding affinity Kd. For time-dependent binding kinetics, CRANAD-147 (1.0 μM) and $A\beta_{40}$ (500 nm) were mixed in the PBS and the fluorescence emission intensity was recorded every 1 min. for 60 minutes.

Photoreaction of CRANAD-147: To a solution of CRANAD-147 (500 nM) in PBS, 250 nM (0.5 eq), 500 nM (1 eq), 1.0 μM (2 eq), 2.5 μM (5 eq) $A\beta_{40}$ aggregates were added and incubated for 1 h. The resulting solution was then irradiated by 470 nm LED light (4 mW/cm^{-2}, 30 s), and the fluorescence intensity was recorded every 30 s. After that, the data were recorded and fitted by GraphPad software. After the experiments, the samples were kept in the dark overnight. For rescue experiments, different concentrations of fresh AD-147 were added to the above samples, and the emission intensity was recorded again. To check whether the emission intensity of the above samples changes after irradiating by the 470 nm again (4 mW/cm^{-2}, 30 s), the emission intensity was recorded again after the addition of fresh solutions of CRANAD-147. Fort he photoreaction experiments, Thioflavin T (ThT) was used as a control probe that is not photosensitive.

Seeding experiments with different $A\beta_{40}$ aggregates: The $A\beta_{40}$ aggregates (300 μL, 25 μM) were treated as following:
Group 1: The $A\beta_{40}$ aggregates were kept in a dark environment;
Group 2: The $A\beta_{40}$ aggregates were irradiated by 470 nm LED light (4 mW/cm^{-2}, 60 s);
Group 3: The $A\beta_{40}$ aggregates were mixed with CRANAD-147 (1 eq), and the resulting mixture was kept in the dark environment;
Group 4: The $A\beta_{40}$ aggregates were mixed with CRANAD-147 (1 eq) and then incubated for 1 h. Then the resulting mixture was irradiated by 470 nm LED light (4 mW/cm^{-2}, 60 s);
Group 5: The PBS group had no seeds of $A\beta_{40}$ aggregates.

After the treatments, all these samples were added to a solution of $A\beta_{40}$ monomers (6 mL, 25 μM) separately. In every time-point, a 500 μL sample was pipetted out, followed by the addition of ThT (500 nM). Then the emission intensity was recorded at 495 nm (Ex = 435 nm). The emission intensities were recorded twice daily until no significant intensity change could be observed. The emission peak intensities were normalized by the last data for each group.

Protease K digest experiments: Six groups of $A\beta_{40}$ aggregates in PBS (3.0 mL, 5 μM) were treated as below, respectively.
Group 1: The $A\beta_{40}$ aggregates were kept in a dark environment;
Group 2: The $A\beta_{40}$ aggregates were irradiated by 470 nm LED light (4 mW/cm^{-2}, 60 s);
Group 3: The $A\beta_{40}$ aggregates were mixed with CRANAD-147 (1 eq), and the resulting mixture was kept in the dark environment;
Group 4: The $A\beta_{40}$ aggregates were mixed with CRANAD-147 (1 eq) and then incubated for 1 h. Then the resulting mixture was irradiated by 470 nm LED light (4 mW/cm^{-2}, 60 s);
Group 5: The $A\beta_{40}$ aggregates was mixed with mixed with ADLumin-4 (10 eq);
Group 6: The $A\beta_{40}$ aggregates were mixed with CRANAD-147 (1 eq) and then incubated for 1 h, followed by the addition of ADLumin-4 (10 eq).

After these treatments, all these samples were incubated overnight. Then protease K (0.1 mg/mL) was added to each sample, and all of these samples were incubated at 37 ℃ humidity incubator. The Aβ aggregates concentrations were measured by the ThT, and the data was recorded every 30 min.

SDS-PAGE gel electrophoresis: The samples of $A\beta_{40}$ aggregates were treated as same as the above six groups. The treated samples were loaded into 12-20% SDS-Page gel with a volume of 20 μL/well. The gel was run at 200 V for 50 min. Then the gel was visualized by silver stain kit, and the image was captured by the IVIS. For the western blot experiment, the primary antibody was 6E10.

MALDI-TOF: The samples of $A\beta_{40}$ aggregates were treated as same as the above six groups. Then the treated samples were diluted with the HFIP (Hexafluoro isopropanol) (5 μM final concentration) to dissociate no-covalent binding between Aβs.

Molecular light for CRET between ADLumin-4 and CRANAD-147: CRANAD-147 (50 μM) and ADLumin-4 (50 μM) was mixed in a DMSO solution, and the chemiluminescent emission spectrum was recorded on an IVIS imaging system with filters of 20nm-band width from 500nm to 700nm. For CRET with Aβs, CRANAD-147 (25 μM) was added to a solution of $A\beta_{40}$ oligomers (25 μM) in PBS buffer (pH7.4), and the resulting mixture was incubated for 1 h. Then, ADLumin-4 (25 μM) was added to the above mixture, and the chemiluminescent emission was recorded by the IVIS imaging system.

Cell toxicity/viability measurement: SH-SY5Y cells were seeded in a 96-well plate and incubated for 24 h. To the cell media, different Aβs that were treated as the above groups were added. Then the cells were further incubated 48 h. Upon the completion of culture, ADP kit and ATP kit were used to measure the concentrations of ADP and ATP in the medium separately.

3D organoids cell culture: For the 3D brain cell spheroids: about 4×10^3 per well SH-SY5Y cells were seeded into the 96-well spheroid bottom plate and incubated in an incubator at 37 ℃ for 3 days. Then the cell was treated as the above six groups respectively. The cell spheroids' diameter was recorded every two days. After two weeks of measurement, cell spheroids were dissociated and subjected to ATP assay. For 3D brain organoids: The iPS cell was obtained from Applied StemCell, Inc. (cat. No. ASE-9321K). The detailed experimental condition to prepare iPS 3D organoids was referred to the application nort of neural organoid culture from gibco (www.thermofisher.com/organoid). Brefiely, about 10^3 per well iPSCs were seeded into the 96-well spheroid bottom plate.The organoid culture in the presence of extracellular matrices and incubated in 37 ℃ incubators for 3 days. Then the organoids were subjected to a treatment of two weeks as the above six groups respectively. After the organoids were fixed and embedded in O.T.C., then the organoids were sliced into slides of 7-micron thickness. The tissue slices were stained with kits of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

***In vivo* therapeutic studies**: 5xFAD mice (12 weeks, female) were randomly separated into 6 groups (n=5 per group). For *in vivo* imaging, on day 0, 5, 12, 17, 23, 28 and 32, the mice were i.p injected with ADLumin-1 (10 mg/kg, formulation: 2.0 mg/mL, 5% DMSO, 15% Castor oil and 80% saline), and chemiluminescence imaging was performed on the IVIS imaging system with an open filter at 30 minutes post the injection, and semi-quantitative imaging analysis was conducted with an average intensity of an ROI of the brain area. For therapeutic studies, the mice were treated on day 1, 3, 6, 8, 10, 13, 15, 18, 20, 22, 24, 26, and 29. After the last imaging session, all the mice were sacrificed, and the brains were extracted and frozen in -78°C freezer. For each group, the mice were independently treated as described below:
Group 1: intraperitoneal injection of vehicle of 100 μL of the mixture of 5% DMSO, 15% Castor oil and 80% saline.
Group 2: intraperitoneal injection of vehicle of 100 μL of the mixture of 5% DMSO, 15% Castor oil and 80% saline, 30 min later, the brain areas of the mice were exposed under the 470 nm LED light (2 mW/cm^2) for 15 min.
Group 3: intraperitoneal injection of 100 μL of a dose of 2.5 mg/Kg CRANAD-147 (Formulation: 0.5 mg/mL, 5% DMSO, 15% Castor oil and 80% saline).
Group 4: intraperitoneal injection of 100 μL of a dose of 2.5 mg/Kg CRANAD-147 (Formulation: 0.5 mg/mL, 5% DMSO, 15% Castor oil and 80% saline), 30 min later, the mice head were exposed under the 470 nm LED light (2 mW/cm^2) for 15 min.
Group 5: intraperitoneal injection of 100 μL of a dose of 10 mg/Kg ADLumin-4 (Formulation: 2.0 mg/mL, 5% DMSO, 15% Castor oil and 80% saline).
Group 6: intraperitoneal injection of 100 μL of a dose of 2.5 mg/Kg CRANAD-147 (Formulation: 0.5 mg/mL, 5% DMSO, 15% Castor oil and 80% physiological saline), 30 min later, the mice were intraperitoneal injected (i.p.) of 100 μL of a dose of 10 mg/Kg ADLumin-4 (Formulation: 2.0 mg/mL, 5% DMSO, 15% Castor oil and 80% saline).

Ethics Statement: All experimental protocols have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital. (Approval No.: 2011N000161).

Supporting Figures

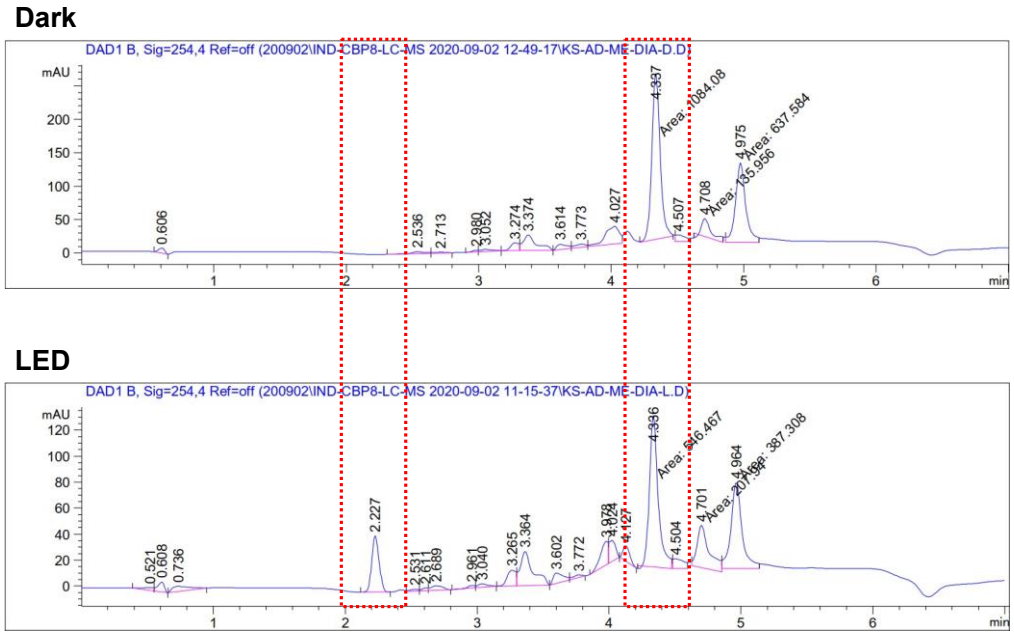


Figure S1. HPLC of CRANAD-147 before (upper) and after (bottom) LED-irradiation.

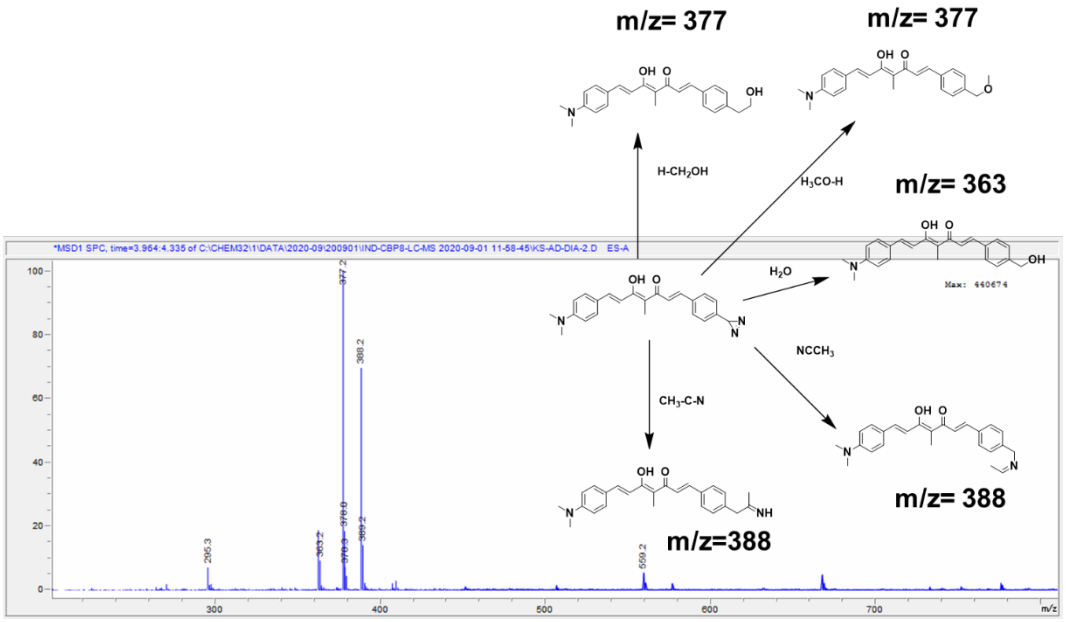


Figure S2. MS of CRANAD-147 after LED irradiation

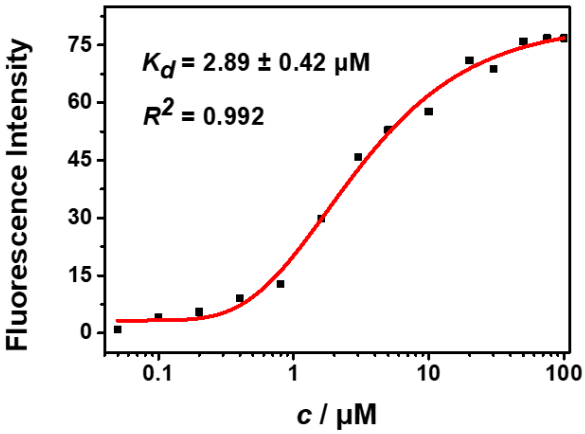


Figure S3. Binding constant Kd measurement of CRANAD-147 with Aβ aggregates.

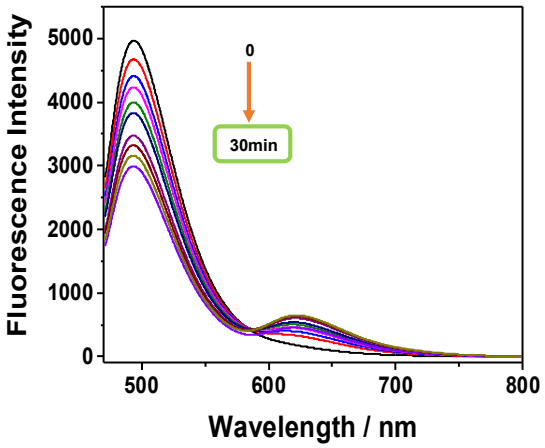


Figure S4. Fluorescence emission spectra of Thioflavin T after LED irradiation at different time points.

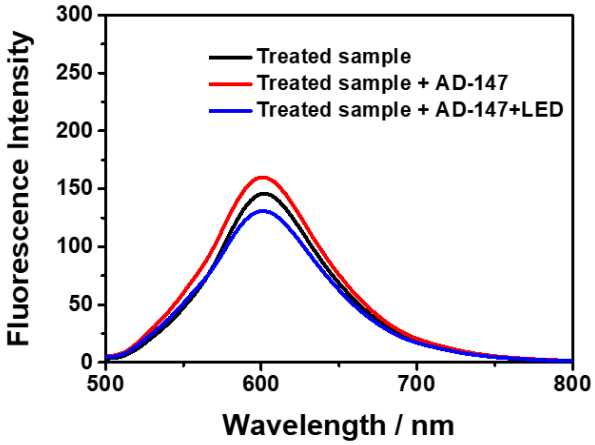


Figure S5. Fluorescence spectra of CRANAD-147 after LED irradiation and after adding fresh CRANAD-147 for rescuing study.

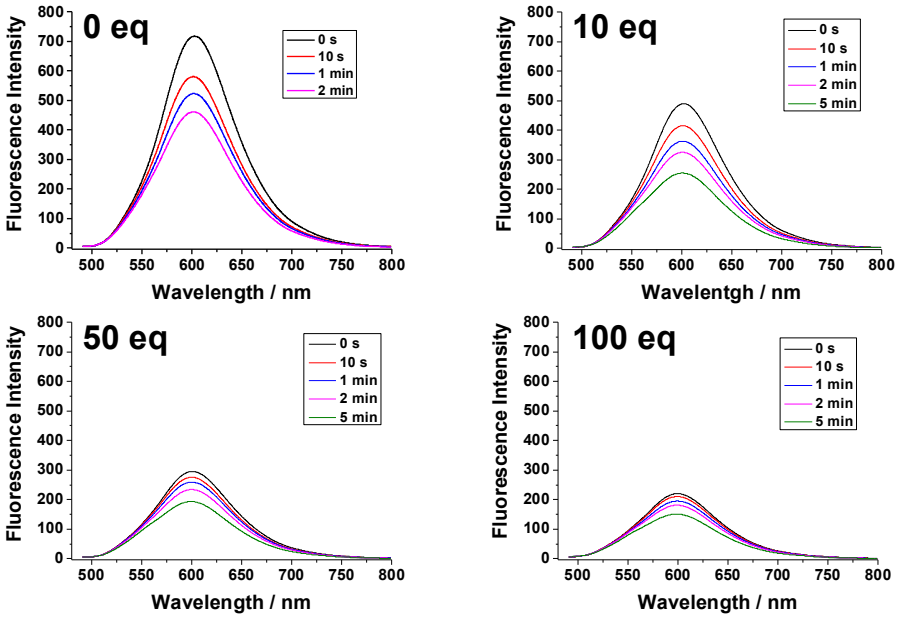


Figure S6. Fluorescence emission spectra of CRANAD-147 and $A\beta_{40}$ aggregates with different concentrations of catechol at different time points after LED irradiation.

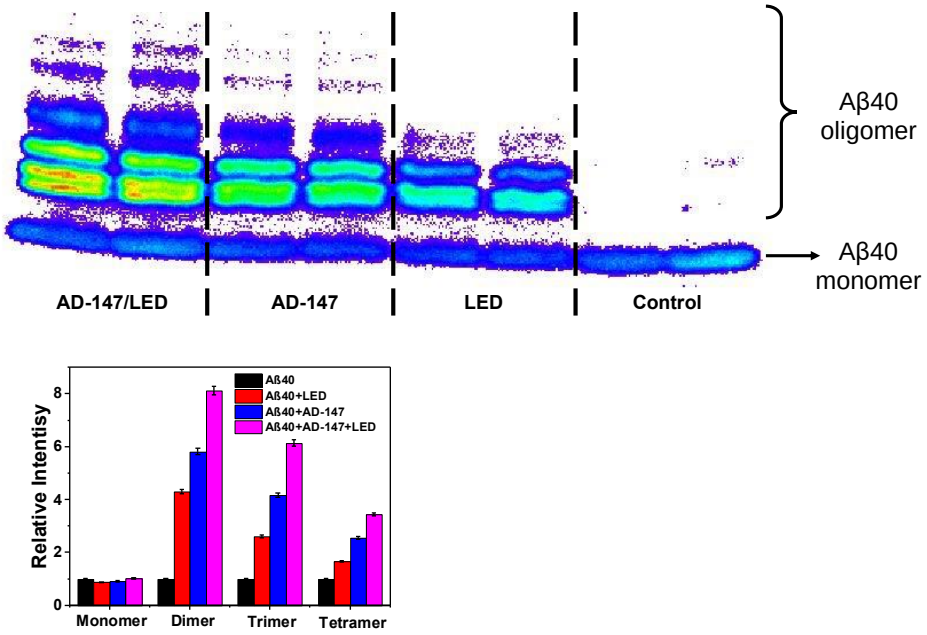


Figure S7. Western blot of Aβ_{40} aggregates with different treatments (upper) and quantitative analysis of the bands (bottom).

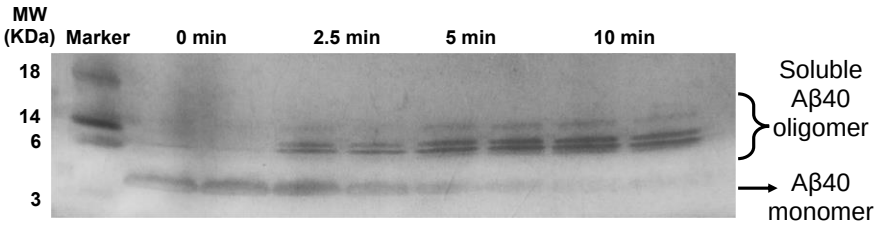


Figure S8. SDS-Page gel of Aβ_{40} aggregates with CRANAD-147 under LED irradiation of different durations. The gel was visualized by silver staining.

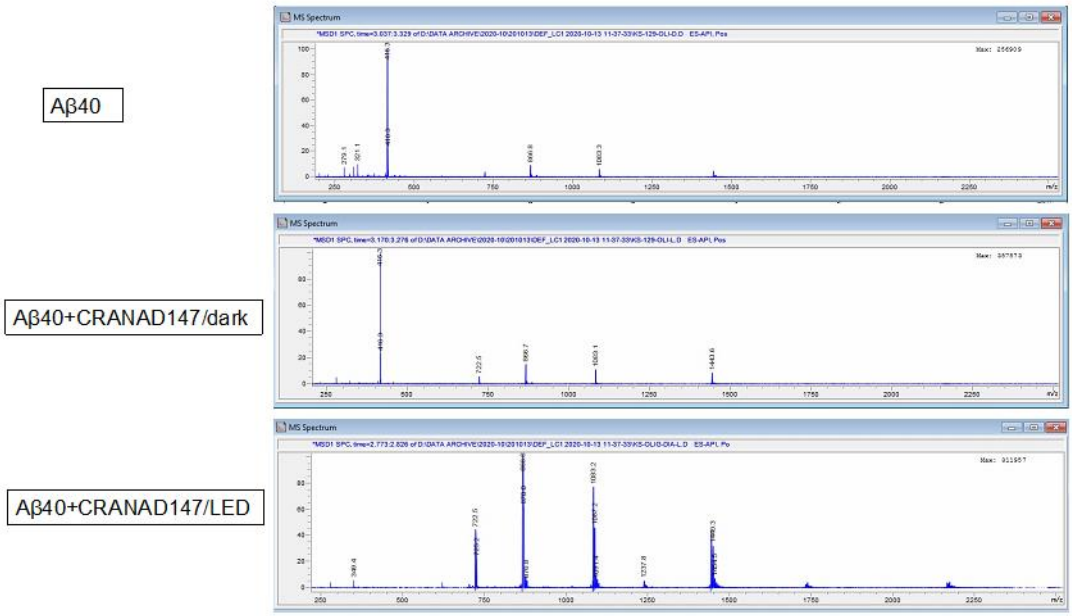


Figure S9. LC-MS spectra of $A\beta_{40}$ after different treatments. Several clusters of m/z could be seen in the group of $A\beta_{40}$/CRANAD-147/LED.

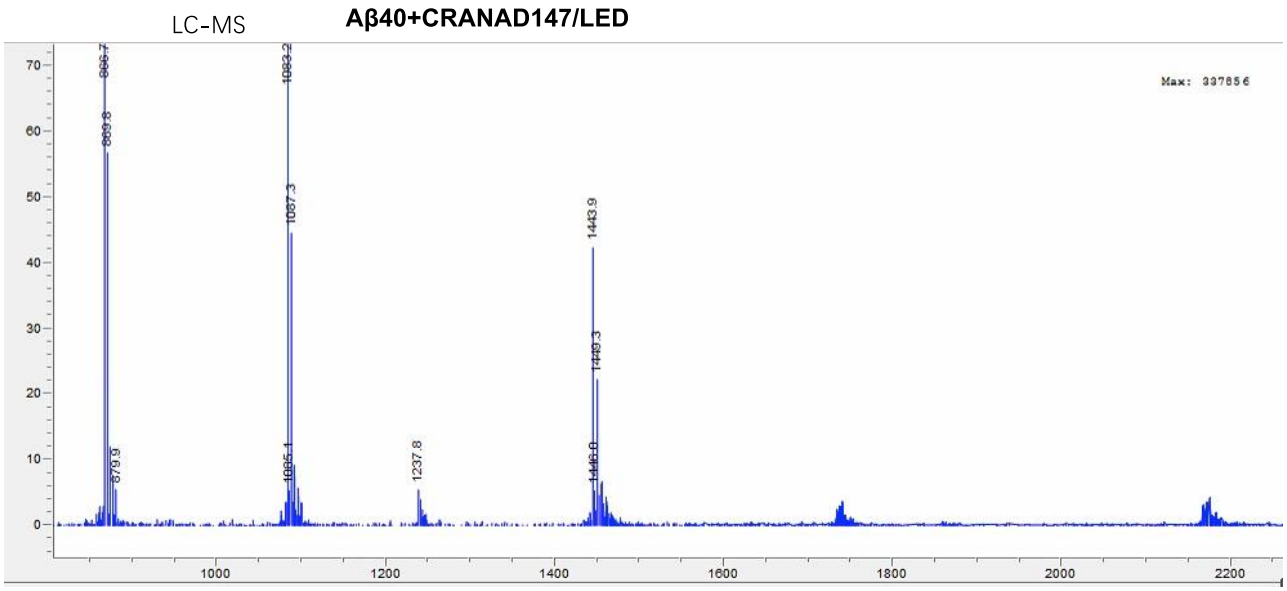


Figure S10. Zoomed-in MS spectra of Aβ species for the group of $A\beta_{40}$/CRANAD-147/LED. Several clusters can be clearly seen.

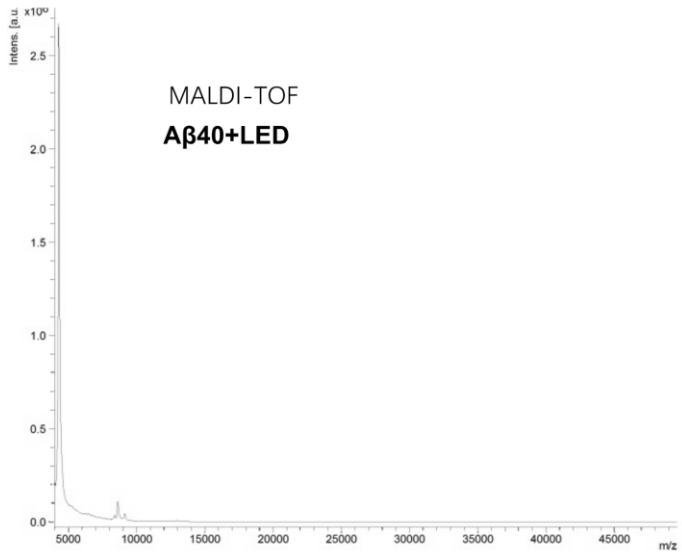


Figure S11. MALDI-TOF MS of $A\beta_{40}$ after LED irradiation but without CRANAD-147.

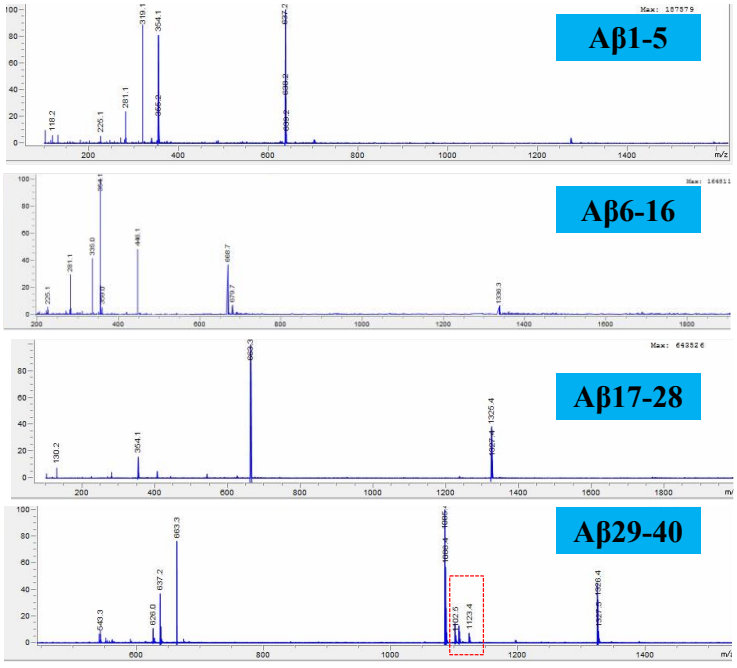


Figure S12. LC-MS of Aβ fragments from trypsinization treatment of the mixture of $A\beta_{40}$/CRANAD-147 and LED irradiation.

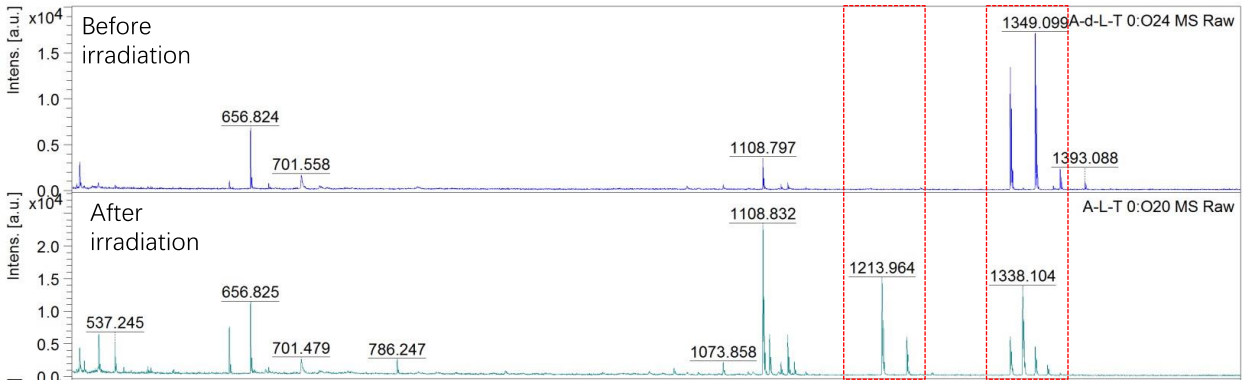


Figure S13. MALDI-TOF MS of Aβ fragments from trypsinization treatment of the mixture of $A\beta_{40}$/CRANAD-147 and LED irradiation.

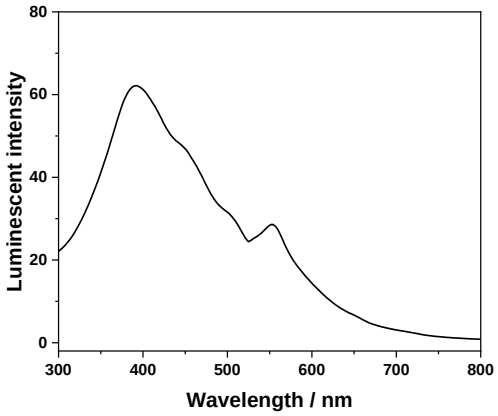


Figure S14. Luminescent spectrum of ADLumin-4 (25 μM) after incubation with Aβ aggregates (25 μM).

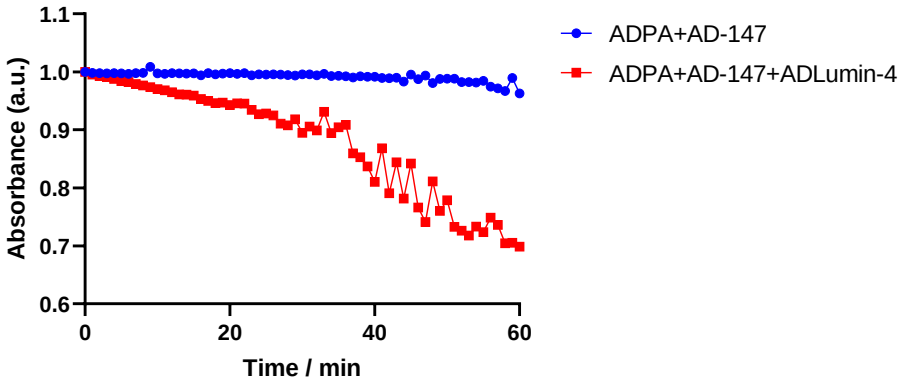
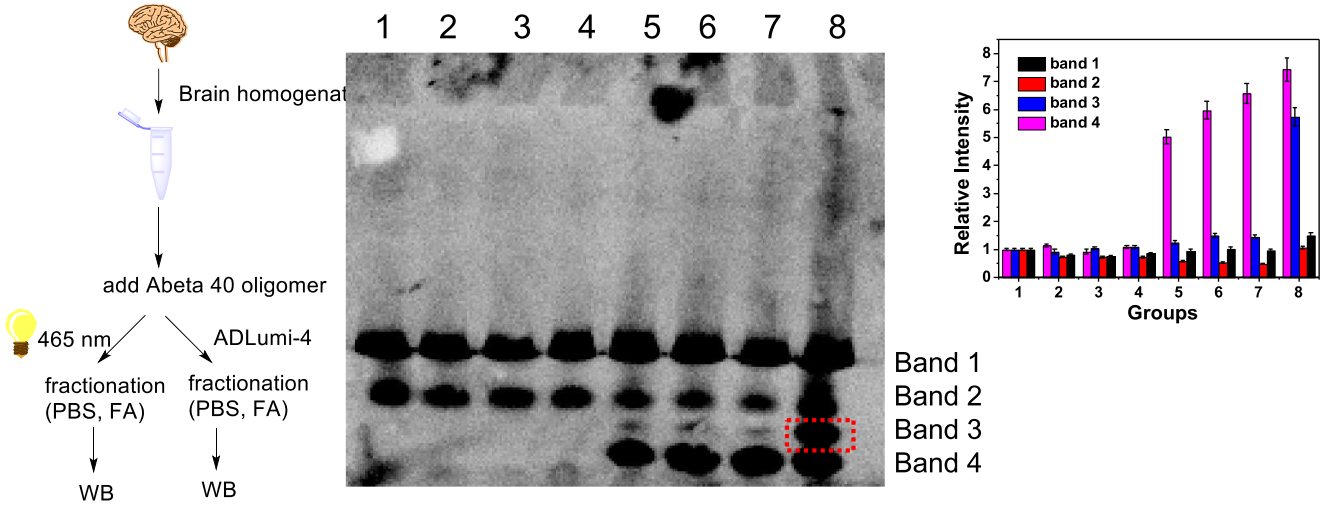


Figure S15. UV-Vis spectra of ADPA after being treated with CRANAD-147 or the mixture of CRANAD-147 and ADLumin-4 for different time durations.



Group	1	2	3	4	5	6	7	8
Aβ 40	-	-	-	-	+	+	+	+
AD-147	-	-	+	+	-	-	+	+
Light	-	+	-	+	-	+	-	+

Figure S16. *In vitro* mimic experiment with externally added $A\beta_{40}$ aggregates that were treated with CRNAD-147/LED or other conditions. Western blot showed a new band (No. 3) that was absent from groups without $A\beta_{40}$ addition.

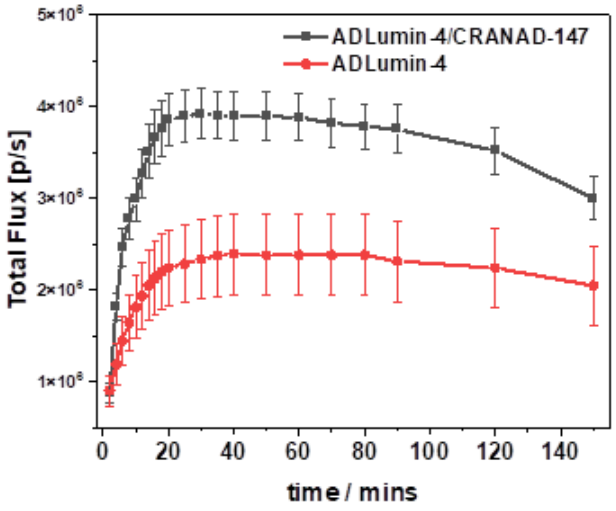


Figure S17. Time-dependent quantitative analysis of chemiluminescence intensity of images captured from 2 min to 150 min after i.p injection of ADLumin-4 (2.5 mg/Kg) only and the mixture of ADLumin-4 (2.5 mg/Kg) and CRANAD-147 (10.0 mg/Kg) (the CRET pair). There are significant differences between the two groups, n=5, *** P < 0.001. The higher signals from the CRET pair group are due to the better tissue penetration from CRANAD-147 upon CRETing. The results indicate the stable co-existence of ADLumin-4/CRANAD-147 and the good stability of both compounds in vivo for over 150 minutes.

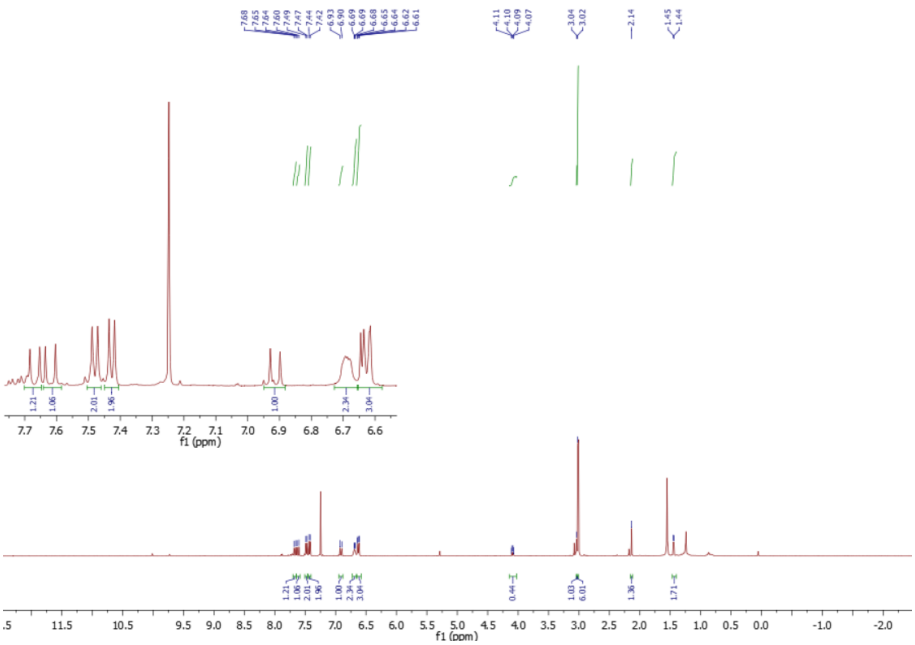


Figure S18. ^{1}H NMR spectrum of CRANAD-147 in $CDCl_3$.

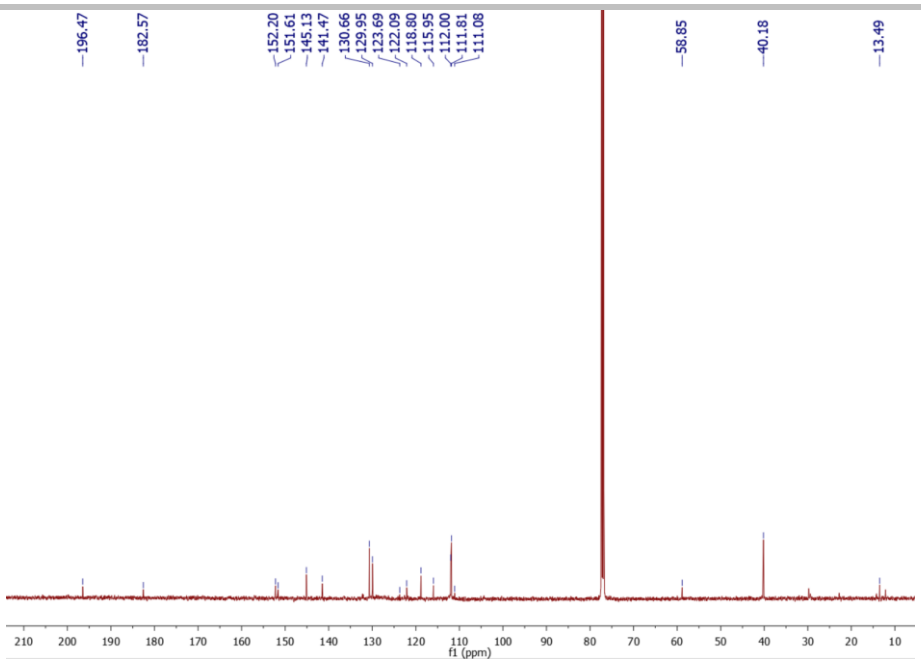


Figure S19. ^{13}C NMR spectrum of CRANAD-147 in $CDCl_3$.

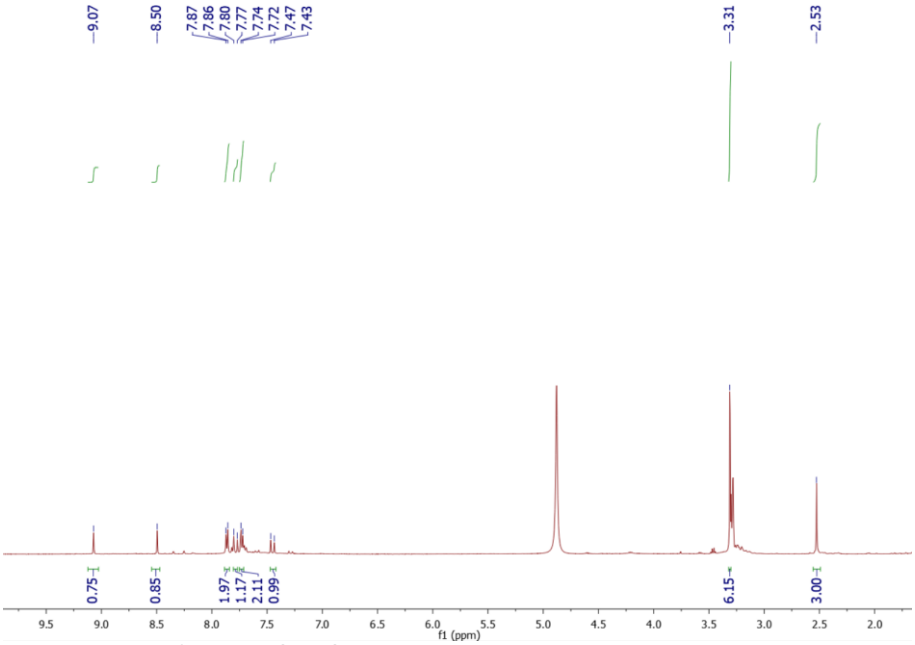


Figure S20. ^{1}H NMR spectrum of ADLumin-4 in CD_3OD.

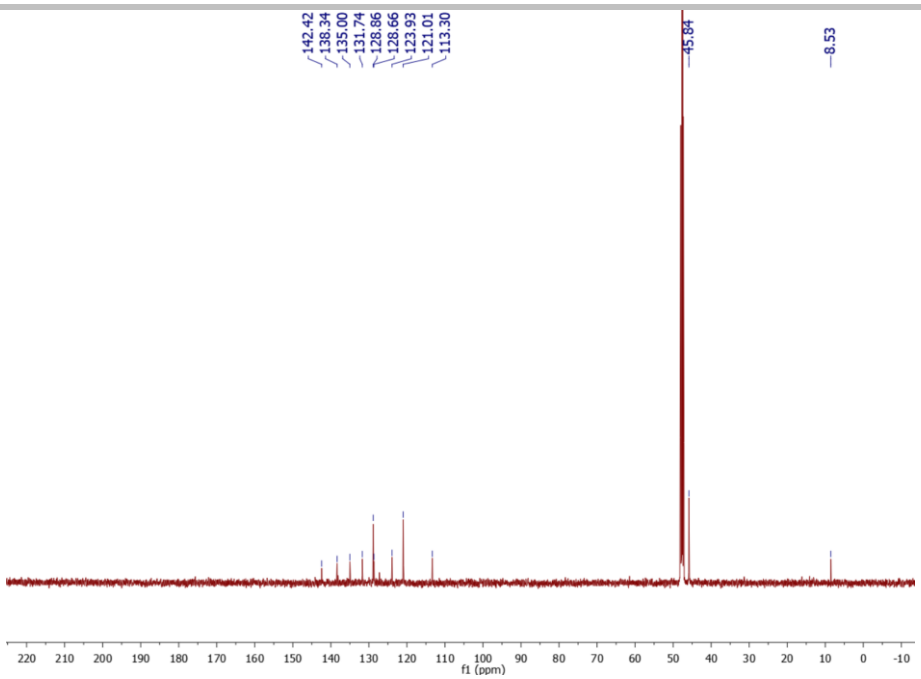


Figure S21. ^{13}C NMR spectrum of ADLumin-4 in CD_3OD.

References

[1] J. Yang, W. Yin, R. Van, K. Yin, P. Wang, C. Zheng, B. Zhu, K. Ran, C. Zhang, M. Kumar, Y. Shao, C. Ran, *Nat. Commun.* **2020**, *11*, 4052.