Supporting Information

Design of NO Fluorescent Probes and Application in Cellular Inflammation and Apoptosis Analysis

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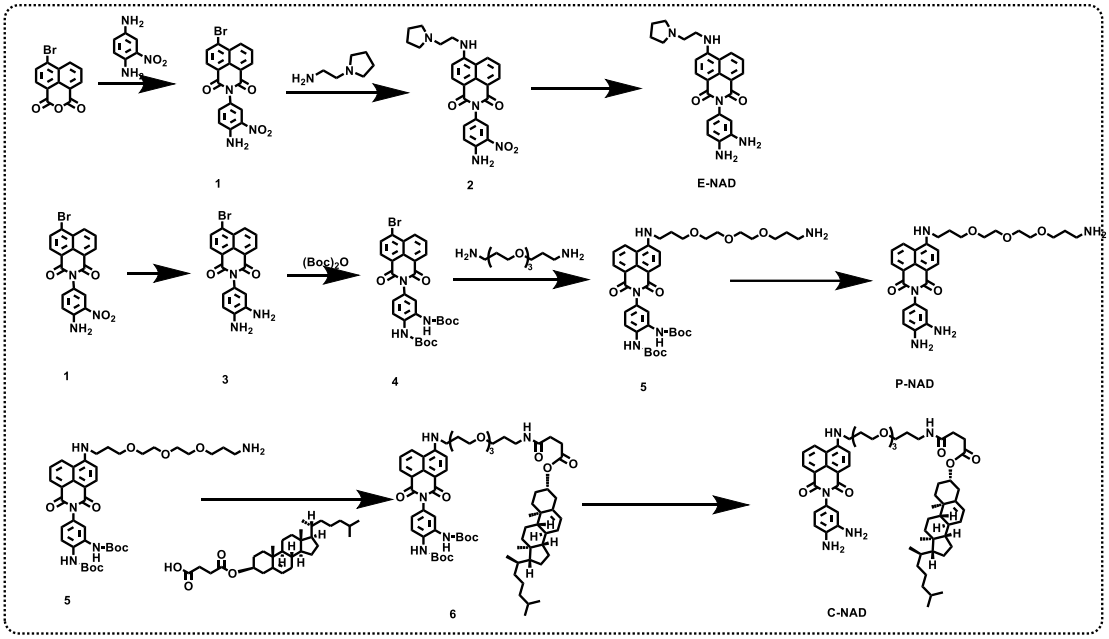
Experimental Section



Scheme S1. synthetic routes and structures of NADs.

Synthesis of Compound **1**: 4-Bromo-1,8-naphthalic anhydride (1.108 g, 4 mmol), 2-nitro-1,4-phenylenediamine (0.735 g, 4.8 mmol), and sodium acetate (0.328 g, 4 mmol) were weighed and transferred into a 100 mL three-necked flask. To this, 15 mL of anhydrous ethanol and 15 mL of ice acetic acid were added, thoroughly agitated until a suspension formed, and then heated under reflux at 100°C under nitrogen protection for 6 hours. The mixture changed from reddish-black to dark brown. After cooling to room temperature, distilled water was added, followed by filtration. The resulting deep yellow solid was washed with anhydrous ethanol and dichloromethane, dried, and compound 1 was obtained as a yellow-green product (1.517 g, yield 95%). MS(ESI)：m/z$[M+H]^+$ = calcd. for $C_{11}H_{18}BrN_3O_4^+$ 411.9927；found 411.9924.^{1}H NMR (400 MHz, DMSO-d6) δ 8.68 – 8.55 (m, 2H), 8.36 (d, J = 8.0, 0.7 Hz, 1H), 8.27 (d, J = 7.9, 0.7 Hz, 1H), 8.09 – 7.99 (m, 2H), 7.64 (s, 2H) 7.42 (dd, J = 8.9, 2.4 Hz, 1H), 7.13 (d, J = 8.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d6) δ 163.87, 163.82, 146.52, 137.11, 133.21, 132.10, 131.87, 131.46, 130.43, 130.18, 129.64, 129.34, 129.27, 126.34, 123.95, 123.51, 123.18, 119.84.

Synthesis of Compound **2**: Compound **1** (0.410 g, 1 mmol) and 1-(2-

aminoethyl)pyrrolidine (1.141 g, 10 mmol) were added to a 50 mL three-necked flask, along with 15 mL of anhydrous DMSO. The mixture was heated under reflux under nitrogen protection and an oxygen-free environment at 85°C for 4 hours. Upon cooling to room temperature, it was extracted with distilled water and dichloromethane. After drying and concentration, silica gel column chromatography was performed using a gradient of dichloromethane/methanol/triethylamine = (v/v/v=100:1:5), yielding an orange product (0.356 g, 80%). MS(ESI)：m/z[M+H]$^+$ = calcd. for $C_{24}H_{24}N_5O_4^+$ 446.1823；found 446.1826. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.63 – 8.56 (m, 2H), 8.35 (d, J = 7.9 Hz, 1H), 8.26 (d, J = 7.9 Hz, 1H), 8.09 – 8.01 (m, 2H), 7.65 (s, 2H), 7.42 (dd, J = 8.9, 2.4 Hz, 1H), 7.13 (d, J = 9.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d6) δ 164.62, 163.70, 151.02, 146.29, 137.49, 134.75, 131.28, 130.30, 130.16, 129.31, 126.20, 124.74, 124.33, 122.68, 120.70, 119.69, 108.46, 104.32, 54.11, 53.80, 23.54.

Synthesis of E-NAD: Compound **2** (0.223 g,0.5 mmol) was added to a 50 mL three-necked flask, followed by 10 mL of concentrated hydrochloric acid. Tin powder was gradually added during stirring, and the mixture was heated to 50°C. Then, 5 mL of anhydrous ethanol was added, and the reaction was refluxed at 85°C for 6 hours. The color changed from orange to yellow. After adjusting to alkalinity with sodium hydroxide and extracting with dichloromethane, the dried and concentrated material underwent silica gel column chromatography using a gradient of dichloromethane/methanol/triethylamine = (v/v/v=10:1:1), yielding an orange product (0.176 g, 85%). MS(ESI)：m/z[M+H]$^+$ = calcd. for $C_{24}H_{24}N_5O_4^+$ 416.2081；found 416.2089.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (d, J = 8.4 Hz, 1H), 8.42 (d, J = 7.3, 2.5 Hz, 1H), 8.25 (d, J = 8.6, 2.7 Hz, 1H), 7.80 (s, 1H), 7.70 (t, J = 8.0, 2.6 Hz, 1H), 6.84 (d, J = 8.7, 2.6 Hz, 1H), 6.56 (d, J = 8.4, 2.6 Hz, 1H), 6.36 (s, 1H), 6.25 (d, J = 8.0 Hz, 1H), 3.66 – 3.55 (m, 2H), 3.02 – 2.91 (m, 2H), 2.84 – 2.68 (m, 4H), 1.85 – 1.62 (m, 4H).

Synthesis of Compound **3**: Compound **1** (0.824 g, 2 mmol) was introduced into a 50 mL three-necked flask, to which 10 mL of concentrated hydrochloric acid was added. Tin powder was slowly added during stirring, and the mixture was heated to 50°C. Then, 5 mL of anhydrous ethanol was added, and the reaction was refluxed at 85°C for 10

hours, changing from yellow-green to light yellow. Upon cooling to room temperature, the reaction was neutralized with sodium hydroxide solution, the precipitate was separated and dried, providing crude product **3** ready for the next step.

Synthesis of Compound **4**: The entire amount of crude product **3** was placed in a 100 mL round-bottom flask, dissolved in 10 mL DMSO, and di-tert-butyl dicarbonate (4.365 g, 20 mmol) was added to the flask. Next, 30 mL of anhydrous ethanol was added, and the reaction was allowed to proceed at room temperature under nitrogen protection for 24 hours. After filtration, the filtrate was subjected to vacuum distillation, followed by extraction with dichloromethane and water. The extract was dried, concentrated, and purified via silica gel column chromatography using a gradient of ethyl acetate/petroleum ether = (v/v=3:1), yielding a pale yellow product (0.990 g, 85%). MS(ESI)：m/z$[M+Na]^+$ = calcd. for $C_{28}H_{28}BrN_3NaO_6^+$ 604.1054；found 604.1062.^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.69 (d, *J* = 7.2, 1.2 Hz, 1H), 8.63 (d, 2H), 8.44 (d, *J* = 7.9 Hz, 1H), 8.26 (d, *J* = 8.3, 1.2 Hz, 1H), 8.07 (d, *J* = 7.9 Hz, 1H), 7.88 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.78 (t, *J* = 7.8 Hz, 1H), 7.65 (s, 2H), 7.10 – 7.03 (m, 1H), 1.54 (s, 9H), 1.49 (s, 9H). ^{13}C NMR (101 MHz, CDCl3) δ 163.67, 163.63, 153.64, 151.43, 134.28, 133.65, 132.49, 131.63, 131.22, 130.78, 130.69, 129.31, 128.18, 127.02, 123.21, 122.33, 81.20, 28.28, 28.25.

Synthesis of Compound **5**: compound **4**(0.582 g, 1 mmol) and 4,7,10-trioxa-1,13-tridecanediamine (2.804 g, 10 mmol) were added to 50 mL of a three-port flask and dissolved in 15 mL of anhydrous DMSO; Under the protection of nitrogen, the reaction was finished after heating anhydrous and oxygen-free to 85 °C for 6 hours. Cool to room temperature and extract with distilled water and dichloromethane. After dry concentration, the product was separated by silica gel column chromatography and eluted with a gradient of dichloromethane methanol triethylamine = (v/v/v = 10:1:5) to obtain an orange product (0.577 g, 80%). MS (ESI): m/z $[M+H]^+$ = calcd. for $C_{38}H_{52}N_5O_9^+$ 722.3760； found 722.3759. ^{1}H NMR (400 MHz, Methanol-d4) δ 8.24 (t, J = 7.8 Hz, 2H), 8.12 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 8.5 Hz, 1H), 7.56 (s, 1H), 7.44 (t, J = 7.9 Hz, 1H), 7.04 (dd, J = 8.5, 2.4 Hz, 1H), 6.60 (d, J = 8.8 Hz, 1H), 3.65 – 3.54 (m,

12H), 3.46 – 3.42 (m, 2H), 2.92 – 2.82 (m, 2H), 2.05 – 1.96 (m, 2H), 1.84 – 1.75 (m, 2H), 1.54 (s, 9H), 1.50 (s, 9H). ^{13}C NMR (101 MHz, Methanol-d4) δ 164.75, 164.13, 154.41, 154.28, 151.24, 134.66, 132.56, 131.22, 130.86, 130.04, 129.84, 128.12, 125.02, 124.33, 123.86, 121.66, 120.02, 107.42, 103.65, 80.26, 80.20, 70.15, 70.02, 69.97, 69.93, 69.75, 68.77, 40.56, 38.51, 28.37, 27.99, 27.34.

Synthesis of P-NAD: Compound **5** (0.217 g, 0.3 mmol) was added to a 25 mL round-bottom flask, along with 10 mL trifluoroacetic acid and 20 mL dichloromethane. The mixture was allowed to react at room temperature for 6 hours. Following the reaction, vacuum distillation was performed. Anhydrous methanol was added repeatedly under reduced pressure to remove trifluoroacetic acid. The solvent was evaporated under vacuum, and the product was subjected to silica gel column chromatography using a gradient of dichloromethane/methanol/triethylamine = (v/v/v=40:10:1). This resulted in a yellow product (0.141 g, 90%). MS(ESI)：m/z[M+H]$^+$ = calcd. for $C_{28}H_{36}N_5O_5^+$ 522.2711； found 522.2710. ^{1}H NMR (400 MHz, Methanol-d4) δ 8.28 – 8.12 (m, 3H), 7.74 (d, J = 8.5 Hz, 1H), 7.63 (d, J = 1.9 Hz, 1H), 7.42 (d, J = 7.9 Hz, 1H), 7.15 (dd, J = 8.6, 1.9 Hz, 1H), 6.56 (d, J = 8.7 Hz, 1H), 3.59 – 3.39 (m, 14H), 2.92 – 2.81 (m, 2H), 2.02 – 1.94 (m, 2H), 1.80 – 1.69 (m, 2H). ^{13}C NMR (101 MHz, Methanol-d4) δ 165.30, 164.66, 151.21, 146.40, 146.03, 141.28, 140.59, 130.81, 129.85, 128.12, 124.24, 123.83, 123.31, 121.78, 121.55, 120.02, 118.87, 117.34, 116.76, 107.61, 103.60, 70.02, 69.84, 69.78, 69.61, 68.70, 65.51, 40.51, 38.43, 28.35, 26.85, 14.06, 8.17.

Synthesis of Compound **6**: Cholesterol succinate monoester (0.146 g, 0.3 mmol), N-hydroxy succinimide (0.104 g, 0.9 mmol), and 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (0.173 g, 0.9 mmol) were combined in a 25 mL three-necked flask, to which 10 mL of anhydrous dichloromethane was added for dissolution. The mixture was allowed to react at room temperature for 12 hours. After the reaction, saturated ammonium chloride aqueous solution and dichloromethane were used for extraction. The resulting dried and concentrated product was the activated ester of cholesterol succinate monoester. This activated ester, along with Compound **5** (0.217 g, 0.3 mmol) and 10 mL of anhydrous dichloromethane, was added to a new 25 mL three-

necked flask. Azodicarboxylic acid diisopropyl ester (0.404 g, 2 mmol) was also added, and the reaction proceeded for 12 hours, with completion determined by silica gel plate chromatography results. The reaction mixture was concentrated, and the product was purified through silica gel column chromatography using a gradient of ethyl acetate/acetonitrile/triethylamine = (v/v=8:1:1), resulting in a yellow product (0.304 g, 85%). MS(ESI)：m/z$[M+H]^+$ = calcd. for $C_{65}H_{100}N_5O_{12}^+$ 1190.7363；found 1190.7351. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.43 – 8.27 (m, 3H), 7.66 (s, 1H), 7.51 (t, J = 7.9 Hz, 2H), 7.00 (dd, J = 8.6, 2.3 Hz, 1H), 6.85 (s, 1H), 6.68 – 6.56 (m, 2H), 5.31 (dd, J = 6.2, 4.2 Hz, 1H), 4.67 – 4.52 (m, 1H), 3.71 – 3.46 (m, 14H), 3.37 – 3.23 (m, 2H), 2.62 – 2.59 (m, 2H), 2.49 – 2.37 (m, 2H), 2.33 – 2.22 (m, 2H), 2.05 – 1.92 (m, 4H), 1.85 – 1.78 (m, 3H), 1.75 – 1.67 (m, 2H), 1.56 – 1.33 (m, 29H), 1.18 – 0.96 (m, 13H), 0.92 – 0.90 (m, 3H), 0.88 – 0.85 (m, 6H), 0.66 (s, 3H). ^{13}C NMR (101 MHz, CDCl3) δ 172.49, 171.62, 171.54, 164.75, 164.18, 153.80, 153.63, 150.72, 139.58, 135.11, 132.31, 131.30, 130.18, 127.55, 124.26, 122.58, 122.47, 120.39, 108.70, 103.79, 80.50, 74.20, 70.49, 70.32, 70.15, 70.08, 70.02, 69.63, 69.44, 56.64, 56.09, 53.90, 49.97, 46.09, 42.26, 42.01, 40.81, 39.68, 39.48, 38.40, 38.04, 37.50, 36.92, 36.52, 36.15, 35.75, 34.41, 31.86, 31.79, 30.97, 30.91, 29.93, 29.67, 29.63, 29.26, 29.06, 28.91, 28.45, 28.33, 28.31, 28.20, 27.98, 27.70, 24.25, 23.81, 23.17, 22.83, 22.57, 20.98, 19.27, 18.70, 14.75, 14.13, 11.83, 8.64.

Synthesis of C-NAD: Compound **6** (0.238 g, 0.2 mmol) was added to a 25 mL round-bottom flask, together with 10 mL trifluoroacetic acid and 20 mL dichloromethane. The mixture was allowed to react at room temperature for 2 hours. After the reaction, vacuum distillation was performed, followed by extraction with distilled water and dichloromethane. The dried and concentrated material was subjected to silica gel column chromatography using a gradient of dichloromethane/triethylamine = (v/v=10:1), leading to a yellow product (0.178 g, 90%). MS(ESI)：m/z$[M+H]^+$ = calcd. for $C_{59}H_{84}N_5O_8^+$ 990.6314；found 990.6295. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.55 (d, J = 7.3 Hz, 1H), 8.44 (d, J = 8.4 Hz, 1H), 8.24 (d, J = 8.4 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 6.78 (d, J = 8.1 Hz, 1H), 6.65 (d, J = 8.3 Hz, 2H), 6.39 (t, J = 5.7 Hz, 1H), 5.32 (d, J = 4.8 Hz, 1H), 4.58 (dp, J = 12.0, 4.2 Hz, 1H), 3.75 – 3.65 (m, 8H), 3.58

– 3.46 (m, 7H), 3.32 – 3.27 (m, 2H), 2.66 – 2.56 (m, 2H), 2.45 – 2.38 (m, 2H), 2.32 – 2.25 (m, 2H), 2.09 – 1.93 (m, 4H), 1.86 – 1.78 (m, 3H), 1.74 – 1.68 (m, 2H), 1.55 – 1.30 (m, 11H), 1.19 – 0.97 (m, 13H), 0.91 (d, J = 6.6 Hz, 3H), 0.86 (dd, J = 6.6, 1.8 Hz, 6H), 0.66 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 172.55, 171.56, 165.29, 164.69, 150.33, 135.45, 135.08, 134.99, 131.38, 130.27, 128.00, 127.13, 124.47, 123.11, 122.65, 120.52, 120.05, 116.90, 109.59, 103.82, 74.33, 70.52, 70.50, 70.48, 70.03, 69.78, 56.67, 56.13, 50.00, 42.50, 42.30, 39.71, 39.52, 38.08, 37.73, 36.95, 36.57, 36.18, 35.79, 31.93, 31.89, 31.83, 31.04, 29.95, 29.70, 29.03, 28.25, 28.23, 28.02, 27.74, 24.28, 23.84, 22.84, 22.70, 22.58, 21.01, 19.30, 18.72, 11.86.

2. Experimental Section

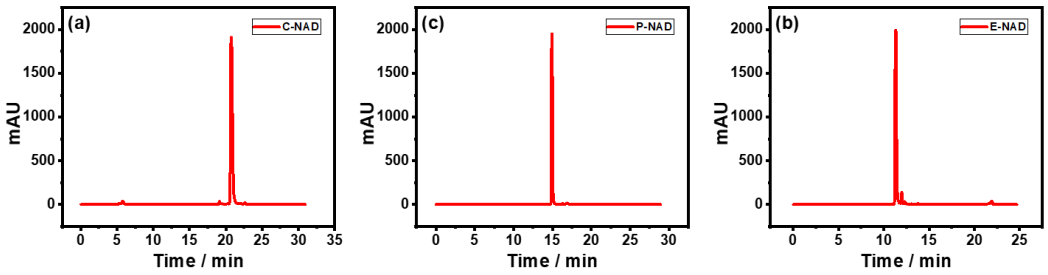


Figure S1. HPLC chromatogram of NADs. (detection wavelength at 450 nm).

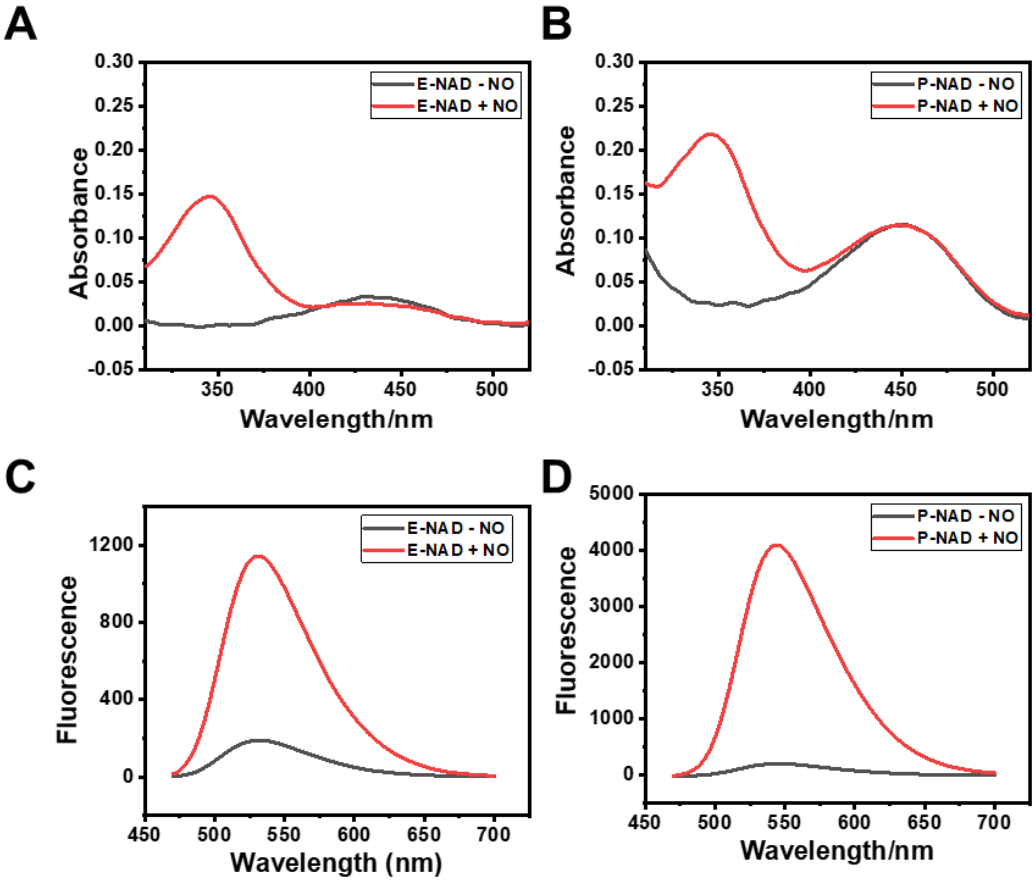


Figure S2. (A, B)UV–vis absorption spectrum of E-NAD (A) and P-NAD (B) before and after the addition of NO (50 μM). (C, D) Fluorescence spectra spectrum of E-NAD (A) and P-NAD (B) before and after the addition of NO (50 μM). E-NAD: λex = 430 nm. P-NAD: λex = 452 nm.

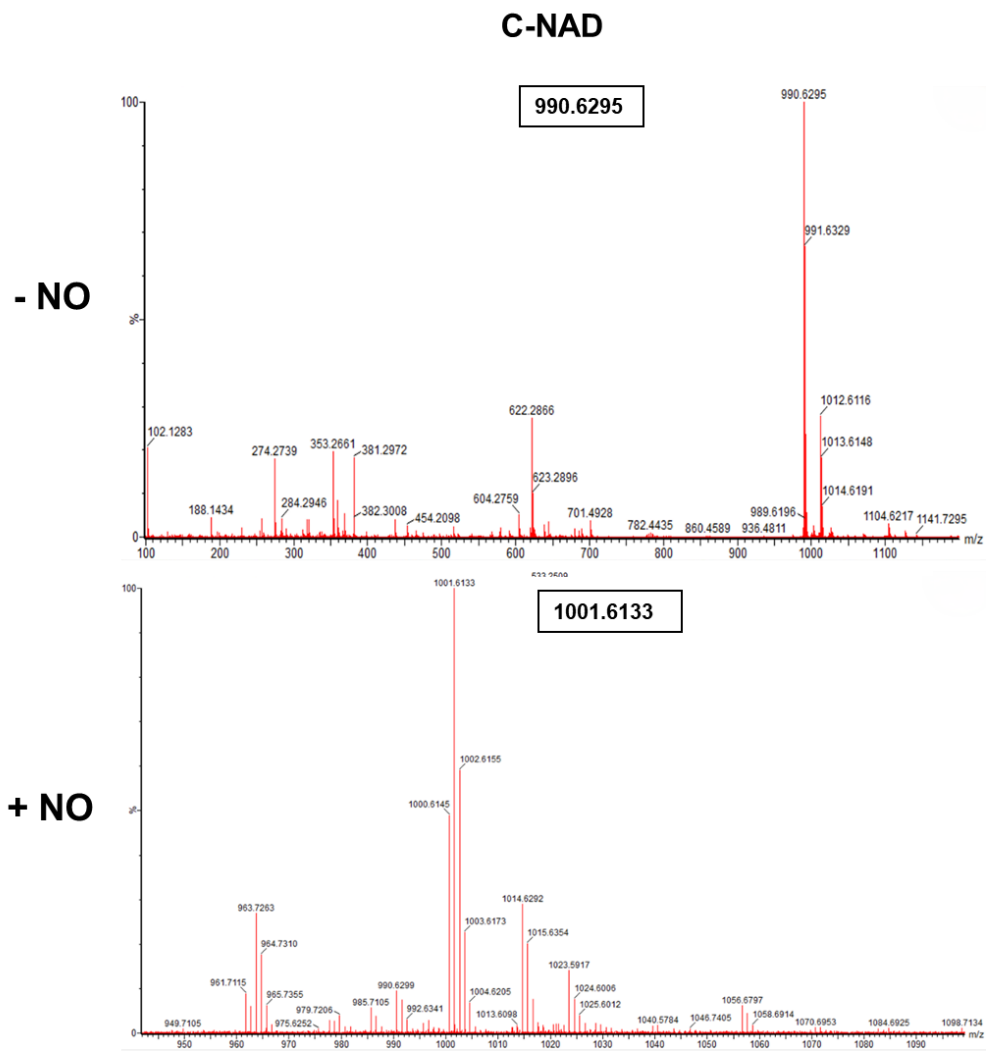


Figure S3. High-resolution mass spectrometry of the reaction between C-NAD and NO.

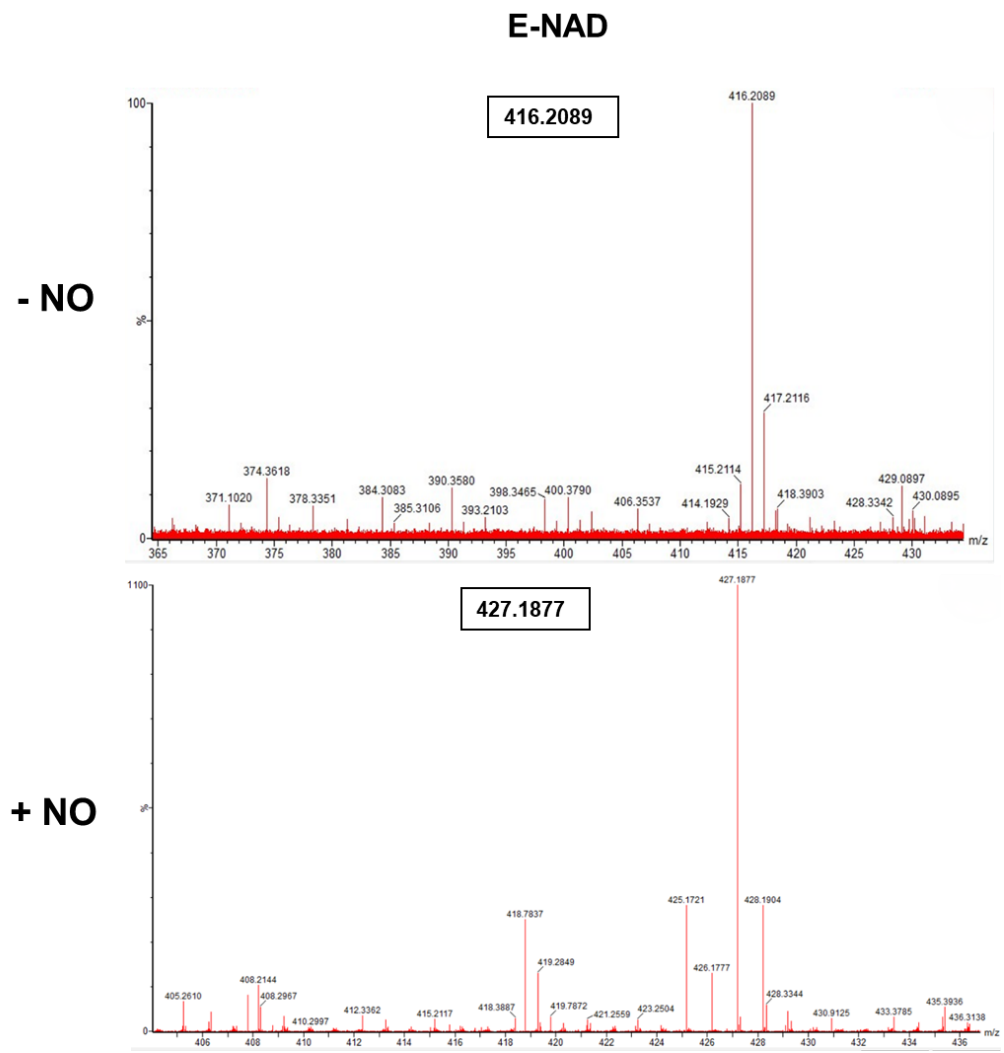


Figure S4. High-resolution mass spectrometry of the reaction between E-NAD and NO.

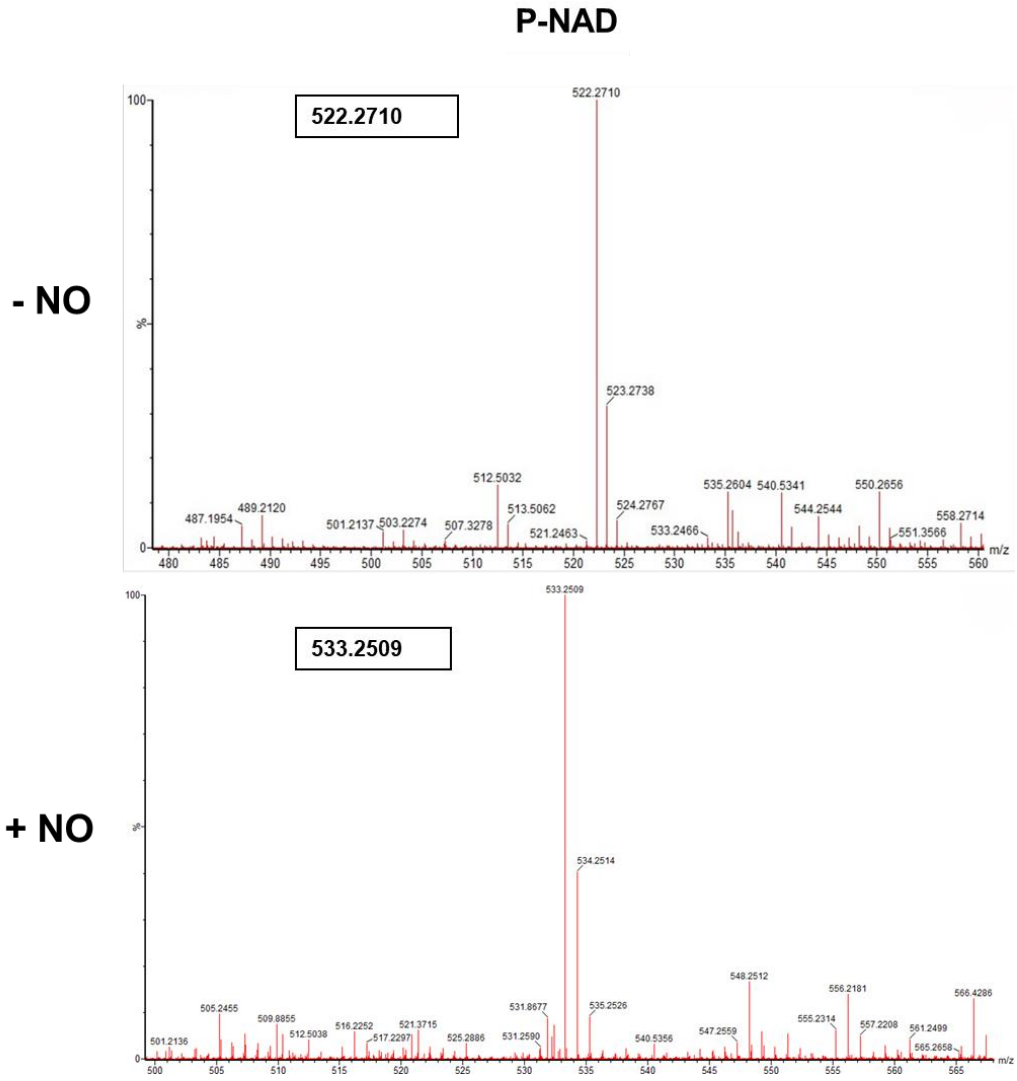


Figure S5. High-resolution mass spectrometry of the reaction between P-NAD and NO.

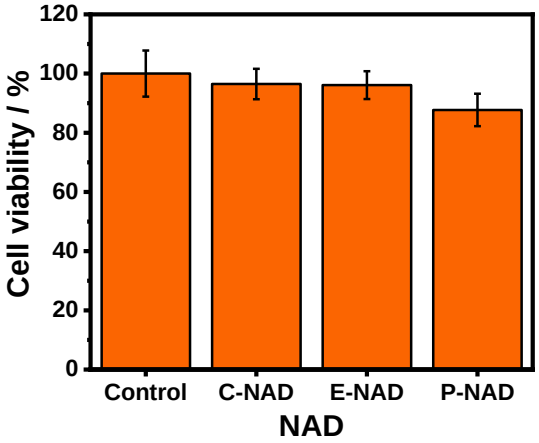


Figure S6. MTT analysis of HeLa cells treated with NADs. The concentration of NADs was 10 μM, while the concentration of NO was 20 μM.

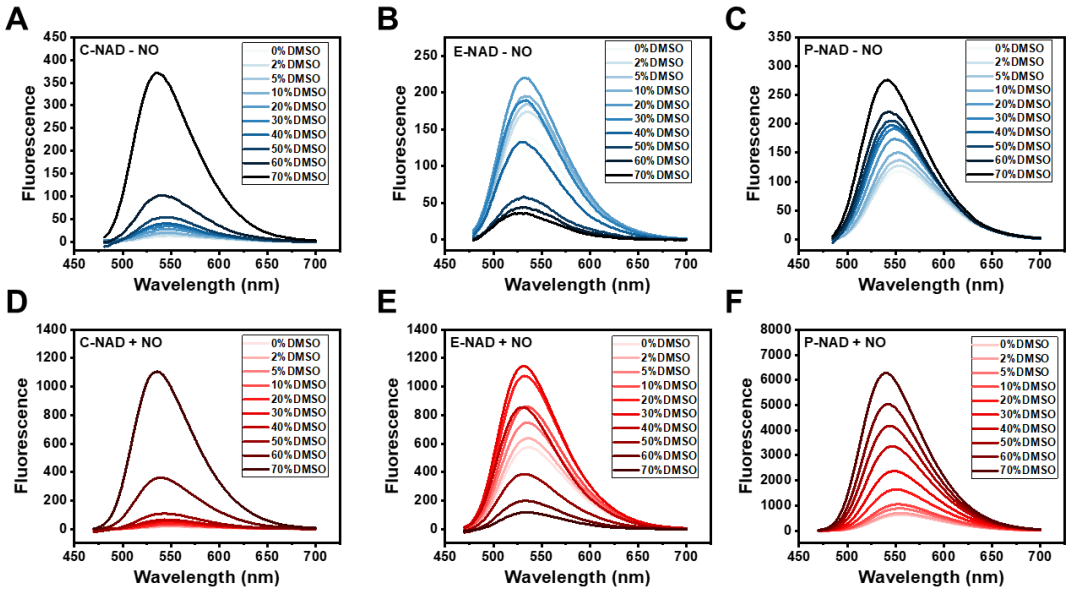


Figure S7. Fluorescence spectra of NAD series probes in solvents with varying volume proportions of DMSO. (A, B, C) Fluorescence spectra of C-NAD (A), E-NAD (B), P-NAD (C) without the addition of NO in various DMSO concentrations. (D, E, F) Fluorescence spectra of C-NAD (D), E-NAD (E), P-NAD (F) with the addition of NO in various DMSO concentrations. The colors range from light to dark, representing an increasing proportion of DMSO in the solvent, specifically: 0%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, and 70% DMSO. The concentration of NAD series probes was 10 μM, while the concentration of NO was 50 μM. C-NAD: λex = 452 nm. E-NAD: λex = 430 nm. P-NAD: λex = 452 nm. λem = 470-700 nm.

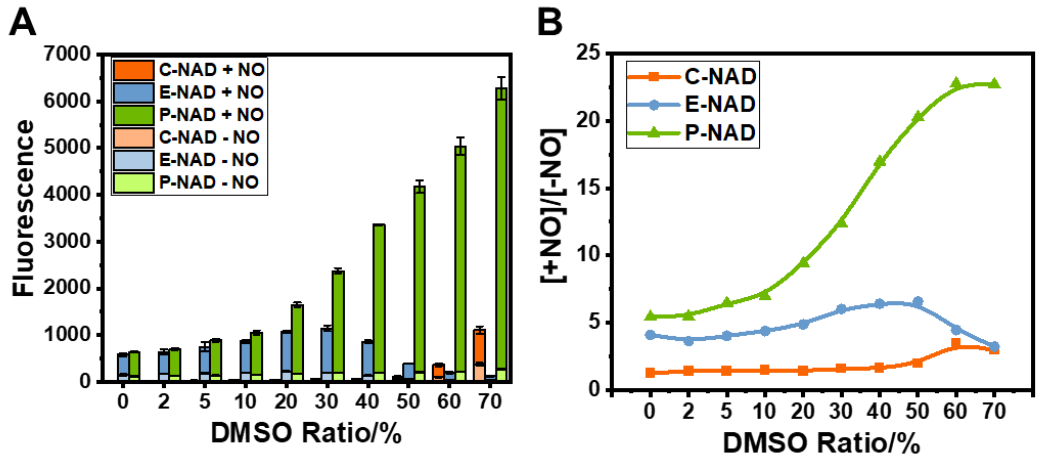


Figure S8. (A) Fluorescence intensity of different series probes in solvents with varying volume proportions of DMSO from Figure S3. (B) Fluorescence signal-to-noise ratios

of NADs in solvents with varying proportions of DMSO from Figure S7. C-NAD: λex = 452 nm. E-NAD: λex = 430 nm. P-NAD: λex = 452 nm. λem = 470-700 nm.

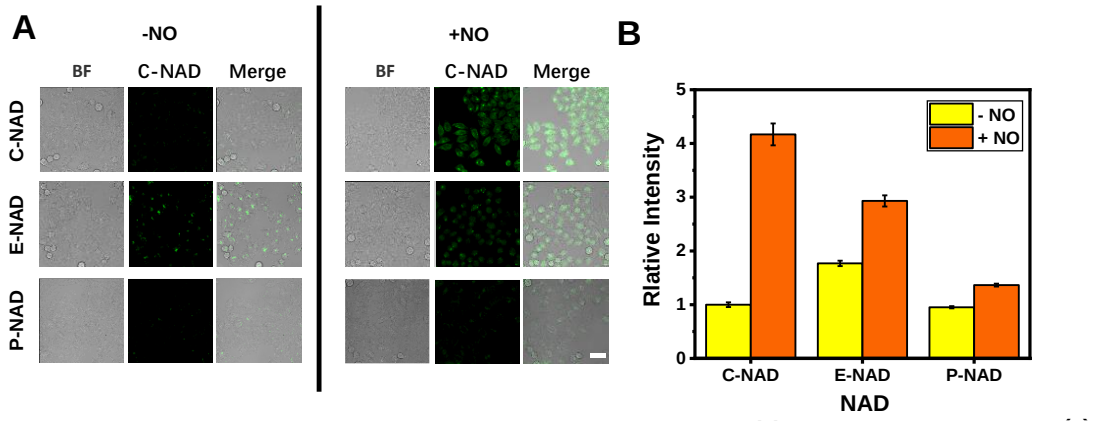


Figure S9. Fluorescence imaging of NO in Hela cells. (A) Fluorescence imaging of NADs (10 μM) without and with NO (20μM) in Hela cells. (B) Fluorescence intensity at ROI from A. Fluorescence signals were collected at 500–600 nm for the green channel (λex = 488 nm).

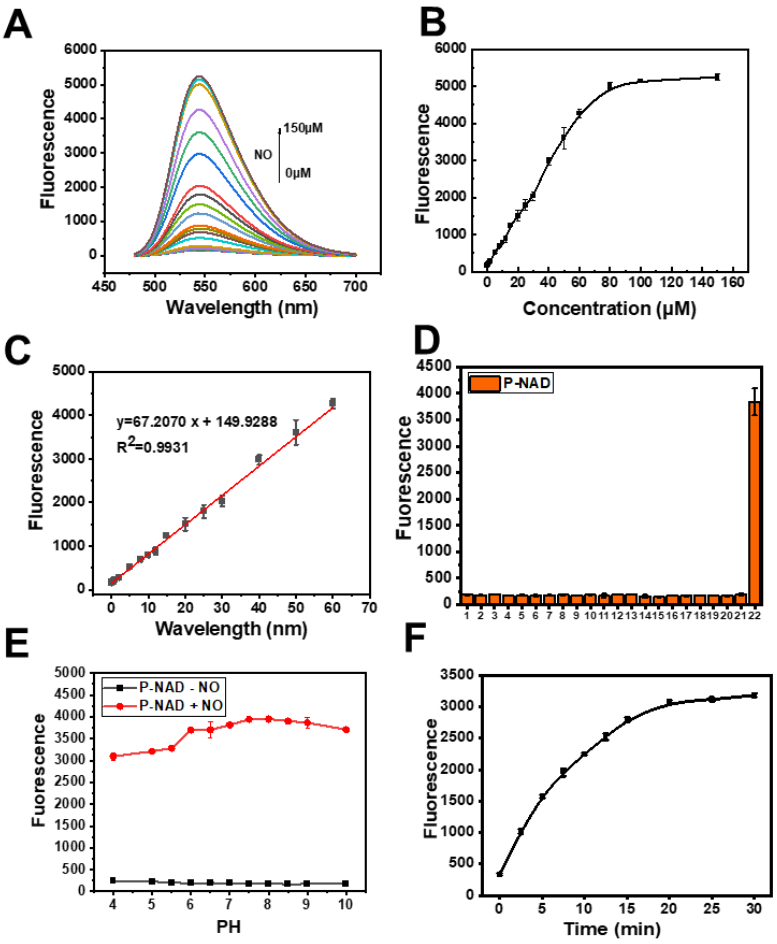


Figure S10. P-NAD's detection of NO in vitro (A) Fluorescence spectra of P-NAD (10 μM) with increasing NO concentration (0, 0.1, 0.2, 0.5, 1, 2, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100 μM). (B) Fluorescence intensity of P-NAD (10 μM) with NO concentration. (C) Linear fit to the plot of the NO emission and NO concentration (0–2 μM). (D) Fluorescence intensity of P-NAD (10 μM) upon the addition of other analytes (100 μM) with or without NO (100 μM). Analytes: (1) Probe only (2) Ca^{2+} (3) Mg^{2+} (4) Zn^{2+} (5) Fe^{2+} (6) Fe^{3+} (7) Cu^{2+} (8) NO_2^- (9) NO_3^- (10) $ONOO^-$(11) H_2O_2 (12)·OH (13) L-Arg (14) DHA (15) LPS (16) ATP (17) GSH (18) Cys (19) Hcy (20) H_2S (21) Formaldehyde (22) DEA·NONOate. (E) Effect of pH on the fluorescence intensity of P-NAD (10 μM) in the absence and presence of NO (100 μM) in an DMSO–(PBS) (1 : 1 vol%) solution. (F) Time-dependent changes in the fluorescence of P-NAD (10 μM) upon the addition of NO (50 μM). Sensitivity and selectivity of P-NAD toward NO detection. λex = 452 nm. λem = 470-700 nm.

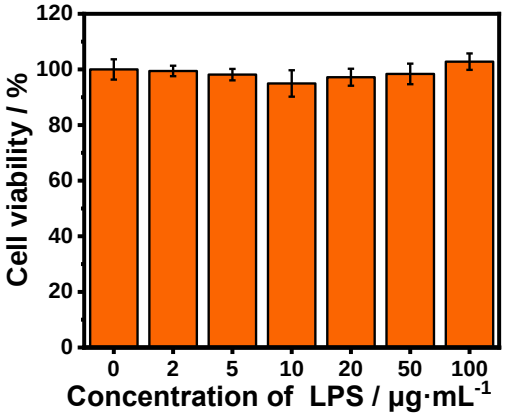
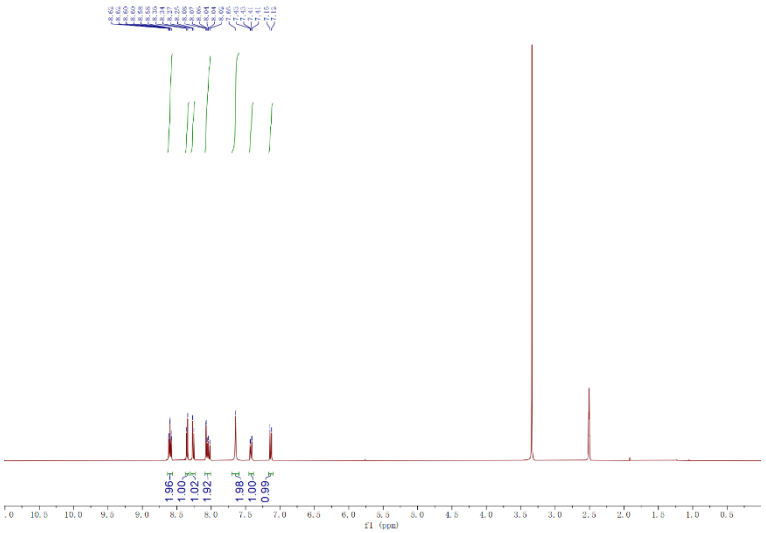
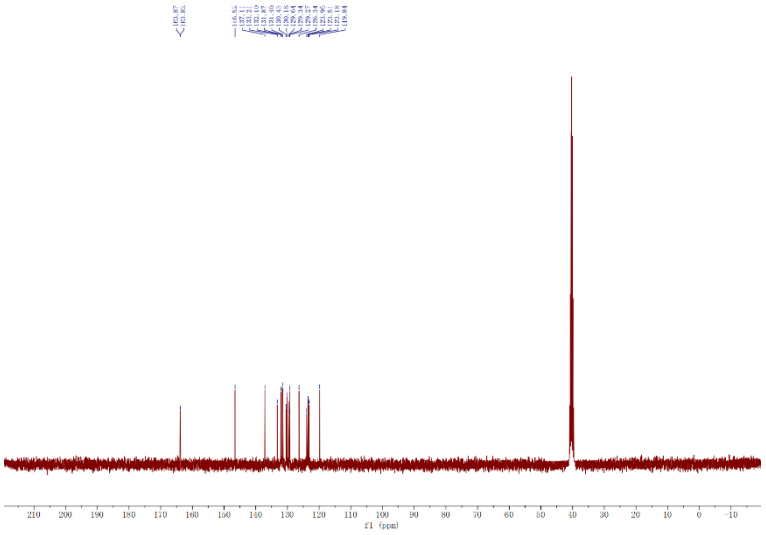


Figure S11. MTT analysis of HeLa cells treated with LPS.

3. Structural Identifications of the Compounds

Figure S12. ^{1}H NMR ^{13}C NMR and MS spectrum of Compound **1**.





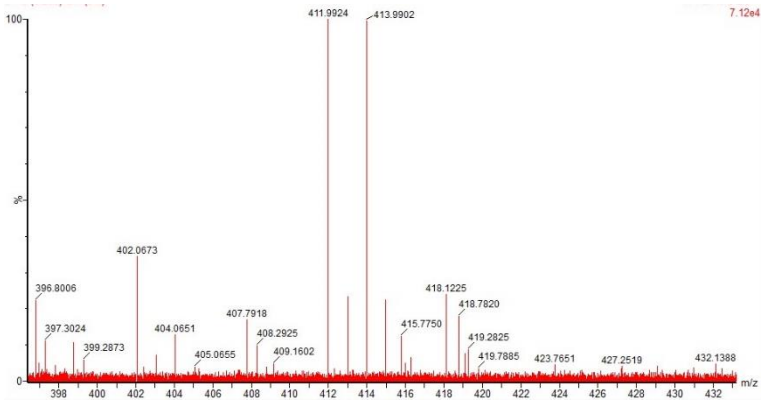
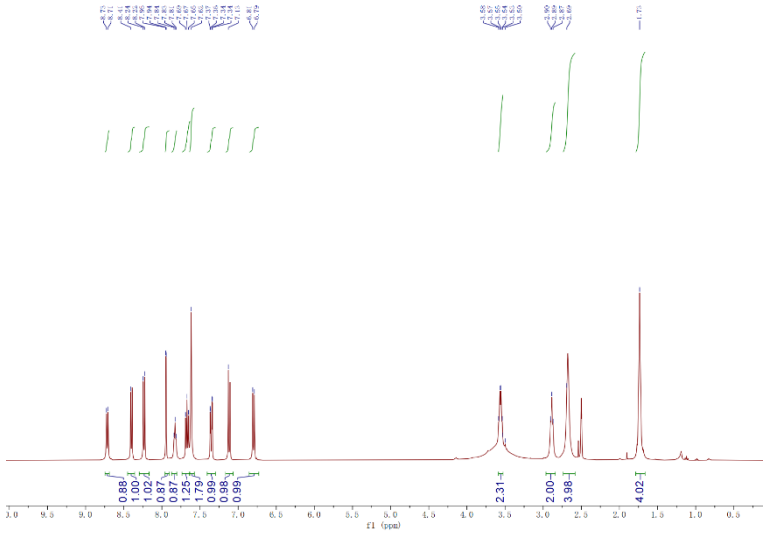
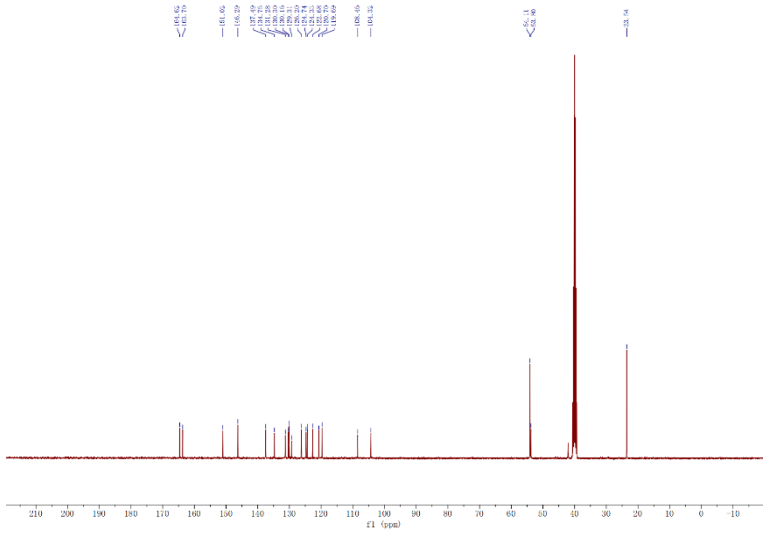


Figure S13. ^{1}H NMR ^{13}C NMR and MS spectrum of Compound **2**.





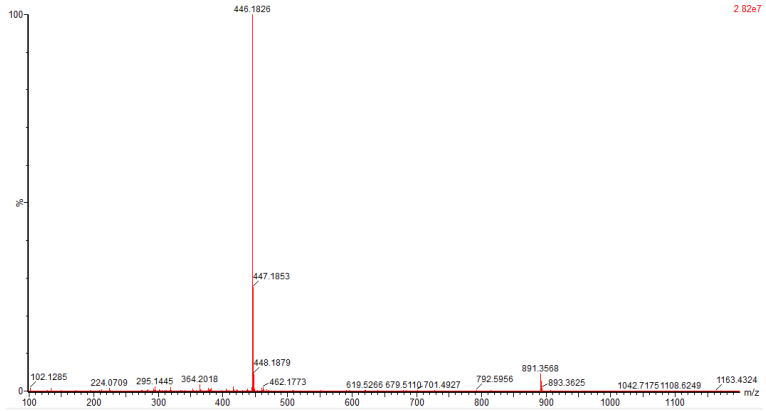


Figure S14. ^{1}H NMR ^{13}C NMR and MS spectrum of E-NAD.

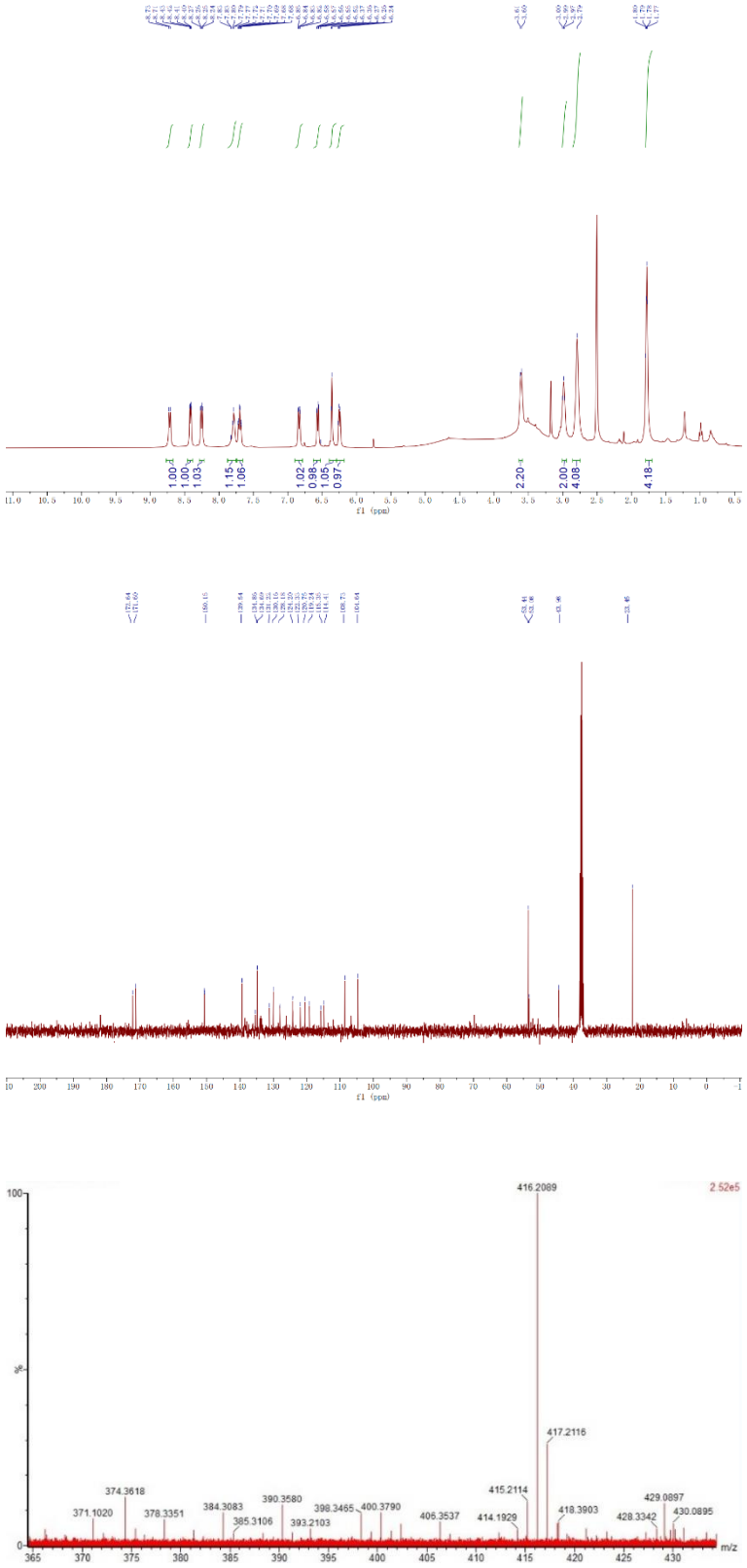


Figure S15. ^{1}H NMR ^{13}C NMR and MS spectrum of Compound **4**.

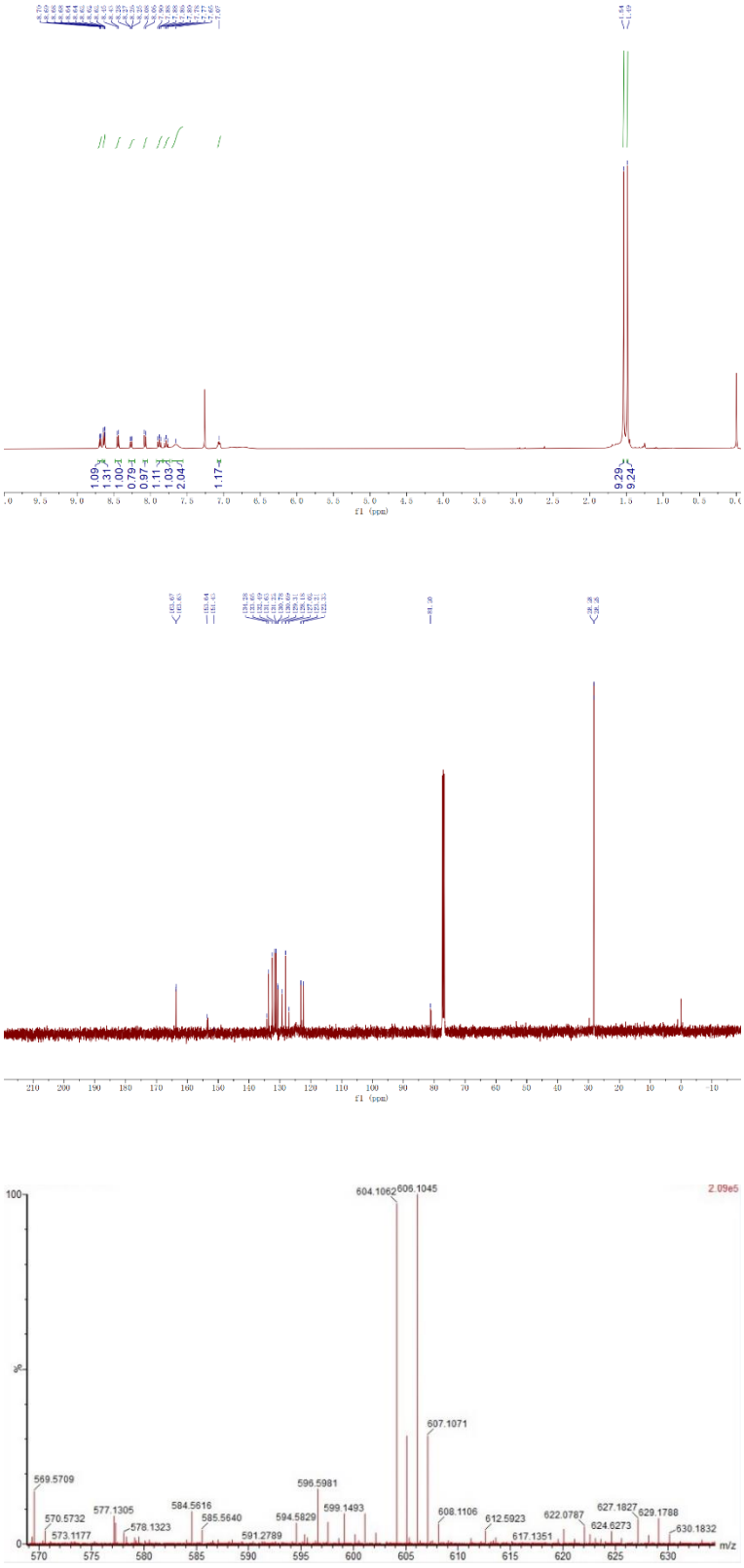


Figure S16. ^{1}H NMR ^{13}C NMR and MS spectrum of Compound **5**.

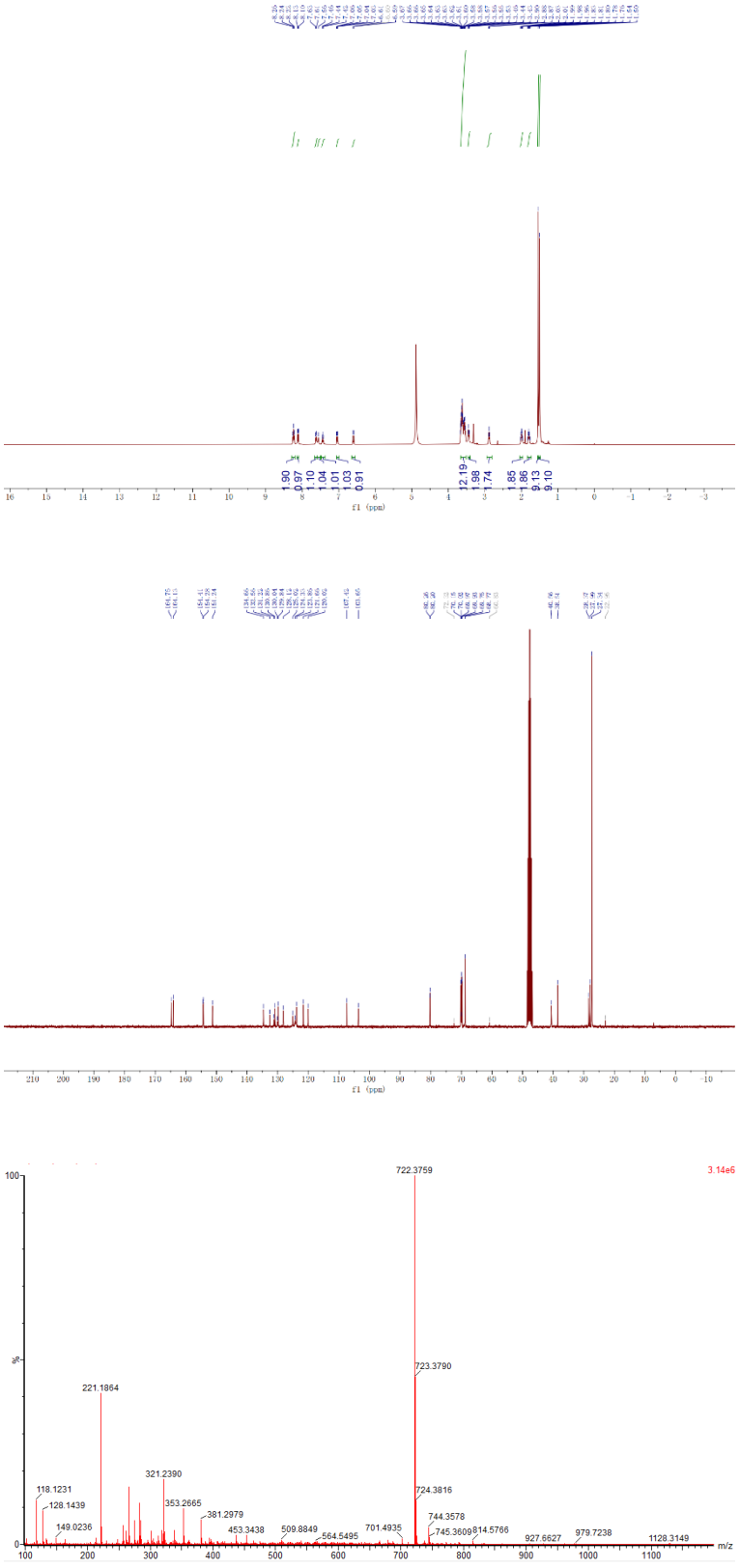


Figure S17. ^{1}H NMR ^{13}C NMR and MS spectrum of P-NAD.

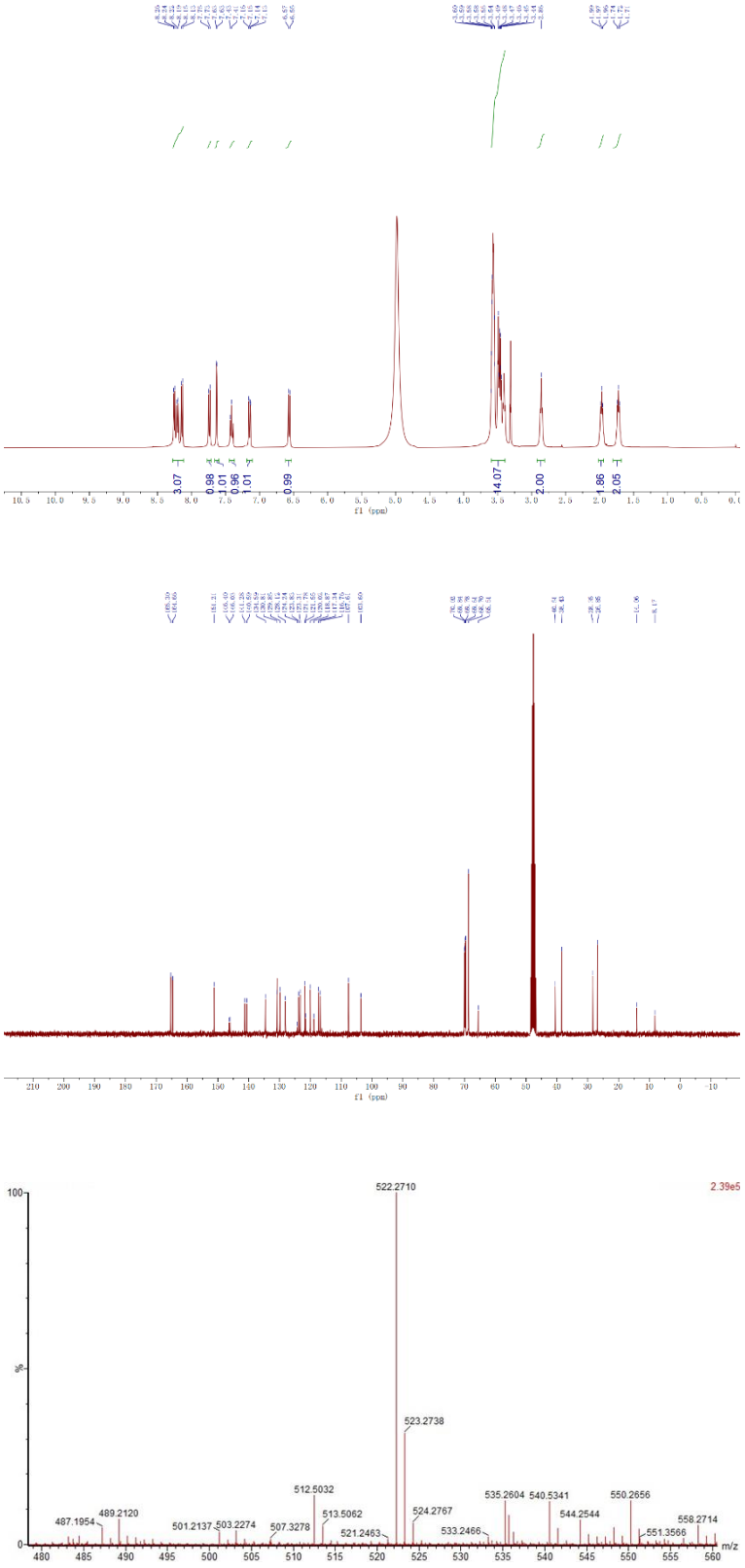


Figure S18. ^{1}H NMR ^{13}C NMR and MS spectrum of Compound **6**.

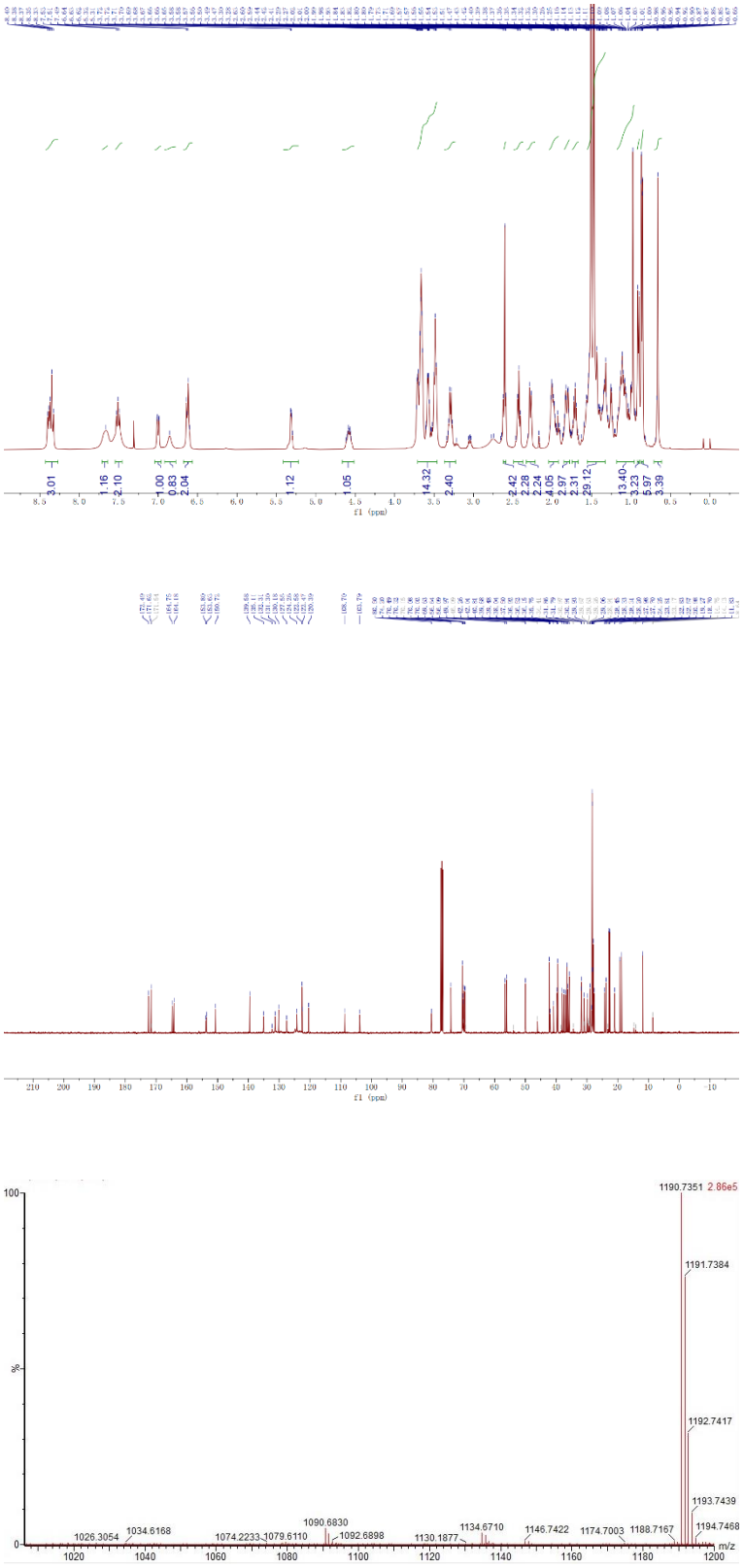


Figure S19. ^{1}H NMR ^{13}C NMR and MS spectrum of C-NAD.