Supplementary Information

for

Stimuli-Responsive Biotin-Anchored Prodrug for the Targeted Delivery of Anti-Cancer Agent NBDHEX with Turn-On NIR Fluorescence

Rahul Kesarwani,[a] Nikita Pal,[b] and Krishna P. Bhabak[a,*]

[a]Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India.
Email: kbhabak@iitg.ac.in

Author contribution

K.P.B. designed the project and supervised the research work as well as data analysis. R.K. optimized the experimental condition, synthesized the prodrug, purified, characterized, and carried out spectroscopic studies and related assays using UV-Vis, Fluorescence spectroscopy and HPLC. N.P. carried out cellular studies (anti-proliferative activities and fluorescence microscopic experiments). The manuscript was prepared by K.P.B., R.K. and N.P. Original draft of the manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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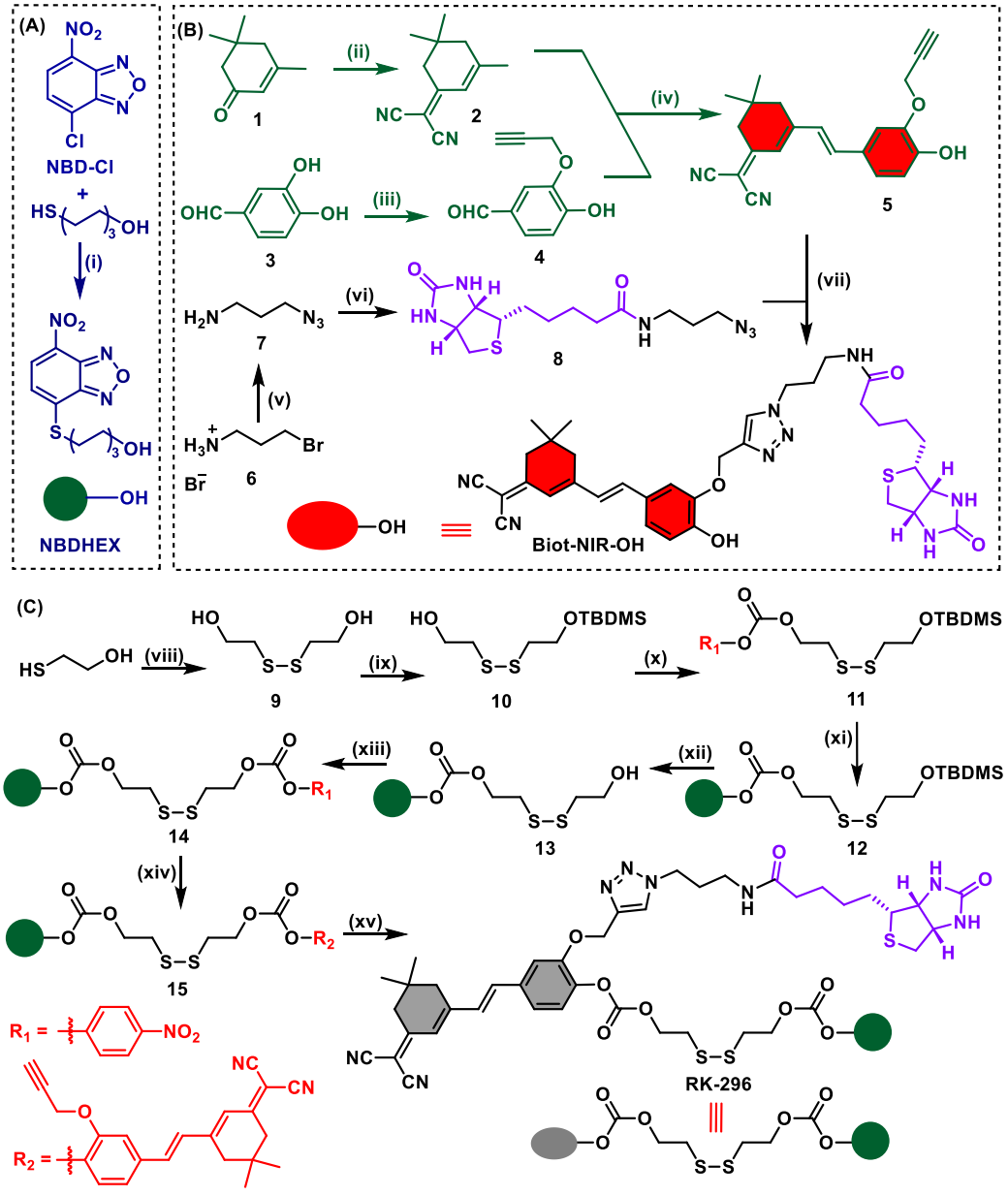
Abbreviations

Met, L-Methionine; Ala, L-Alanine, Ser, L-Serine; Gly, Glycine; Arg, L-Arginine; Glu, L-Glutamic acid; Asp, Aspartic acid; NaCl, Sodium chloride; NADPH, β-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt; H_2O_2, Hydrogen peroxide; *t*-BuOOH, *tert*-butyl hydroperoxide; BSA, Bovine serum albumin; 2-ME, 2-Mercaptoethanol; $Na_2S{\cdot}9H_2O$, Sodium sulfide nonahydrate; PhSH, Thiophenol; Cys, L-Cysteine; Hcy, Homocysteine; GSH, Glutathione reduced; NBDHEX, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol; DCM, dichloromethane; DMAP, 4-Dimethylaminopyridine; CDI, Carbonyl diimidazole; DMF, *N*,*N*-dimethylformamide; NaH, Sodium hydride; EtOAc, Ethyl acetate; ACN, Acetonitrile; PTSA, *p*-Toluene sulfonic acid; DMSO, Dimethyl sulfoxide; TLC, Thin layer chromatography; NEM, *N*-ethylmaleimide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide.

Experimental Section



Scheme 1. Synthetic outlines of (A) **NBDHEX**, (B) **Biot-NIR-OH** and (C) the prodrug **RK-296**. Reagents and conditions: (i) EtOH: sodium phosphate buffer (1:1), r.t., 6 h; (ii) Malononitrile, EtOH, Piperidine, 85 °C, 12 h; (iii) Propargyl bromide, NaH, DMF, 0 °C – r.t., 4 h; (iv) Piperidine, ACN, 80 °C, 6 h; (v) NaN_3, H_2O, 100 °C, 24 h; (vi) (a) Biotin, DMF, 55 °C, (b) CDI, r.t., 15 h; (vii) $CuSO_4{\cdot}5H_2O$, Sodium ascorbate, DCM:H_2O (1:1), r.t., 6 h; (viii) NaI, H_2O_2, EtOAc, r.t., 30 min; (ix) TBDMSCl, Imidazole, THF, 0 °C – r.t., 6 h; (x) *p*-

Nitrophenyl chloroformate, Pyridine, DCM, 0 °C – r.t., 6 h; (xi) **NBDHEX**, Pyridine, DCM, DMAP, 0 °C – r.t., 24 h; (xii) *p*-Toluene sulfonic acid, MeOH, r.t., 12 h; (xiii) *p*-Nitrophenyl chloroformate, Pyridine, DCM, 0 °C – r.t., 6 h; (xiv) **5**, Pyridine, DCM, 0 °C – r.t., 24 h; (xv) **8**, $CuSO_4 \cdot 5H_2O$, Sodium ascorbate, DCM:H_2O (1:1), r.t., 6 h.

Materials and methods

All chemicals were procured either from Sigma Aldrich or from reputable local suppliers and were utilized without additional purification, unless otherwise stated. TLC analyses were carried out on pre-coated silica gel on aluminium sheets and the compounds were visualized by irradiation with UV or fluorescent light. Organic solvents employed for chromatographic separations were distilled prior to use. Nuclear Magnetic Resonance (NMR) spectra (^{1}H and ^{13}C) were recorded on Bruker (600 MHz) NMR spectrometers and the chemical shifts are reported relative to TMS (Me_4Si) as an internal standard. UV-Vis spectroscopic analyses were carried out using a Lambda 365$^+$. UV-Visible spectrophotometer and fluorescence emission spectra were recorded on a Fluoromax-4 spectrophotometer (FluoroMax-4 - Horiba). Melting points were determined using a Büchi B-540 apparatus and are uncorrected. Mass spectra were obtained using an Agilent 1100/1200/1260/1290 LC Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS spectrometer.

Synthesis of NBDHEX[1]

NBDHEX was synthesized according to the previously reported literature. Briefly, NBD-Cl (1.00 g, 5.00 mmol) was dissolved in ethanol and 0.1 M sodium phosphate buffer (1:1, v/v, 100 mL), of pH = 7. Then 6-mercaptohexan-1-ol (0.73 g, 5.50 mmol) was added and the pH of the medium was adjusted to 7 using 1M NaOH solution. The reaction was stirred for 3 h at room temperature, and the resultant precipitate was filtered off and washed with water (2 × 15 mL) and dried to afford the crude compound. The crude compound was purified by 230-400 silica gel column chromatography using 60% ethyl acetate in petroleum ether as the eluent to afford the pure product as light brown solid. R_f = 0.5 (60% Ethyl acetate in petroleum ether), Yield: (1.40 g, 81%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.41 (d, *J* = 7.9 Hz, 1H), 7.15 (d, *J* = 7.9 Hz, 1H), 3.68 (t, *J* = 6.5 Hz, 2H), 3.28 (t, *J* = 7.4 Hz, 2H), 1.91-186 (m, 2H), 1.63-1.57 (m, 4H), 1.50-1.45 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 149.2, 142.5, 141.9, 132.5, 130.6, 120.2,62.6, 32.4, 31.7, 28.6, 27.8, 25.3.

Synthesis of compound 2[2]

To a solution of compound **1** (0.20 g, 1.47 mmol) and malononitrile (0.11 g, 1.72 mmol) in absolute ethanol was added piperidine (13.00 mg, 0.15 mmol) and acetic acid (9.00 mg, 0.15 mmol) under argon atmosphere. The reaction mixture was refluxed for 12 h upon which the reaction mixture turned dark black in color. The progress of the reaction was monitored by TLC analysis. After completion, the mixture was cooled to room temperature and the solvent was evaporated under reduced pressure and the residue obtained was dissolved in CH_2Cl_2. The organic layer was washed with water, brine and dried over anhydrous sodium sulfate. The solvent was evaporated to afford the crude product, which was subjected to silica gel column chromatography using dichloromethane and petroleum ether as eluents to afford the pure product **2** as a white solid. R_f = 0.5 (20% DCM in petroleum ether). Yield: (0.20 g, 74%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 6.62 (s, 1H), 2.51 (s, 2H), 2.18 (s, 2H), 2.03 (s, 3H), 1.01 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 170.4, 159.8, 120.6, 113.2, 112.4, 78.2, 45.6, 42.6, 32.4, 27.8, 25.3.

Synthesis of compound 4

In an oven dried double neck round bottom flask with an argon inlet, NaH (1.39 g, 57.92 mmol) was added into anhydrous DMF (10 mL) in small portions. While stirring and cooling this solution to 0 °C, anhydrous DMF (5 mL) containing 3,4-dihydroxybenzaldehyde **3** (2.00 g, 14.48 mmol) was added dropwise into the flask. This reaction mixture was stirred at room temperature for 30 min. Propargyl bromide (1.10 mL, 14.48 mmol) was then added dropwise via syringe and the mixture was stirred at room temperature overnight. The mixture was poured into ice, neutralized by 1 M HCl solution and the product was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed with brine (3 × 50 mL) and dried over sodium sulfate. After filtration the solvent was removed under reduced pressure to afford crude product as a brownish powder. The crude compound was purified using silica gel column chromatography using 20% ethyl acetate in petroleum ether as eluent to afford compound **4** as a white powder. R_f = 0.5 (20% Ethyl acetate in petroleum ether). Yield: (1.58 g, 62%). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 9.84 (s, 1H), 7.53 (d, *J* = 1.8 Hz, 1H), 7.48 (dd, *J* = 8.1, 1.8, Hz, 1H), 7.09 (d, *J* = 8.1 Hz, 1H), 6.22 (s, 1H), 4.84 (d, *J* = 2.4 Hz, 2H), 2.60 (t, *J* = 2.4, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 190.6, 151.9, 145.1, 129.8, 127.9, 115.1, 111.0, 77.1, 76.9, 57.0. ESI-MS: *m/z* calcd. for $C_{10}H_9O_3$ $[M+H]^+$: 177.0547; obs. $[M+H]^+$: 177.0548.

Synthesis of compound 5

To a solution of compound **2** (1.00 g, 5.36 mmol) in ACN (60 mL) was added compound **4** (0.66 g, 3.75 mmol) and piperidine (9.00 mg, 1.07 mmol). The reaction mixture was refluxed under the inert atmosphere and after 6 h the reaction mixture turned red in color. The progress of the reaction was monitored by TLC analysis. Upon completion, the solvent was removed under reduced pressure. The resulting residue was dissolved in DCM, washed with water, and dried over anhydrous sodium sulfate. The crude product was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluents to afford the pure compound **5** as a red solid. R_f = 0.5 (30% Ethyl acetate in petroleum ether). Yield: (0.85 g, 46%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.17 (d, *J* = 2.0 Hz, 1H), 7.09 (dd, *J* = 8.3, 1.9 Hz, 1H), 6.99 (d, *J* = 16.0 Hz, 1H), 6.96 (d, *J* = 8.3 Hz, 1H), 6.84 (d, *J* = 16.6 Hz, 2H), 5.87 (s, 1H), 4.84 (d, *J* = 2.4 Hz, 2H), 2.62 (t, *J* = 2.4 Hz, 1H), 2.60 (s, 2H), 2.46 (s, 2H), 1.08 (s,6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 169.3, 154.1, 147.9, 144.9, 136.9, 128.3, 127.1, 123.7, 122.8, 115.5, 113.6, 112.9, 110.6, 77.9, 77.5, 57.0, 43.0, 39.2, 32.0, 28.0. ESI-MS: *m/z* calcd. for $C_{22}H_{20}N_2O_2Na$ $[M+Na]^+$: 367.1417; obs. $[M+Na]^+$: 367.1415.

Synthesis of compound 7[3]

To a solution of 3-Bromopropan-1-amine hydrobromide **6** (0.25 g, 1.14 mmol) in water was added sodium azide (0.37 g, 5.70 mmol). The mixture was stirred at 100 °C for 24 h and allowed to cool to room temperature. An aqueous solution of KOH (5.70 mmol) was added dropwise to the reaction mixture and the pH was allowed to raise up to 14 under the temperature below 10 °C. The crude product was extracted with DCM (3 ×15 mL) and the organic layer was washed with brine (3 x10 mL). The organic layer was dried over sodium sulfate, and the solvent was evaporated under reduced pressure to afford compound **7** as a light-yellow oil. Yield: (0.10 g, 92%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 3.38 (t, *J* = 6.8 Hz, 1H), 2.81 (t, *J* = 6.9 Hz, 1H), 1.74 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 49.2, 39.3, 32.4.

Synthesis of compound 8[4]

Biotin (0.05 g, 0.20 mmol) was first dissolved in DMF (1mL) at 55 °C. After cooling to room temperature, CDI (0.06 g, 0.36 mmol) in DMF (0.5 mL) was gradually added and the reaction mixture was stirred for 3 h at room temperature. Subsequently, 1-azido-3-aminopropane **7** (0.06 g,

0.61 mmol) in DMF (1 mL) was added dropwise over approximately 10 min and the reaction mixture was stirred for an additional 12 h. The solvent was removed under reduced pressure and the residues was purified by recrystallization in a mixture of 1-butanol/acetic acid/water (70:7:10, v/v/v). The resulting white solid was washed with diethyl ether three times and dried in a vacuum oven to afford biotin azide **8**. Yield: (0.05 g, 82%). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.83 (t, *J* = 5.7 Hz, 1H), 6.42 (s, 1H), 6.35 (s, 1H), 4.30 (dd, *J* = 7.7, 5.2 Hz, 1H), 4.14-4.11 (m, 1H), 3.35-3.33 (m, 2H), 3.11-3.07 (m, 2H), 2.82 (dd, *J* = 12.8, 5.1 Hz, 1H), 2.58 (s, 1H), 2.56 (s, 1H), 2.20 (t, *J* = 7.4 Hz, 1H), 2.05 (t, *J* = 7.4 Hz, 1H), 1.66-1.43 (m, 6H), 1.37-1.26 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 177.3, 167.9, 66.3, 64.4, 60.6, 53.6, 40.9, 40.4, 38.7, 33.7, 33.2, 30.4, 29.7.

Synthesis of compound Biot-NIR-OH

Compound **5** (0.05 g, 0.14 mmol) and compound **8** (47.00 mg, 0.14 mmol) were dissolved in a mixture of DCM (2 mL) and water (2 mL) followed by the addition of $CuSO_4{\cdot}5H_2O$ (0.07 g, 0.29 mmol) and sodium ascorbate (0.11 g, 0.58 mmol) to get a deep brown reaction mixture. The reaction mixture was stirred at room temperature for 6 h. Upon completion of the reaction as confirmed by TLC analysis, the reaction mixture was diluted with DCM and washed with water (5 × 20 mL) and brine (3 × 10 mL), respectively. The organic layer was collected and dried over sodium sulfate and the solvent was removed under reduced pressure to obtain the crude product. Purification of the crude product was carried out using flash chromatography using silica gel column with 10% methanol in DCM as eluents to afford the pure compound **Biot-NIR-OH** as red solid. R_f = 0.5 (5% Methanol in DCM). Yield: (0.04 g, 41%). ^{1}H NMR (600 MHz, Methanol-d_4) δ (ppm): 8.10 (s, 1H), 7.37 (d, *J* = 1.9 Hz, 1H), 7.17 – 7.04 (m, 2H), 7.02 (d, *J* = 16.0 Hz, 1H), 6.82 (d, *J* = 8.2 Hz, 1H), 6.79 (s, 1H), 5.26 (s, 2H), 4.48-4.44 (m, 3H), 4.29-4.27 (m, 1H), 3.22-3.16 (m, 3H), 2.99 (s, 1H), 2.91 – 2.88 (m, 1H), 2.85 (s, 1H), 2.68 (d, *J* = 12.8 Hz, 1H), 2.59 (s, 2H), 2.52 (s, 2H), 2.19 (t, *J* = 7.4 Hz, 2H), 2.13-2.08 (m, 2H), 1.97 (s, 1H), 1.75 – 1.54 (m, 4H), 1.49-1.40 (m, 2H), 1.07 (s, 6H). ^{13}C NMR (150 MHz, Methanol-d_4) δ (ppm): 176.3, 171.3, 166.1, 164.9, 157.2, 150.5, 147.9, 144.8, 139.2, 129.7, 127.9, 125.7, 124.6, 123.1, 117.3, 114.8, 114.1, 113.8, 77.4, 63.4, 63.3, 61.6, 57.0, 43.9, 41.0, 39.9, 37.4, 36.7, 32.9, 31.1, 29.7, 29.4, 28.1, 26.7. ESI-MS: *m/z* calcd. for $C_{35}H_{46}N_8O_4S$ $[M+H]^+$: 671.3123; obs. $[M+H]^+$: 671.3122.

Synthesis of compound 9[5]

To a solution of 2-mercaptoethanol (6.00 g, 76.92 mmol) in distilled ethyl acetate (60 mL) was added NaI (0.06 g) followed by hydrogen peroxide (8.4 mL, 30% solution in water). The reaction mixture was stirred at room temperature for 20 min and color of the solution turned brownish. The reaction was quenched by addition of saturated solution of $Na_2S_2O_3$ (50 mL). Upon completion of the reaction, the reaction mixture was washed with saturated solution of Na_2CO_3 (3 × 30 mL) followed by brine solution (3 × 30 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was evaporated under vacuum to afford the compound **9** as colorless oil. R_f = 0.5 (40% Ethyl acetate in petroleum ether), Yield: (5.27 g, 89%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.92 (t, *J* = 5.7 Hz, 4H), 2.89 (t, *J* = 5.8 Hz, 4H), 2.08 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 60.4, 41.2.

Synthesis of compound 10[5]

To a solution of compound **9** (3.00 g, 19.44 mmol) and imidazole (1.32 g, 19.44 mmol) in dry THF (100 mL) was added the solution of TBDMSCl (2.93 g, 19.44 mmol) dropwise under inert condition at 0 °C. The reaction mixture was allowed to attain room temperature and stirred for 6 h. Upon completion of the reaction, the solvent was removed under reduced pressure and the residue was dissolved in water. The crude compound was extracted by ethyl acetate (3 × 50 mL). The combined organic layer was washed with brine solution (3 × 50 mL) and dried over anhydrous sodium sulfate. The compound was purified by flash chromatography with silica gel column using 40% ethyl acetate in petroleum ether to afford the pure compound **10** as pale yellow liquid. R_f = 0.4 (10% Ethyl acetate in petroleum ether), Yield: (2.50 g, 50%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.92-3.86 (m, 4H), 2.87-2.83 (m, 4H), 0.93 (s, 9H) 0.10 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 61.8, 60.2, 41.3, 41.3, 25.9, 18.3, -5.2.

Synthesis of compound 11

To a solution of compound **10** (2.50 g, 9.31 mmol) in anhydrous DCM (50 mL) was added pyridine (2.20 g, 27.93 mmol) and the reaction mixture was cooled to 0 °C. A solution of *p*-nitrophenyl chloroformate (2.24 g, 11.17 mmol) in anhydrous DCM (50 mL) was added dropwise to the reaction mixture at 0 °C. The reaction mixture was allowed to attain room temperature and stirred for another 6 h. The progress of the reaction was monitored by TLC analysis. Upon completion,

the reaction mixture was diluted with DCM and subsequently washed with $NaHCO_3$ (3 × 30 mL), water (3 × 30 mL), and brine (3 × 30 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure to afford compound **11**. The crude product was purified by flash chromatography with silica gel column using 22% ethyl acetate in petroleum ether to afford the pure product **11** as pale yellow liquid. R_f = 0.7 (10% Ethyl acetate in petroleum ether), Yield: (3.30 g, 82%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.29 (d, *J* = 4.5 Hz, 2H), 7.39 (d, *J* = 4.5 Hz, 2H), 4.56 (t, *J* = 6.6 Hz, 2H), 3.87 (t, *J* = 6.6 Hz, 2H), 3.02 (t, *J* = 6.6 Hz, 2H), 2.87 (t, *J* = 6.6 Hz, 2H), 0.90 (s, 9H) 0.08 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 155.4, 152.3, 145.4, 125.3, 121.7, 66.9, 61.6, 41.6, 36.6, 25.8, 18.3, -5.2.

Synthesis of compound 12

To a stirred solution of **NBDHEX** (3.00 g, 6.91 mmol) in anhydrous DCM (50 mL) was added pyridine (2.18 g, 27.64 mmol) and the mixture was stirred for 10 minutes and cooled to 0 °C. A catalytic amount of D-MAP (84.5 mg, 10 mol %) was added to the reaction mixture. A solution of compound **11** (1.65 g, 8.29 mmol) in anhydrous DCM (50 ml) was added dropwise to the mixture and the reaction mixture was allowed to attain room temperature and stirred for 24 h. The progress of the reaction was monitored by TLC analysis. Upon completion, the reaction mixture was poured into water and extracted with DCM (3 × 50 mL). The combined organic layer was finally washed with brine solution, and dried over anhydrous sodium sulfate. The solvent was evaporated under the reduce pressure to afford the crude product **12**. The crude product was purified by silica gel column chromatography using 22% ethyl acetate in petroleum ether to obtain the pure product **12** as yellow oil. R_f = 0.5 (20% Ethyl acetate in petroleum ether), Yield: (2.50 g, 61%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.41 (d, *J* = 7.9 Hz, 1H), 7.15 (d, *J* = 7.9 Hz, 1H), 4.39 (t, *J* = 6.7 Hz, 2H), 4.16 (t, *J* = 6.5 Hz, 2H), 3.85 (t, *J* = 6.6 Hz 2H) 3.28 (t, *J* = 7.3 Hz, 2H), 2.94 (t, *J* = 6.6 Hz, 2H), 2.83 (t, *J* = 6.6 Hz, 2H), 1.90-1.85 (m, 2H), 1.74-1.70 (m, 2H), 1.61-1.56 (m, 2H), 1.50-1.45 (m, 2H), 0.90 (s, 9H), 0.08 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 155.0, 149.2, 142.5, 141.8, 132.6, 130.6, 120.3, 67.9, 65.5, 61.7, 41.5, 36.9, 31.6, 28.4, 27.7, 25.8, 25.2, 18.3, -5.2.

Synthesis of compound 13

To a mixture of compound **12** (2.50 g, 4.22 mmol) and PTSA (0.18 g, 1.05 mmol) was added distilled MeOH (30 mL). The reaction mixture was stirred at room temperature for overnight under inert condition. Upon completion of the reaction, the solvent was evaporated, diluted with water,

and extracted by EtOAc (3 × 30 mL). The combined organic phase was washed with brine (2 × 10 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to afford the crude product, which was purified by flash chromatography using silica gel column with 60% ethyl acetate in petroleum ether to afford the pure product **13** as yellow oil. R_f = 0.5 (30% Ethyl acetate in petroleum ether), Yield: (1.45 g, 72%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 8.42 (d, *J* = 8.0 Hz, 1H), 7.15 (d, *J* = 7.9 Hz, 1H), 4.40 (t, *J* = 6.7 Hz, 2H), 4.17 (t, *J* = 6.5 Hz, 2H), 3.89 (t, *J* = 5.8 Hz, 2H), 3.28 (t, *J* = 7.3 Hz, 2H), 2.97 (t, *J* = 6.7 Hz, 2H), 2.92 (t, *J* = 6.5 Hz, 2H), 2.04 (t, *J* = 6.2 Hz, 1H) 1.91-184 (m, 2H), 1.74-1.70 (m, 2H), 1.62-1.55 (m, 2H), 1.51-1.44 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 155.0, 149.2, 142.5, 141.8, 132.6, 130.6, 120.3, 67.9, 65.5, 61.7, 41.5, 36.9, 31.6, 28.4, 27.7, 25.8, 25.2, 18.3. ESI-MS: *m/z* calcd. for $C_{17}H_{23}N_3O_7S_3Na$ $[M+Na]^+$: 500.0591; obs. $[M+Na]^+$: 500.0596.

Synthesis of compound 14

To a solution of compound **13** (1.20 g, 2.51 mmol) in anhydrous DCM (40 mL) was added pyridine (0.79 g, 10.05 mmol). The reaction mixture was cooled to 0 °C. A solution of *p*-nitrophenyl chloroformate (0.76 g, 3.77 mmol) in anhydrous DCM (25 mL) was added in a dropwise manner at 0 °C. The reaction mixture was allowed to attain room temperature and was stirred for overnight. The progress of the reaction was monitored by TLC analysis. Upon completion, the mixture was diluted with DCM and subsequently washed with $NaHCO_3$ (3 × 30 mL) water (3 × 30 mL) and brine (2 × 10 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated under reduced pressure to afford the crude product, which was purified by flash chromatography using silica gel column with 40% ethyl acetate in petroleum ether to afford the pure product **14**. as pale brown liquid. R_f = 0.5 (20% Ethyl acetate in petroleum ether), Yield: (1.27 g, 80%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.41 (d, *J* = 7.9 Hz 1H), 8.29 (d, *J* = 9.1 Hz, 2H), 7.40 (d, *J* = 9.1 Hz, 2H), 7.15 (d, *J* = 7.8 Hz, 1H), 4.55 (t, *J* = 6.5 Hz, 2H), 4.41 (t, *J* = 6.6 Hz, 2H), 4.16 (t, *J* = 6.5 Hz, 2H), 3.28 (t *J* = 7.3 Hz, 2H), 3.05 (t, *J* = 6.5 Hz, 2H), 3.00 (t, *J* = 6.6 Hz 2H), 1.89-1.84 (m, 2H), 1.75-1.67 (m, 2H), 1.62-1.54 (m, 2H), 1.51-1.44 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 155.4, 154.9, 152.3, 149.2, 145.5, 142.5, 141.7, 132.6, 130.6, 125.3, 121.8, 120.3, 68.0, 66.7, 65.4, 37.1, 36.7, 31.6, 28.4, 28.4, 27.7, 25.2. ESI-MS: *m/z* calcd. for $C_{24}H_{26}N_4O_{11}S_3$ $[M+Na]^+$: 665.0653; obs. $[M+Na]^+$: 665.0653.

Synthesis of compound 15

To a stirred solution of compound **5** (0.06 g, 0.18 mmol) in dry DCM (2 mL) was added pyridine (0.01 g, 1.24 mmol) and a catalytic amount of DMAP (2.12 mg, 10 mol%) and the reaction mixture was cooled to 0 °C. A solution of compound **14** (0.10 g, 0.15 mmol) in dry DCM (2 mL) was added dropwise to the reaction mixture at 0 °C. The reaction mixture allowed to attain room temperature and was stirred for 24 h. The progress of reaction was monitored by TLC method. Upon completion of the reaction, the solvent was evaporated under reduced pressure to afford the crude compound, which was purified by flash chromatography using silica gel column and expected product was eluted with 60% ethyl acetate in petroleum ether as sticky orange liquid. R_f = 0.5 (30% Ethyl acetate in petroleum ether). Yield: (0.13 g, 92%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.38 (d, *J* = 7.9 Hz, 1H), 7.24 (d, *J* = 1.8 Hz, 1H), 7.17 (d, *J* = 8.2 Hz, 1H), 7.13 (dd, *J* = 7.8, 3.7 Hz, 2H), 6.99 (d, *J* = 16.1 Hz, 1H), 6.91 (d, *J* = 15.8 Hz, 1H), 6.86 (s, 1H), 4.80 (d, *J* = 2.4 Hz, 2H), 4.52 (t, *J* = 6.6 Hz, 2H), 4.41 (t, *J* = 6.5 Hz, 2H), 4.17 (t, *J* = 6.4 Hz, 2H), 3.26 (t, *J* = 7.4 Hz, 2H), 3.05 (t, *J* = 6.6 Hz, 2H), 3.00 (t, *J* = 6.4 Hz, 2H), 2.62 (s, 2H), 2.60 (d, *J* = 2.0 Hz, 1H), 2.47 (s, 2H), 1.89-1.84 (m, 2H), 1.75-1.70 (m, 2H), 1.59-1.56 (d, *J* = 7.7 Hz, 2H), 1.50-1.45 (m, 2H), 1.09 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 169.2, 155.0, 153.3, 152.7, 149.5, 149.2, 142.5, 141.8, 141.4, 135.6, 134.9, 132.5, 130.7, 129.8, 124.1, 123.1, 121.6, 120.3, 113.3, 112.6, 112.5, 79.2, 77.6, 76.7, 68.0, 66.6, 65.4, 56.8, 43.0, 39.2, 37.2, 36.8, 32.0, 31.6, 29.7, 28.4, 28.0, 27.7, 25.2, 14.2. ESI-MS: *m/z* calcd. for $C_{40}H_{41}N_5O_{10}S_3Na$ $[M+Na]^+$: 870.1908; obs. $[M+Na]^+$: 870.1907.

Synthesis of compound RK-296

Compound **15** (0.05 g, 0.05 mmol) and compound **8** (0.02 g, 0.06 mmol) were dissolved in mixture of DCM (2 mL) and water (2 mL) followed by the addition of $CuSO_4{\cdot}5H_2O$ (0.03 g, 0.18 mmol) and sodium ascorbate (0.05 g, 0.23 mmol) to get a deep brown colored solution, which was stirred at room temperature 6 h. The progress of the reaction was monitored by TLC analysis. Upon completion, the reaction mixture was diluted with DCM and washed with water (5 × 20 mL) and brine (3 × 10 mL), respectively. The organic layer was dried over sodium sulfate and the solvent was evaporated under reduced pressure to obtain the crude product. The crude product was purified by flash chromatography using silica gel column and the expected product was eluted with 30% methanol in ethyl acetate as eluents to afford the pure product **RK-296** as deep yellow solid. R_f =

0.5 (10% Methanol in EtOAc). Yield: (0.03 g, 43%). M.P. = 74 - 76 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.39 (d, *J* = 7.8 Hz, 1H), 7.80 (s, 1H), 7.32 (s, 1H), 7.19 – 7.11 (m, 2H), 7.09 (dd, *J* = 8.3, 1.8 Hz, 1H), 6.99-6.92 (m, 2H), 6.84 (s, 1H), 6.69 (t, *J* = 6.0 Hz, 1H), 6.49 (s, 1H), 5.67 (s, 1H), 5.30 (s, 2H), 4.51-4.47 (m, 3H), 4.43-4.38 (m, 4H), 4.31 (t, *J* = 6.2 Hz, 1H), 4.16 (t, *J* = 6.5 Hz, 2H), 3.27-3.22 (m, 4H), 3.15-3.11 (m, 1H), 3.01-2.96 (m, 4H), 2.90 – 2.87 (m, 1H), 2.70 (d, *J* = 13.2 Hz, 1H), 2.60 (s, 2H), 2.46 (s, 2H), 2.17 (t, *J* = 7.2 Hz, 2H), 2.12 (t, *J* = 6.6 Hz, 2H), 2.04 (d, *J* = 5.3 Hz, 1H), 1.88-1.83 (m, 2H), 1.73-1.67 (m, 3H), 1.66-1.60 (m, 2H), 1.59-1.56 (m, 2H), 1.47-1.43 (m, 2H), 1.42-1.38 (m, 2H), 1.08 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 173.8, 169.4, 164.1, 155.0, 153.6, 152.7, 150.0, 149.2, 143.3, 142.5, 141.8, 141.2, 135.7, 135.2, 132.5, 130.9, 130.1, 124.0, 123.8, 122.9, 121.1, 120.5, 113.4, 113.1, 112.7, 79.0, 68.1, 66.6, 65.4, 62.9, 61.9, 60.2, 55.7, 48.1, 43.0, 40.6, 39.2, 37.2, 36.9, 36.4, 35.7, 32.1, 31.6, 30.1, 29.8, 28.4, 28.4, 28.1, 28.0, 28.0, 27.8, 25.5, 25.2. ESI-MS: *m/z* calcd. for $C_{53}H_{63}N_{11}O_{12}S_4Na$ $[M+Na]^+$: 1196.3433; obs. $[M+Na]^+$: 1196.3423.

UV-Vis and fluorescence spectroscopic studies

All the stock solutions of biologically relevant analytes were prepared in Milli-Q or double distilled water and the stock solutions of prodrug **RK-296**, **NBDHEX** and **Biot-NIR-OH** and were prepared in spectroscopy grade DMSO. All the spectroscopic measurements were performed under physiological pH (7.4) in potassium phosphate buffer saline (PBS, 100 mM) in the presence of 50% DMSO (v/v). Samples for absorption and emission measurements were carried out in quartz cuvettes (1.0 mL). Fluorescence emission spectra were recorded with an excitation wavelength of 580 nm with a slit width of 10/10 nm. To understand the thiol concentration dependency of the prodrug, the concentration of GSH (0–1000 mM) was varied while keeping other parameters constant with an incubation of 30 min. For the studies with different analytes, emission was measured after incubating prodrug (10 μM) with analytes (500 μM) for 30 min under the above optimized condition. The effect of pH on the stability and reactivity of the prodrug towards GSH was studied by the optimized protocol using the PBS (100 mM) of different pH (4-10) with 50% DMSO using fluorescence spectroscopic method.

Determination of the limit of detection (LOD) of the prodrug

The limit of detection (LOD) of the prodrug **RK-296** was determined using the fluorescence titration using the equation, LOD = 3σ/k, where σ is the standard deviation of blank measurements (without GSH) and k is the slope of the plot of emission intensity versus GSH concentration. To determine the standard deviation of the blank measurements, the emission intensity of pure **RK-296** (10 μM) was measured 5 times in the absence of GSH. For the determination of slope, the emission spectra from **RK-296** were measured at different concentrations of GSH (2–70 μM) after incubating the reaction mixture for 30 min.

HPLC analysis

Purity of the synthesized prodrug **RK-296** along with **NBDHEX** and the released fluorophore **Biot-NIR-OH** were analysed using analytical high-performance liquid chromatography (HPLC) Agilent 1220 infinity II LC system using reverse-phase C18 column (Luna®, 150 × 4.6 mm, 5 μm). HPLC grade acetonitrile and water (Finar Ltd) were used as mobile phase and the absorbance profile of compounds were detected by PDA detector at wavelengths of 254 and 430 nm (as applicable). The stock solutions of the samples were prepared in DMSO and were injected into the HPLC system using the in-built autosampler at a flow rate of 1.0 mL/min using acetonitrile/water system as a binary solvent system for 12 min (0-4 min: 50%-90% acetonitrile in water; 4-9 min: 90% acetonitrile in water; 9-12 min: 90%-50% acetonitrile in water). The reaction of **RK-296** (50 μM) was carried out using an organic thiol such as PhSH (1 mM) in the presence of $NaHCO_3$ (1 mM) in acetonitrile and water mixture (3:2). The peaks of analytes, prodrug **RK-296**, reaction intermediates and the released **NBDHEX** and NIR fluorophore **Biot-NIR-OH** were analyzed/identified at different wavelengths (254 and 430 nm) at various time intervals.

Cell culture

The triple-negative breast cancer (TNBC) cells (MDA-MB-231), Human cervical cancer cells (HeLa), Human Embryonic Kidney cells (HEK-293) were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in DMEM medium (Gibco) supplemented with 10% (v/v) FBS (Gibco) and 1% Pen-Strep (Gibco). Cells were cultured as a monolayer in a humidified incubator at 37 ºC in the presence of 5% CO_2 level.

Anti-proliferative activity assay

The synthesized prodrug **RK-296**, **NBDHEX**, **Biot-NIR-OH** and compound **15** were studied for their anti-proliferative activities using the conventional MTT assay in MDA-MB-231 and HEK-293 cell lines. MDA-MB-231 and HEK-293 cells were seeded in 96-well culture plates at a density of 2 $\times 10^4$/100 µL per well and cells were incubated with the freshly prepared test compounds (0.7, 1.5, 3.0, 4.0, 6.0, 12.0 µM) for 0 h (control) and 48 h (experimental), respectively. At the end of the treatment period, 100 µL of 5 mg/mL of MTT was added to the plate (control) and incubated for 4 h. Further, the reagent from the plate was removed and the purple formazan crystals were dissolved using 100 µL of DMSO (Avra Synthesis Pvt. Ltd.) and the absorbance at 570 nm was measured using a microplate reader (Thermo Scientific™ Multiskan™ GO microplate reader). In the experimental set, a similar MTT treatment protocol was followed only after 48 h. The mean ΔOD values were calculated by the subtraction of mean OD values of 0 h plate (control) from the mean OD values of identical wells at 48 h plate (experimental) and the percentage proliferation was calculated keeping the mean ΔOD of untreated control as 100%. The feasibility of drug release from the prodrug over time was studied further by carrying out the dose-dependent anti-proliferative activity of **RK-296** in MDA-MB-231 cells at different time intervals (48 h, 72 h, and 96 h).

Fluorescence microscopic studies

HeLa and HEK-293 cells were cultured in high glucose DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO_2 atmosphere. HeLa and HEK-293 cells were then seeded at a density of 5 × 10^5 and 12 × 10^5 cells/ 2 ml respectively in 35 mm cell culture Petri dishes and incubated at 37 °C under 5% CO_2 for 24 h. The confluent cells were washed with DPBS and finally treated with **RK-296** (4.0 µM) and **Biot-NIR-OH** (2.0 µM) and incubated for 40 min in DPBS (Dulbecco's modified phosphate buffer). After washing the cells with DPBS (3 times), cellular morphology was carefully observed and imaged using Bio-Rad ZOE™ fluorescent cell imager under a bright field and red channel. A negative control experiment was performed in HeLa cells upon the pre-treatment of cells with *N*-ethylmaleimide (NEM, 200 µM) for 1 h to quench the endogenous thiols. The cells were washed with DPBS (3 times) and treated with **RK-296** (4.0 µM). The cellular incorporation of **RK-296** (4.0 µM), **Biot-NIR-OH** (2.0 µM) and the biotin-free fluorophore **5** (2.0 µM) were studied by

measuring the emission intensity both in a biotin-receptor positive (HeLa) and negative (HEK-293) cells under the identical experimental condition.

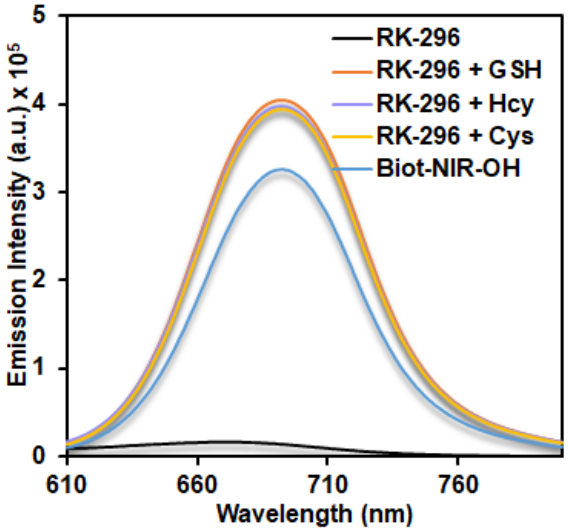


Figure S1. (a) Emission spectra of **RK-296** (10.0 µM) in the presence and absence of different biologically relevant biothiols (500 µM) in PBS (100 mM) of pH 7.4 in the presence of 50% DMSO after incubating for 30 min along with the emission spectrum of pure **Biot-NIR-OH**. λ_{ex} = 580 nm; λ_{em} = 600 - 800 nm; slit width 10/10 nm.

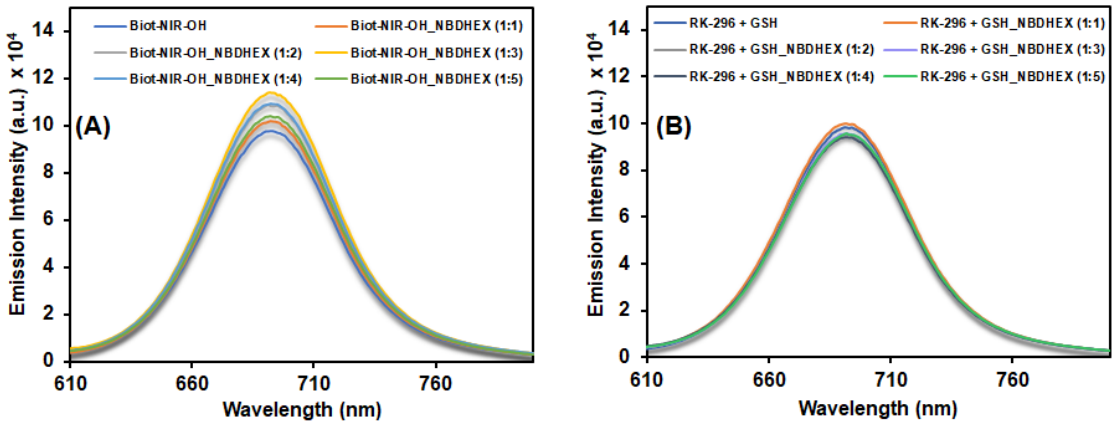


Figure S2. Emission spectra of (A) **Biot-NIR-OH** (5 µM) and (B) **RK-296** (5 µM) + GSH (250 µM) in the presence of varying concentration of **NBDHEX** (5 -25 µM) in PBS (100 mM) of pH 7.4 in the presence of 50% DMSO after incubating for 30 min. λ_{ex} = 580 nm; slit width 10/10 nm.

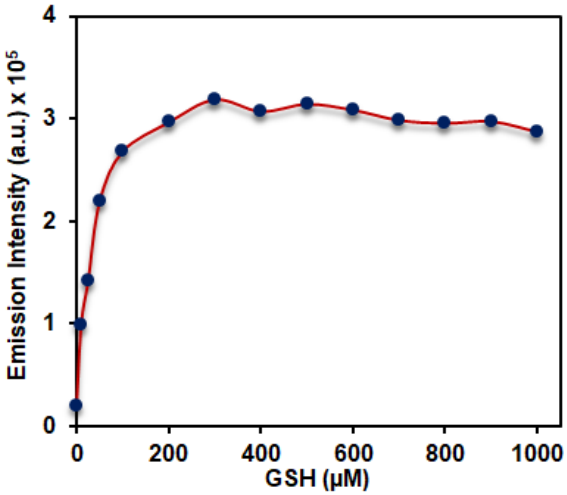


Figure S3. Emission spectra of prodrug **RK-296** (10 μM) in the presence of GSH (0–1000 μM) after an incubation for 30 min in PBS (100 mM, pH 7.4, 50% DMSO v/v).

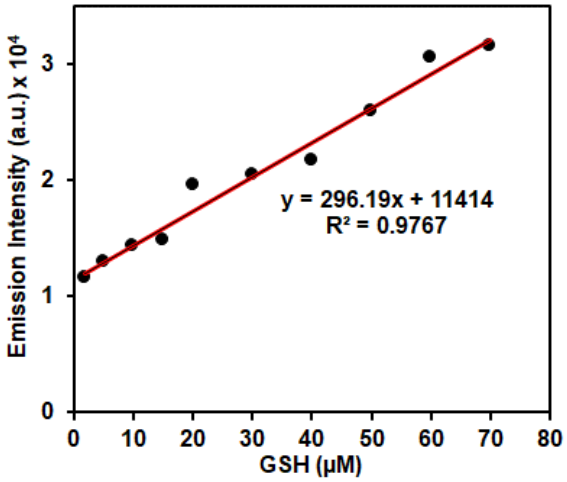


Figure S4. Emission spectra of prodrug **RK-296** (10 μM) in the presence of GSH (2–70 μM) after an incubation for 30 min in PBS (100 mM, pH 7.4, 50% DMSO v/v). From the expression of linear curve fitting, the value of k is **296.19**.

Table S1. Determination of the standard deviation (σ) from blank measurements

Entry	Test 1	Test 2	Test 3	Test 4	Test 5	Mean	SD(σ)
Emission Intensity (690 nm)	7031.81	6984.58	7103.15	7066.38	7185.54	7074.29	68.0051

LOD = 3σ / k = (3 x 68.0051) / 296.19 = **688 nM**

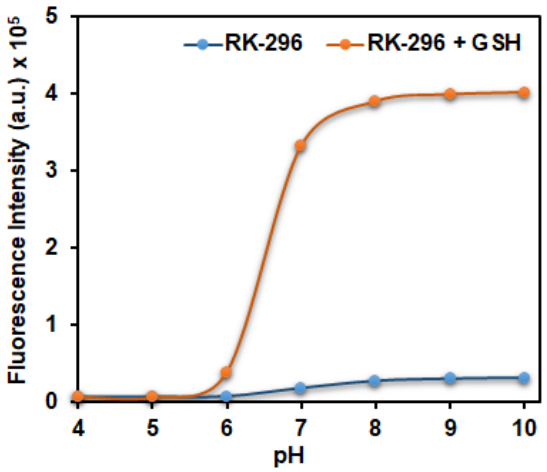


Figure S5. Emission intensity of pH-dependent emission response from **RK-296** (10 μM) in the presence and absence of GSH (500 μM). The reactions were carried out in PBS (100 mM) of different pH in the presence of 50% DMSO (v/v).

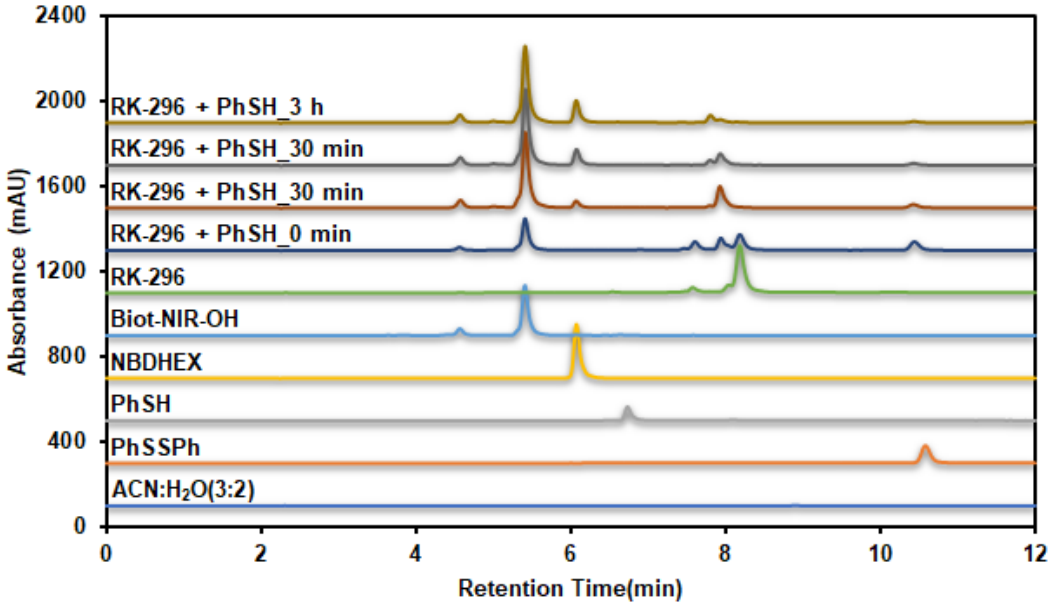


Figure S6. HPLC overlay chromatogram for the reaction of **RK-296** (50 μM) with PhSH (1 mM) over 180 min along with the chromatogram of the released fluorophore **Biot-NIR-OH** and **NBDHEX**. The chromatograms were extracted at 254 nm for PhSH and PhSSPh and 430 nm for pure compounds and **RK-296** + PhSH (0-180 min). Increasing intensity of the released fluorophore **Biot-NIR-OH** (time = 5.4 min) and **NBDHEX** (time = 6.0 min) was observed in the reaction over time.

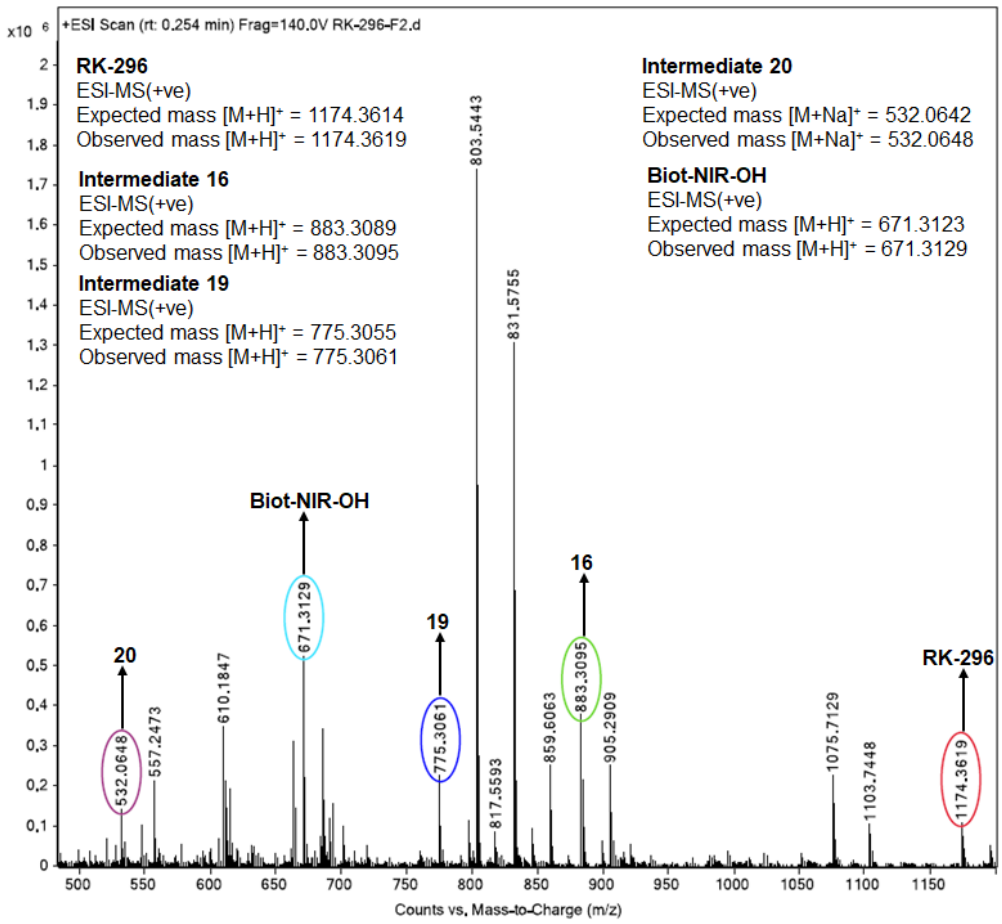


Figure S7. ESI-MS spectrum of the reaction of **RK-296** with PhSH in the presence of $NaHCO_3$ showing the presence of intermediates **16, 19** and **20** along with some unreacted prodrug and the released fluorophore. ESI-MS (+ve) *m/z* calcd for (intermediate **16**) $C_{44}H_{51}N_8O_6S_3$ $[M+H]^+$: 883.3089, obs. 883.3095; for (intermediate **19**) $C_{38}H_{47}N_8O_6S_2$ $[M+H]^+$: 775.3055, obs.775.3061; for (intermediate **20**) $C_{21}H_{23}N_3O_6S_3Na$ $[M+Na]^+$: 532.0642, obs.532.0648; for (**Biot-NIR-OH**) $C_{35}H_{43}N_8O_4S$ $[M+H]^+$: 671.3123, obs.671.3129; and for (prodrug **RK-296**) $C_{53}H_{64}N_{11}O_{12}S_4$ $[M+H]^+$: 1174.3614, Obs. 1174.3619.

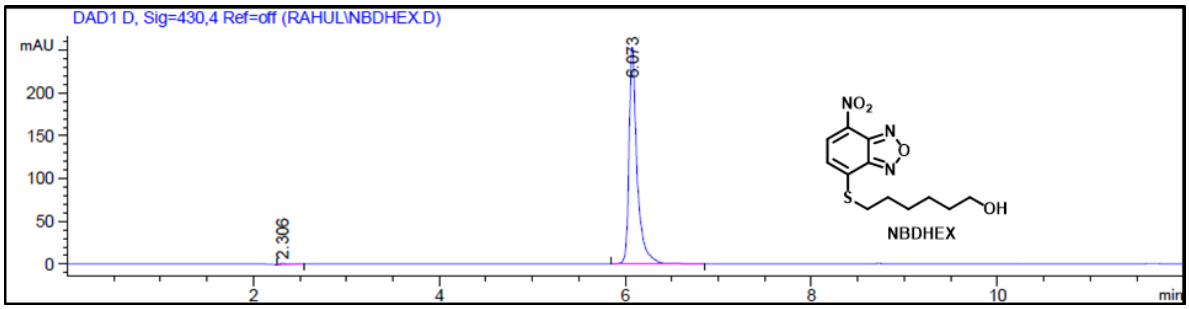


Figure S8. HPLC chromatogram of the **NBDHEX** (6.073 min) showing 99.2% purity.at 430 nm wavelength.

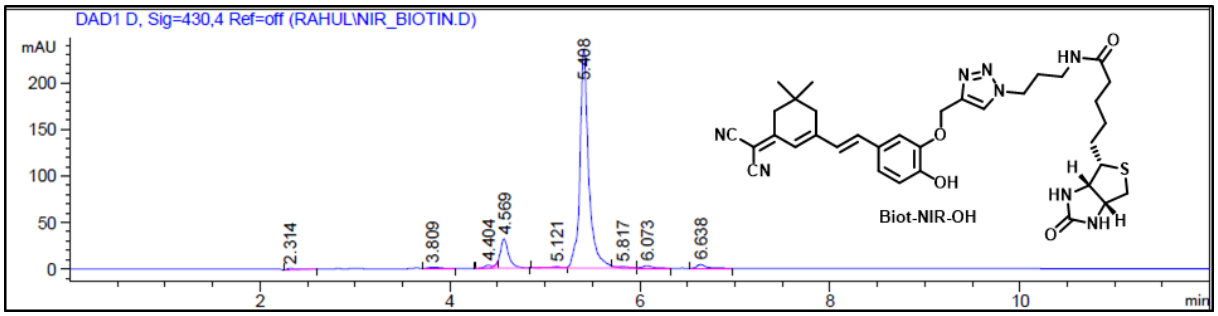


Figure S9. HPLC chromatogram of the **Biot-NIR-OH** exhibits two peaks with retention times of 4.569 min and 5.408 min, respectively, showing relative intensities of 10.94% and 82.73% (two stereoisomers of biotin component) with the cumulative purity of 93.67% at 430 nm wavelength.

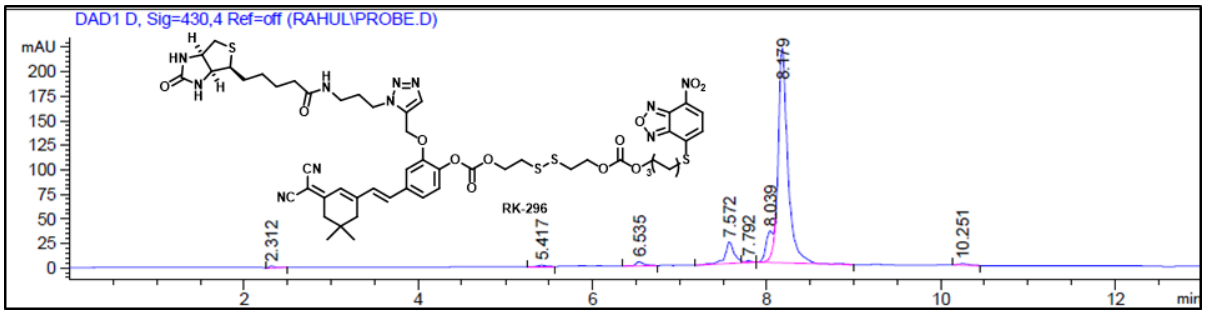


Figure S10. HPLC chromatogram of the **RK-296** exhibiting two peaks with the retention times of 7.572 min and 8.179 min, respectively, with the relative intensities of 7.88% and 82.32%, respectively showing the cumulative purity of 90.2% at 430 nm wavelength.

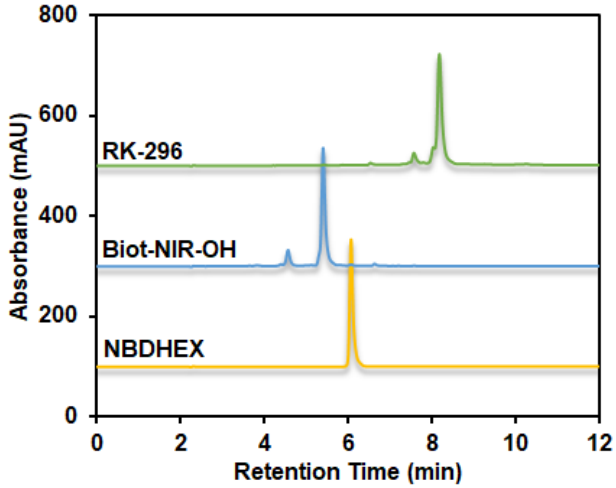


Figure S11. HPLC overlay chromatogram for the pure **RK-296**, **Biot-NIR-OH** and **NBDHEX** at 430 nm wavelength.

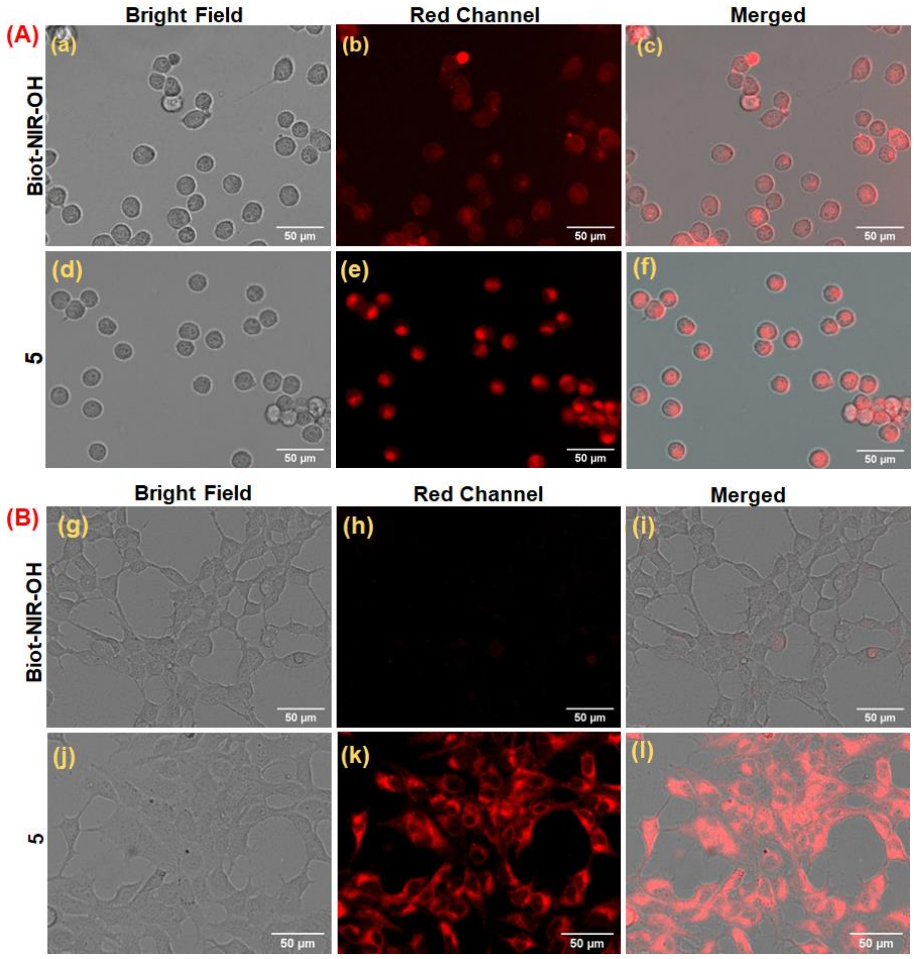


Figure S12. Fluorescence microscopy images (bright, red channel and merged) of (A) HeLa cells and (B) HEK-293 cells in presence of **Biot-NIR-OH** (2.0 µM) and compound **5** (2.0 µM). Scale bar: 50 µm.

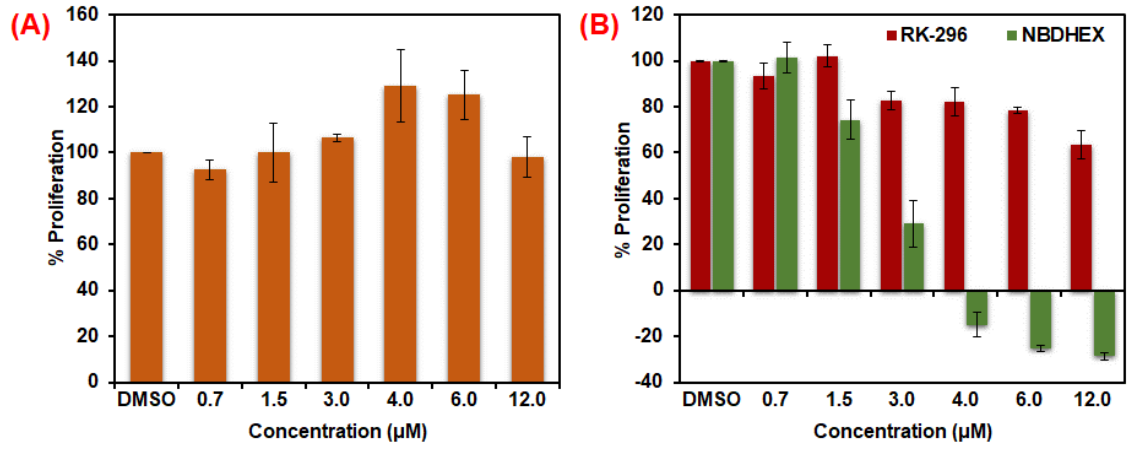


Figure S13. The dose-dependent anti-proliferative activity of (A) **Biot-NIR-OH** in MDA-MB-231 cell line (B) **RK-296** and **NBDHEX** in HEK-293 cell line after an incubation of 48 h.

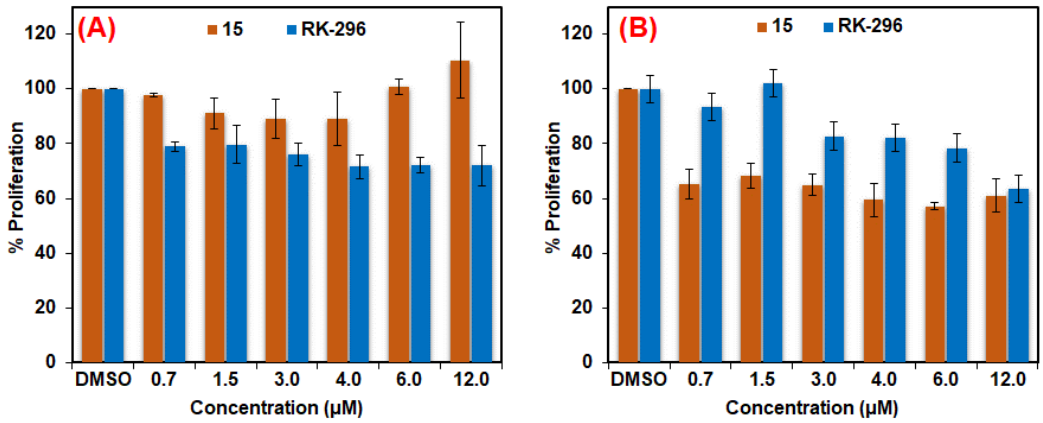


Figure S14. Dose-dependent anti-proliferative activity of compound **15** and **RK-296** in (A) MDA-MB-231 and (B) HEK-293 cell lines with 48 h of incubation.

Spectral data of the synthesized compounds

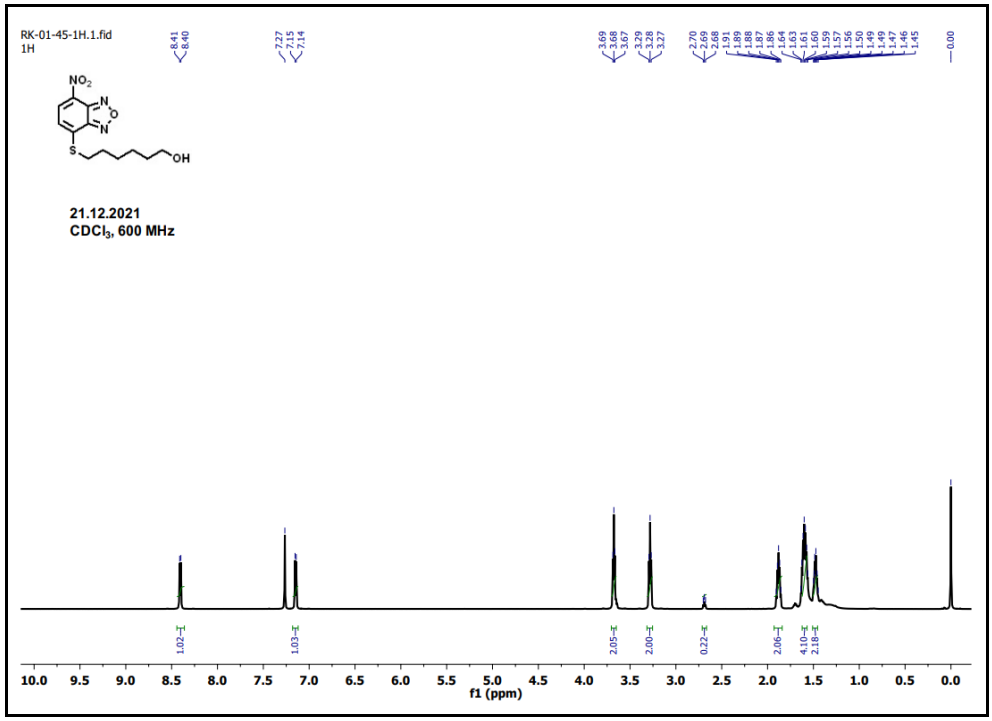


Figure S15. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of **NBDHEX**.

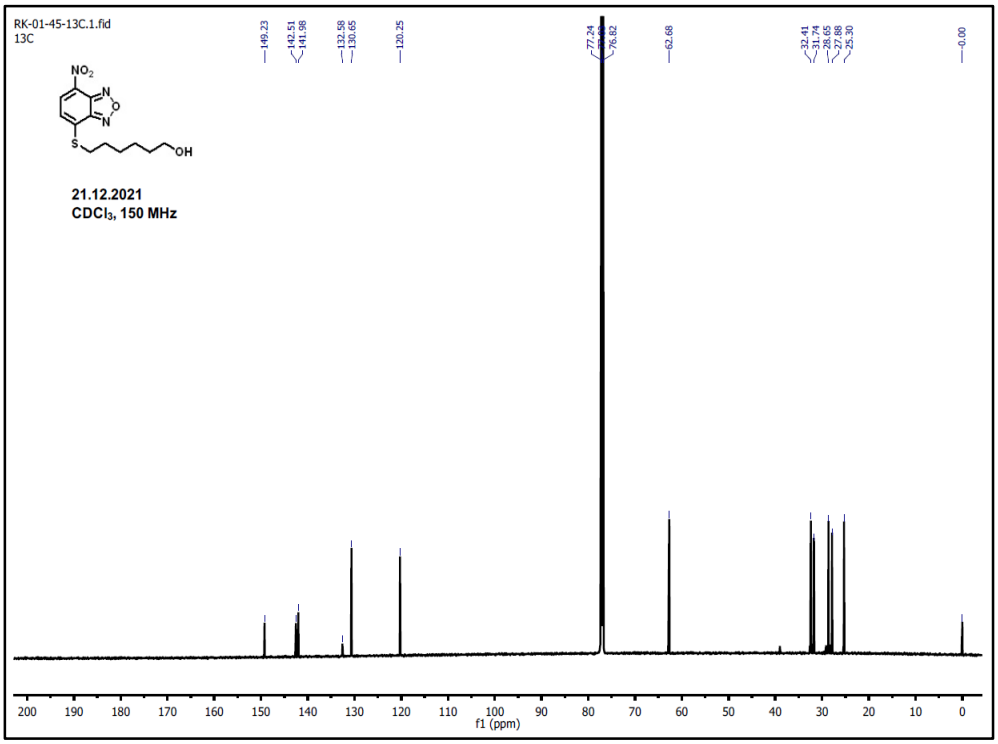


Figure S16. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of **NBDHEX**.

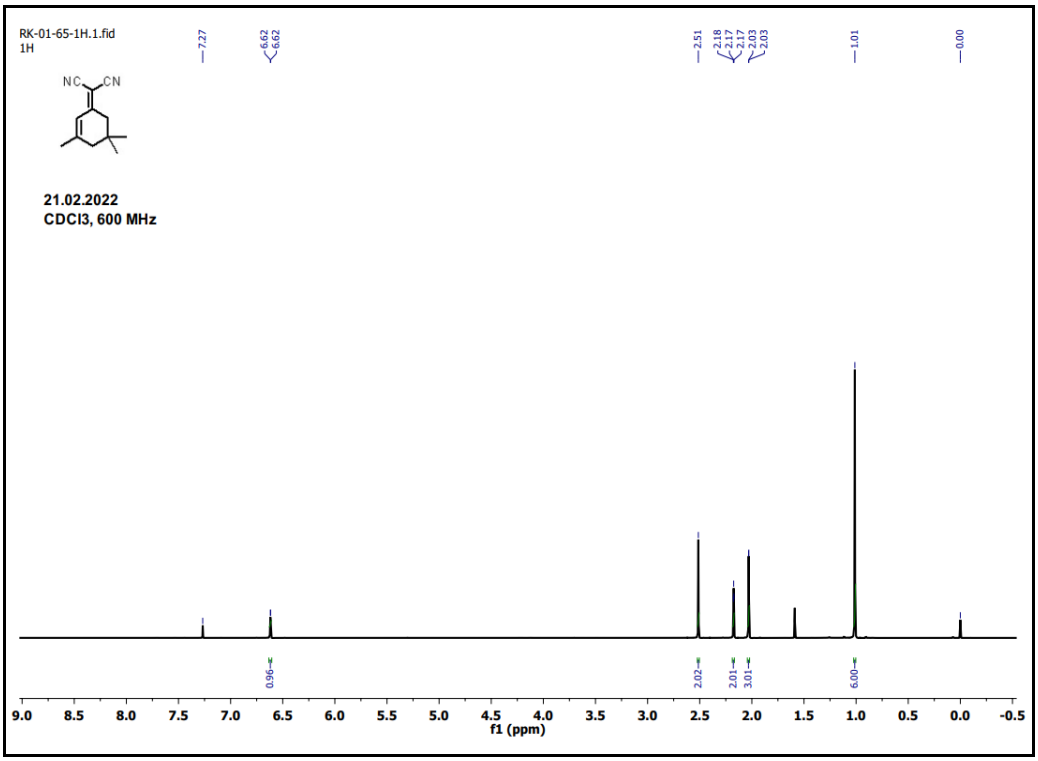


Figure S17. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **2**.

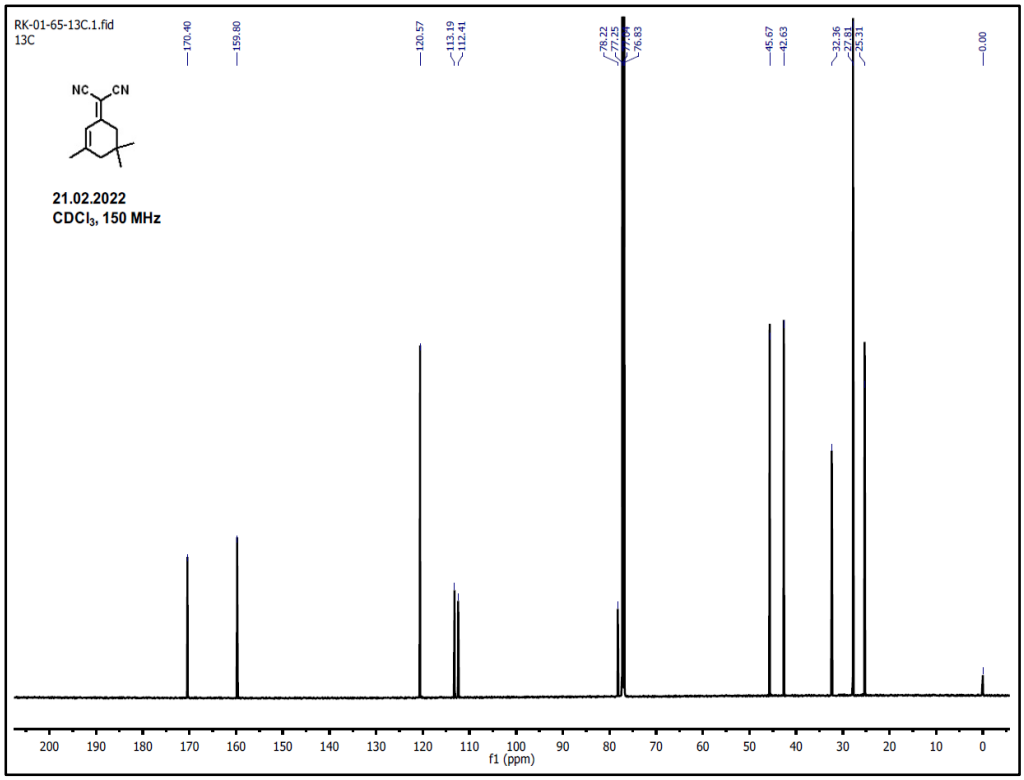


Figure S18. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **2**.

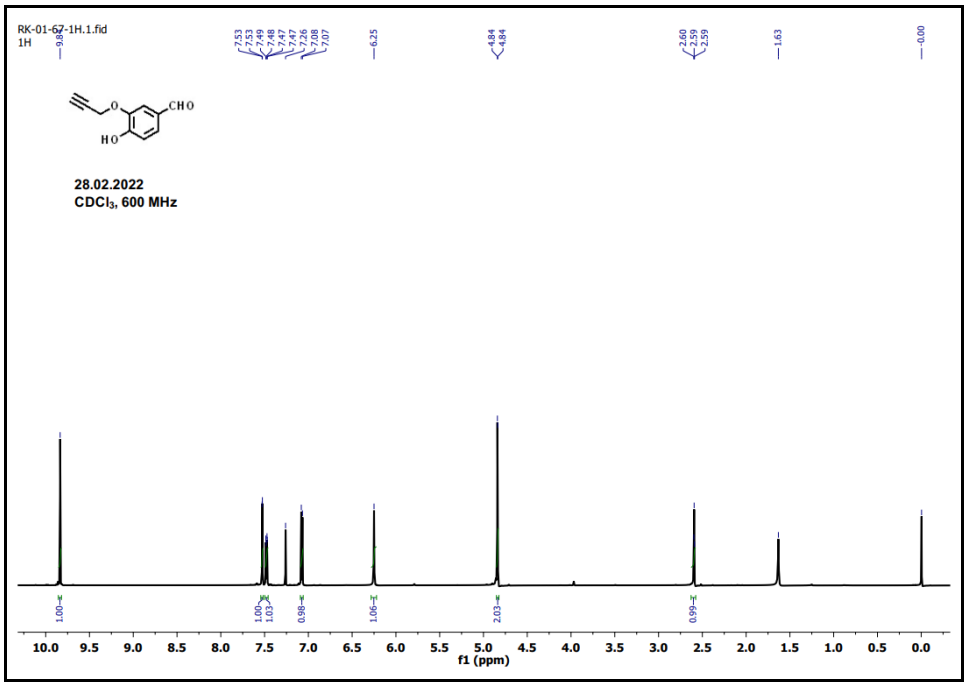


Figure S19. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **4**.

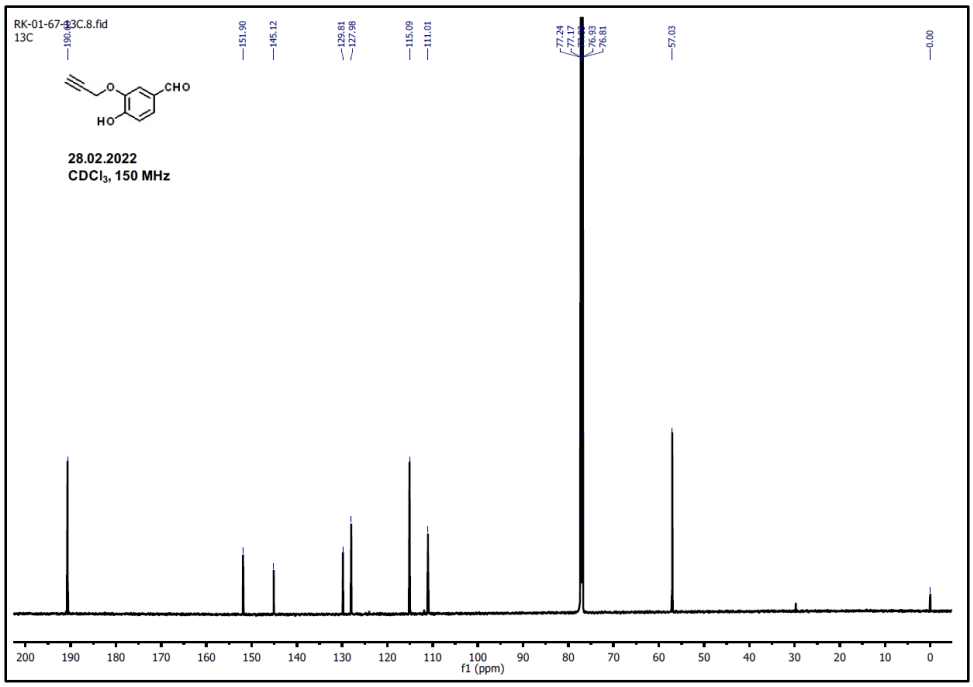


Figure S20. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **4**.

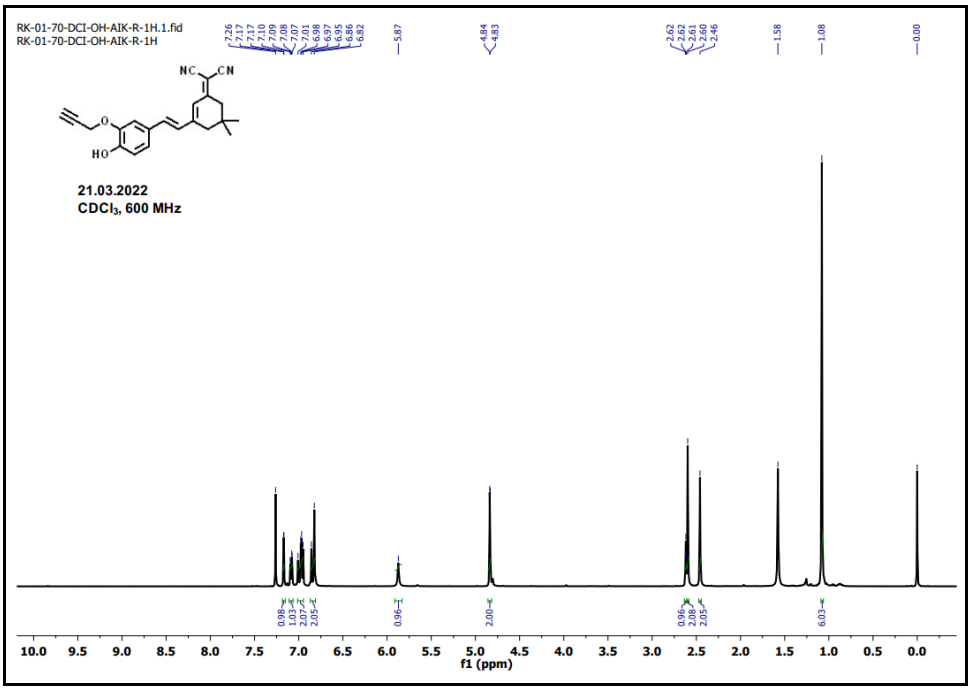


Figure S21. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **5**.

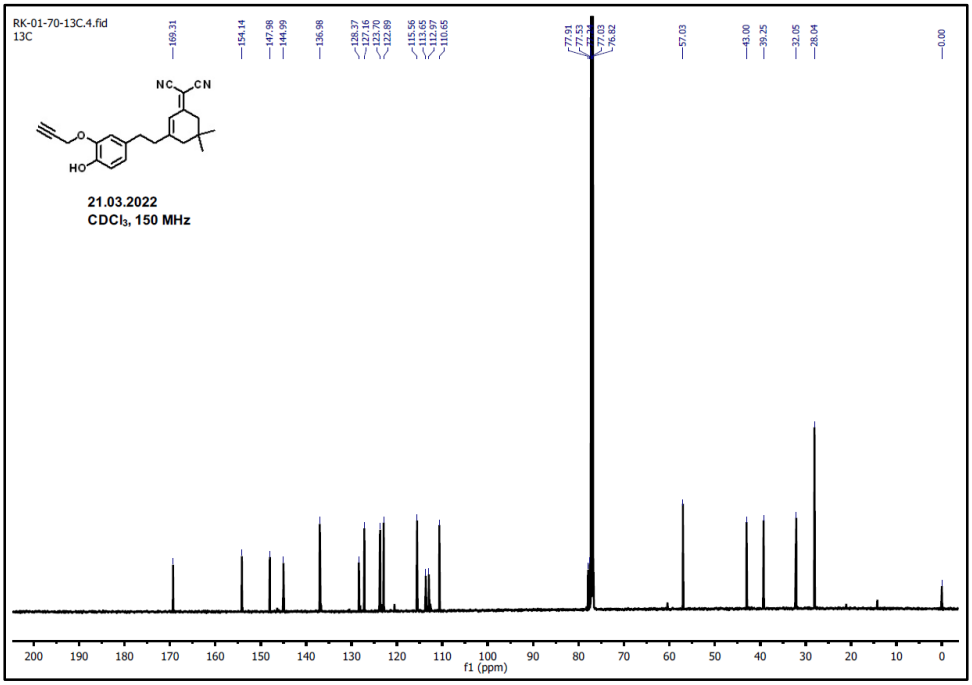


Figure S22. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **5**.

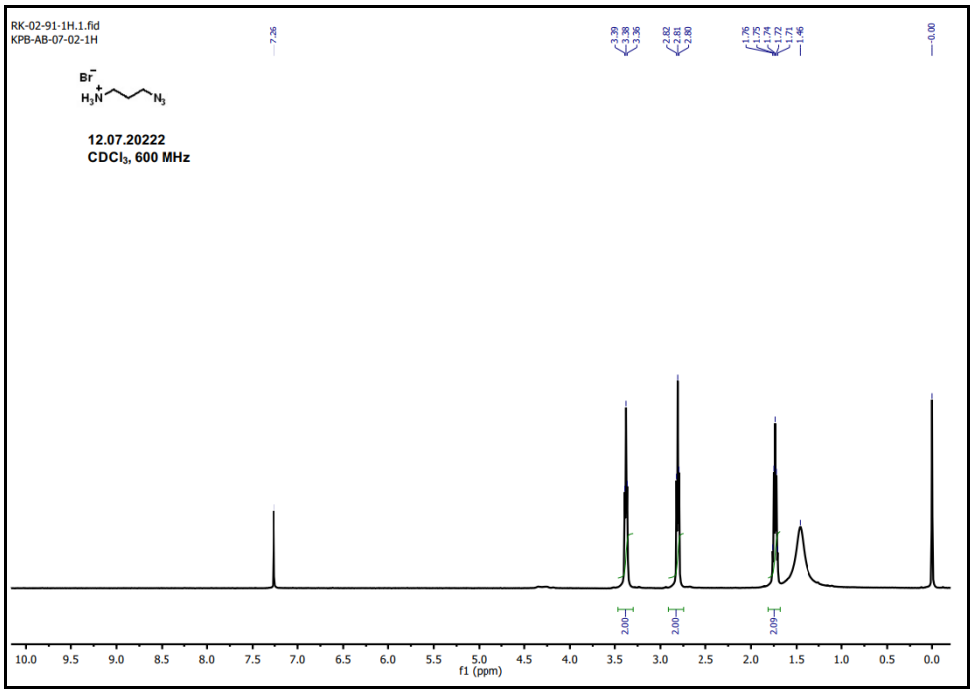


Figure S23. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **7**.

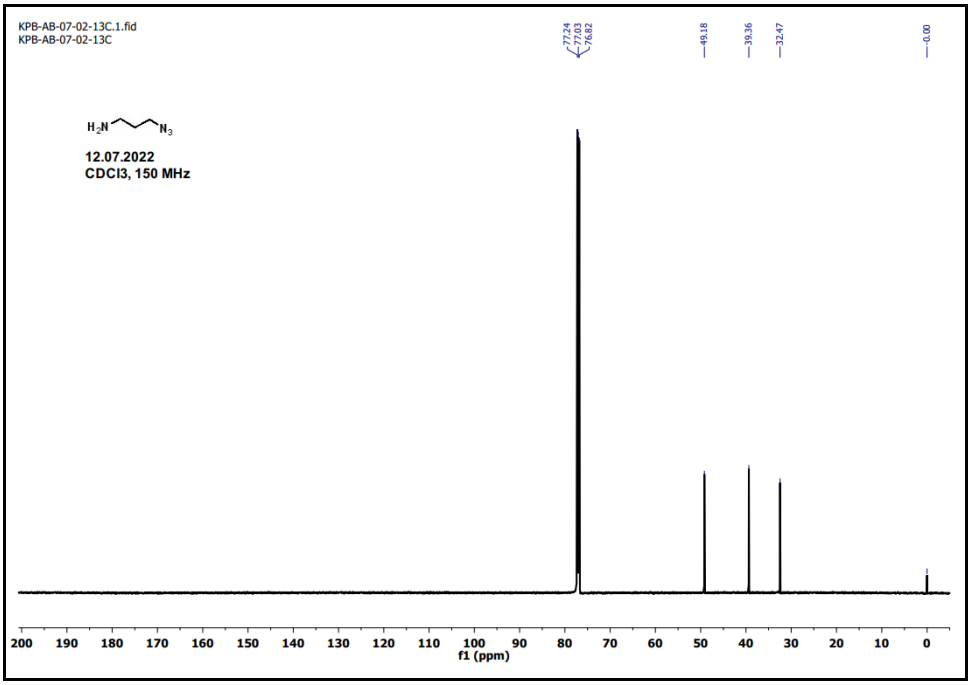


Figure S24. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **7**.

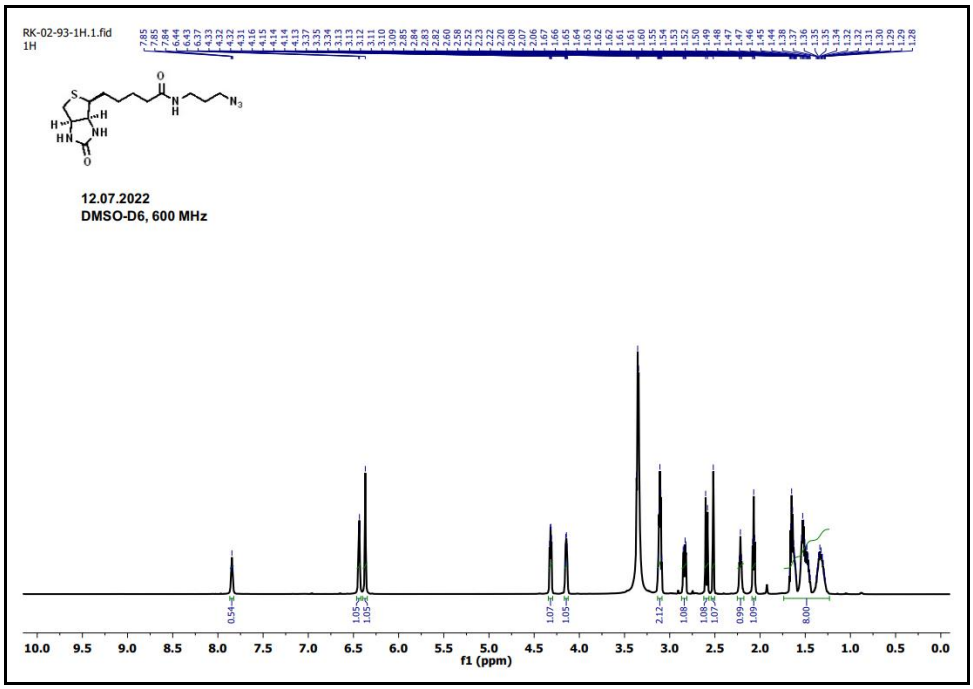


Figure S25. ^{1}H NMR (DMSO-d6, 600 MHz) spectrum of compound **8**.

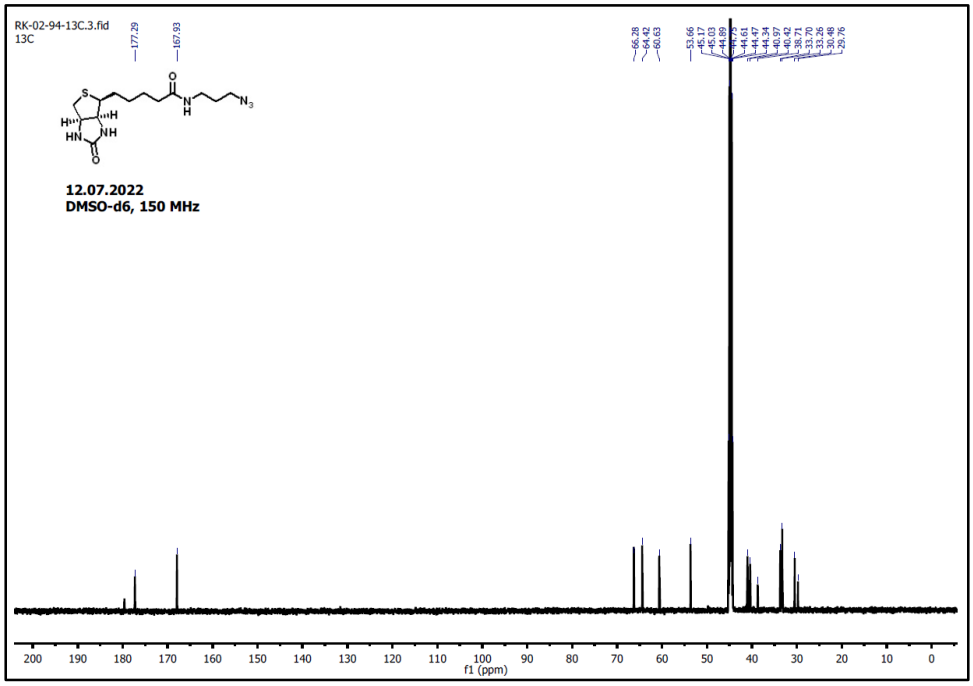


Figure S26. ^{13}C NMR (DMSO-d6, 150 MHz) spectrum of compound **8**.

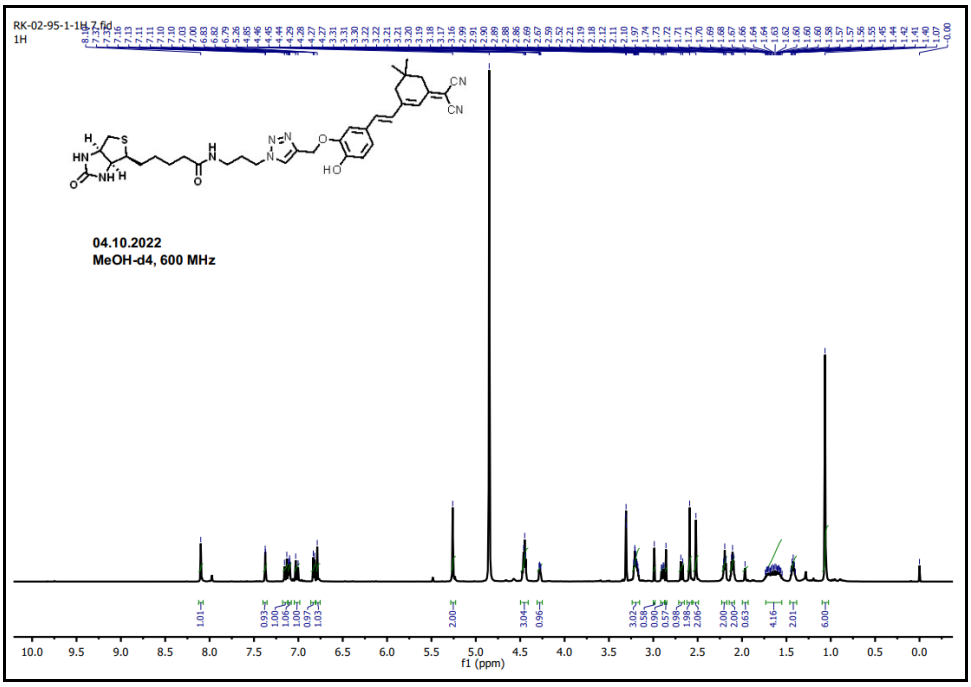


Figure S27. ^{1}H NMR (MeOH-d4, 600 MHz) spectrum of compound **Biot-NIR-OH**.

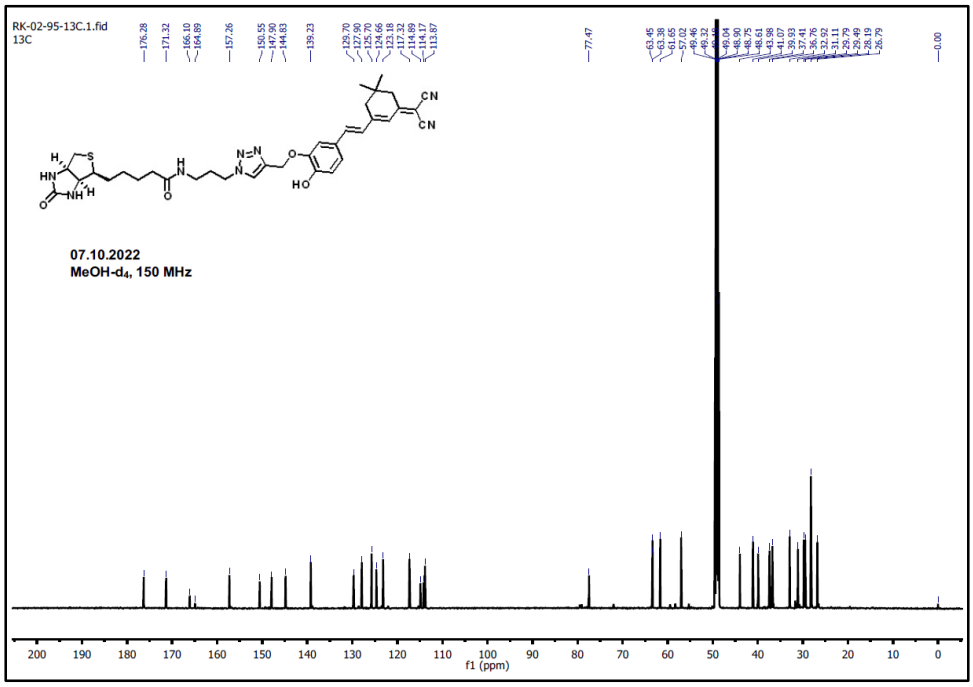


Figure S28. ^{13}C NMR (MeOH-d4, 150 MHz) spectrum of compound **Biot-NIR-OH**.

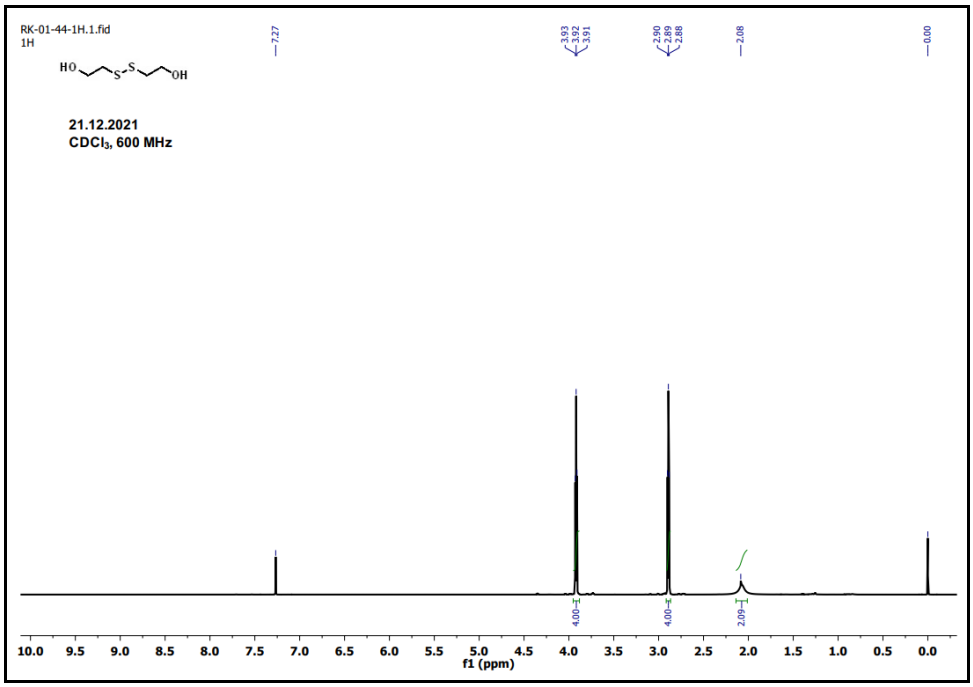


Figure S29. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **9**.

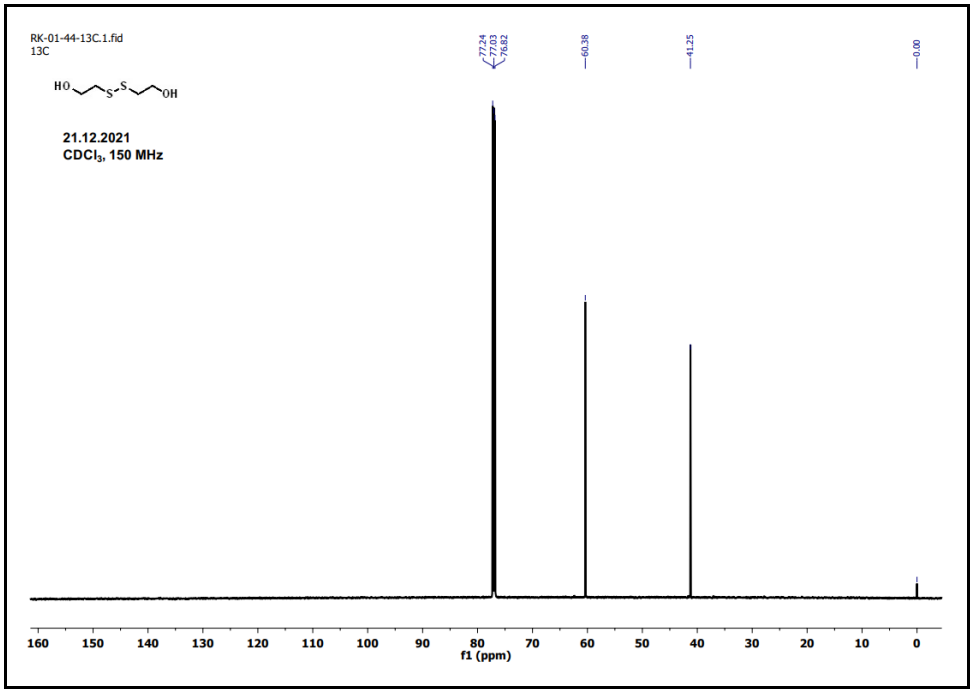


Figure S30. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **9**.

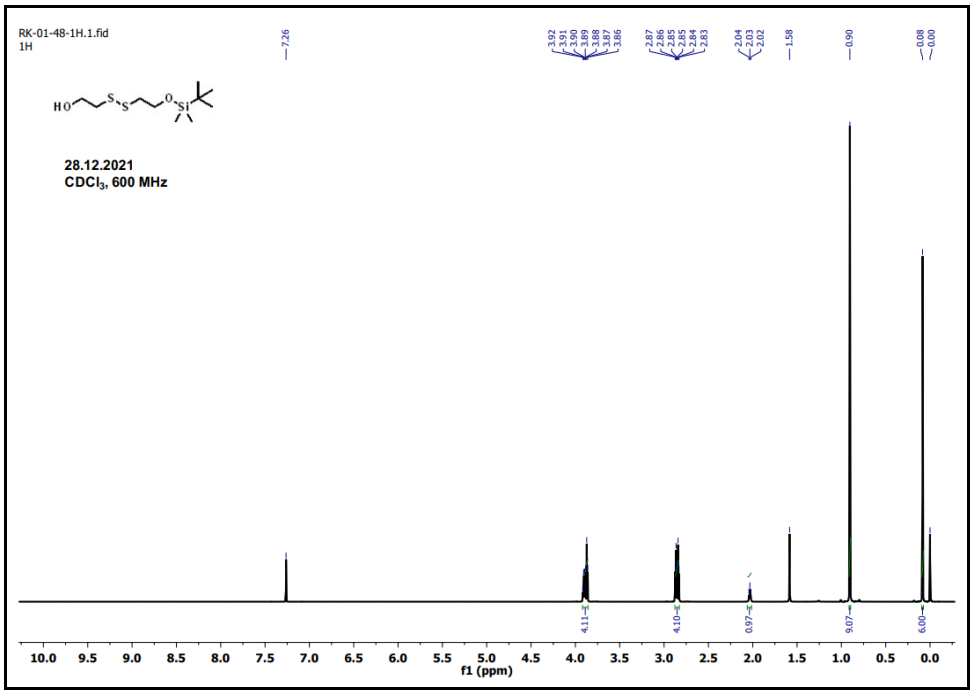


Figure S31. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **10**.

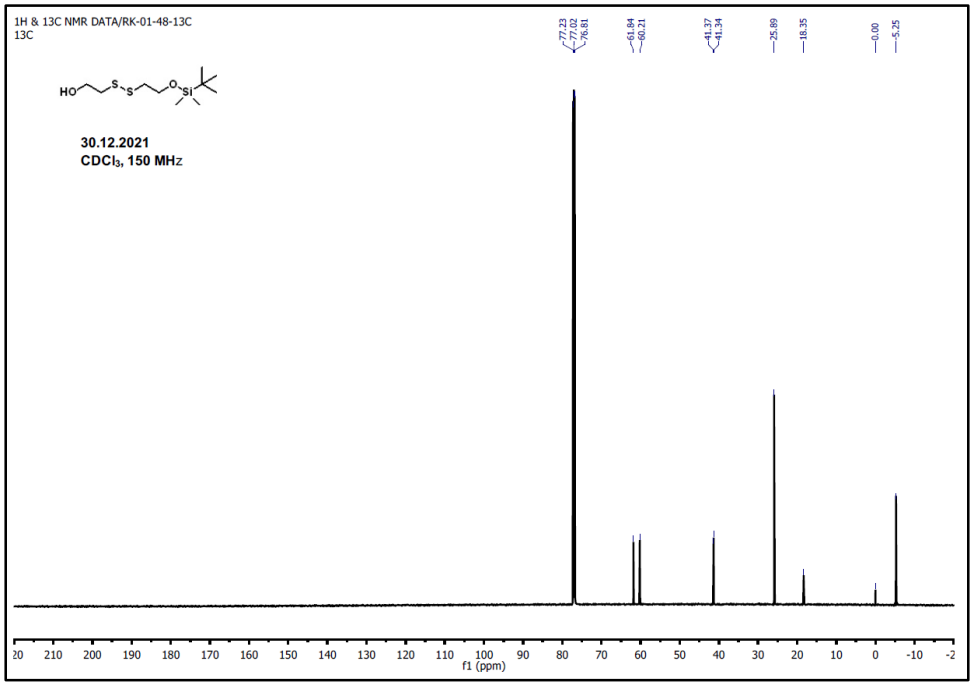


Figure S32. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **10**.

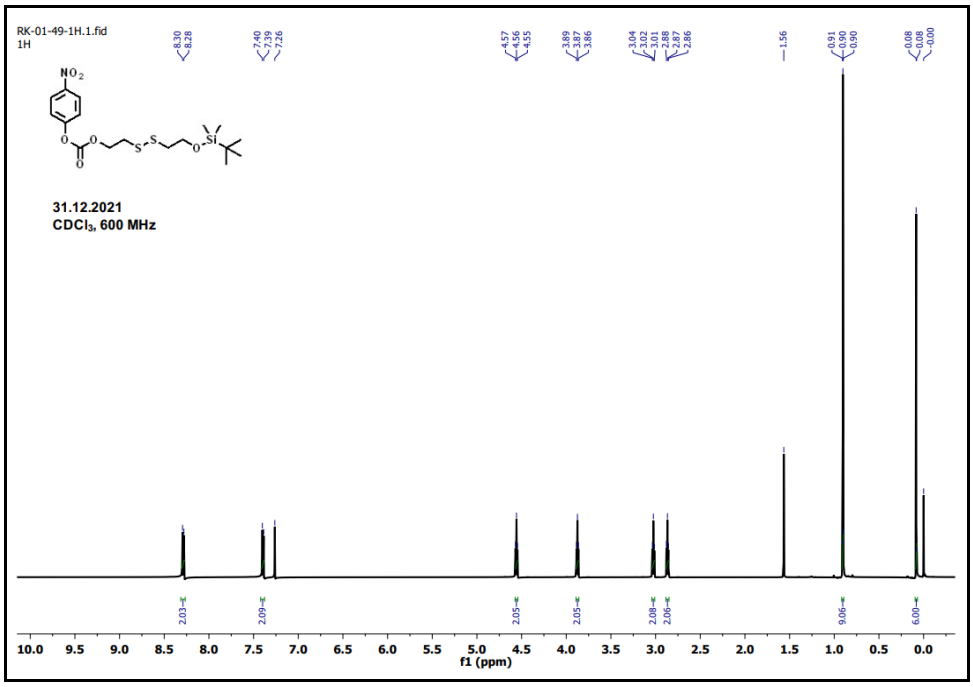


Figure S33. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **11**.

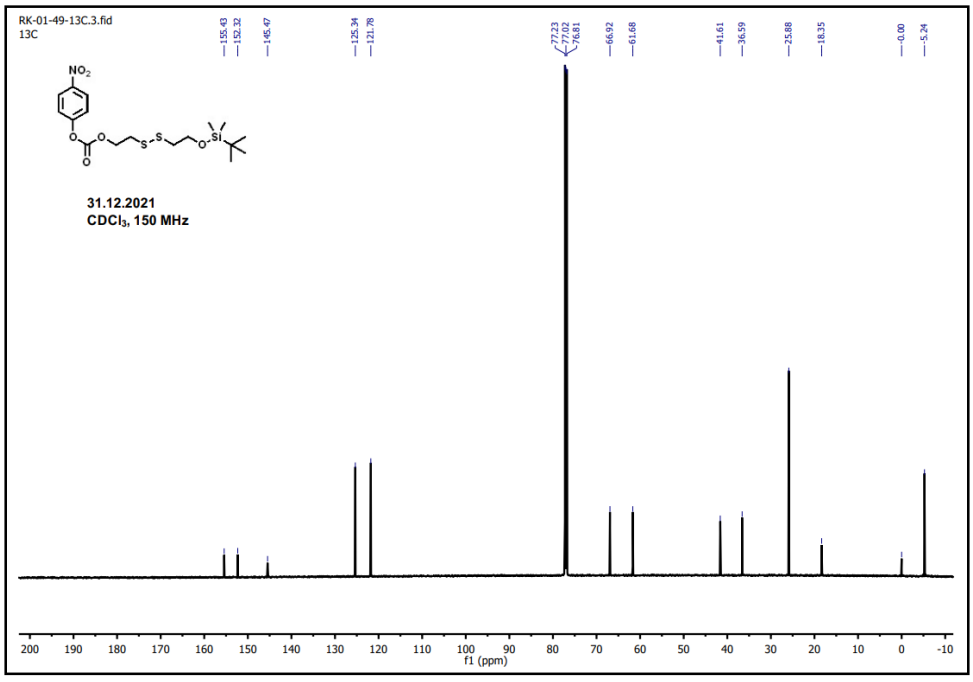


Figure S34. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **11**.

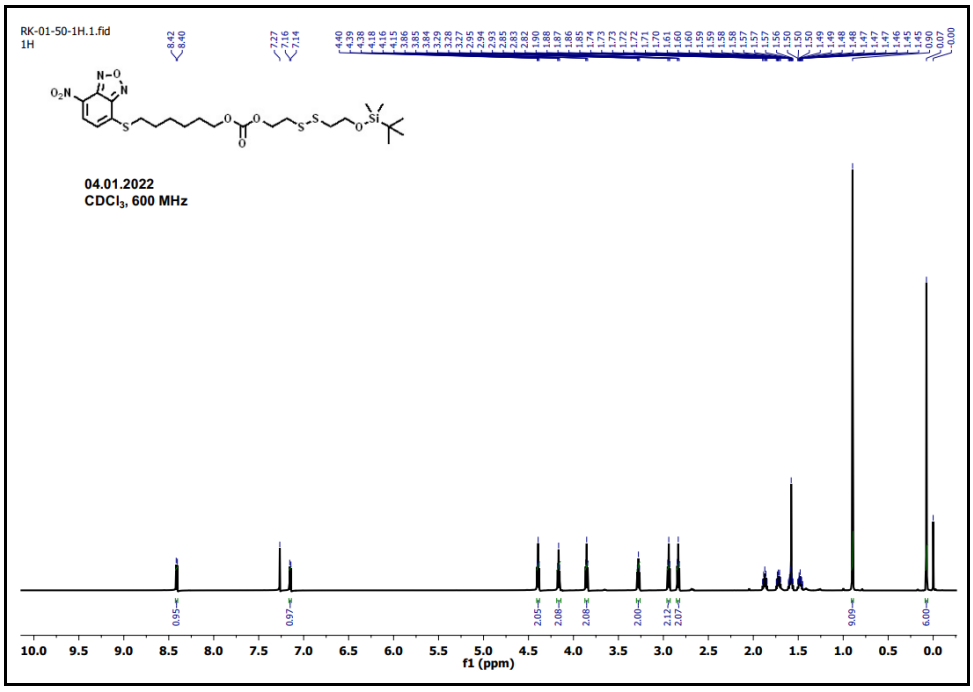


Figure S35. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **12**.

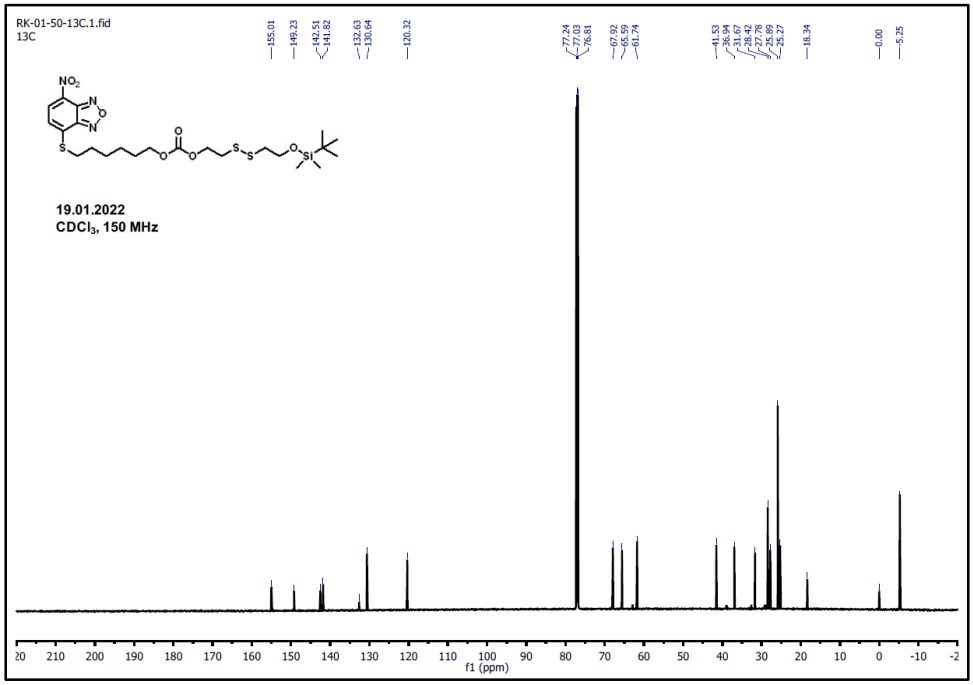


Figure S36. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **12**.

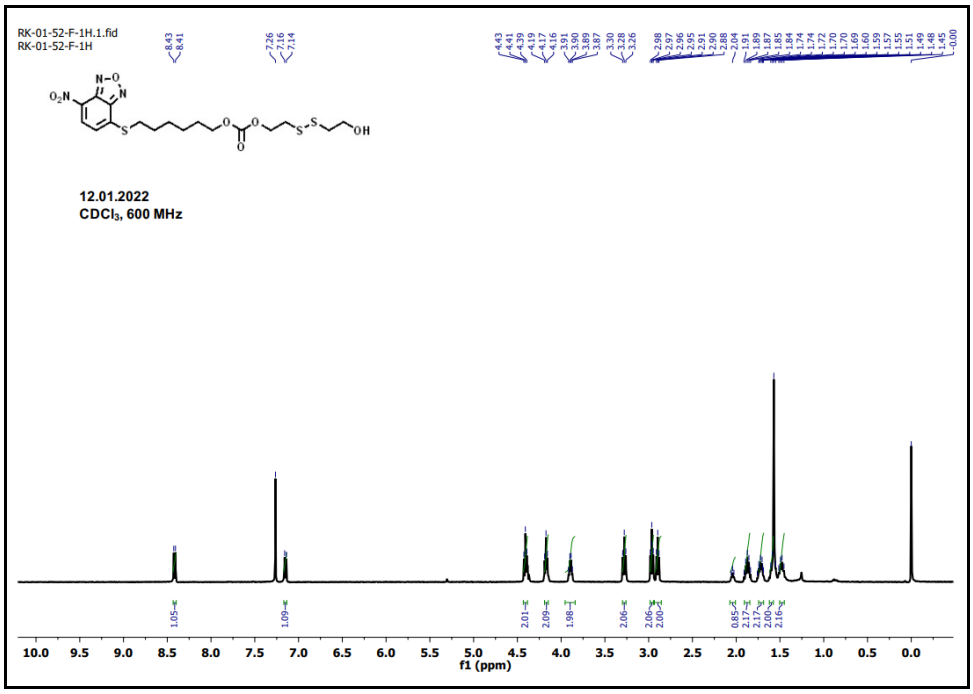


Figure S37. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **13**.

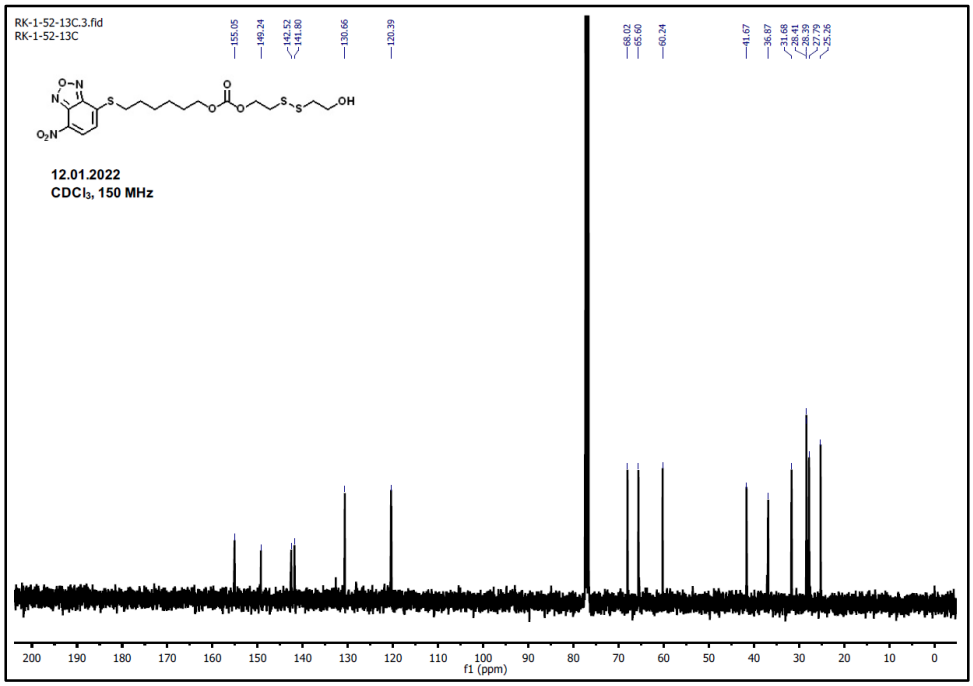


Figure S38. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **13**.

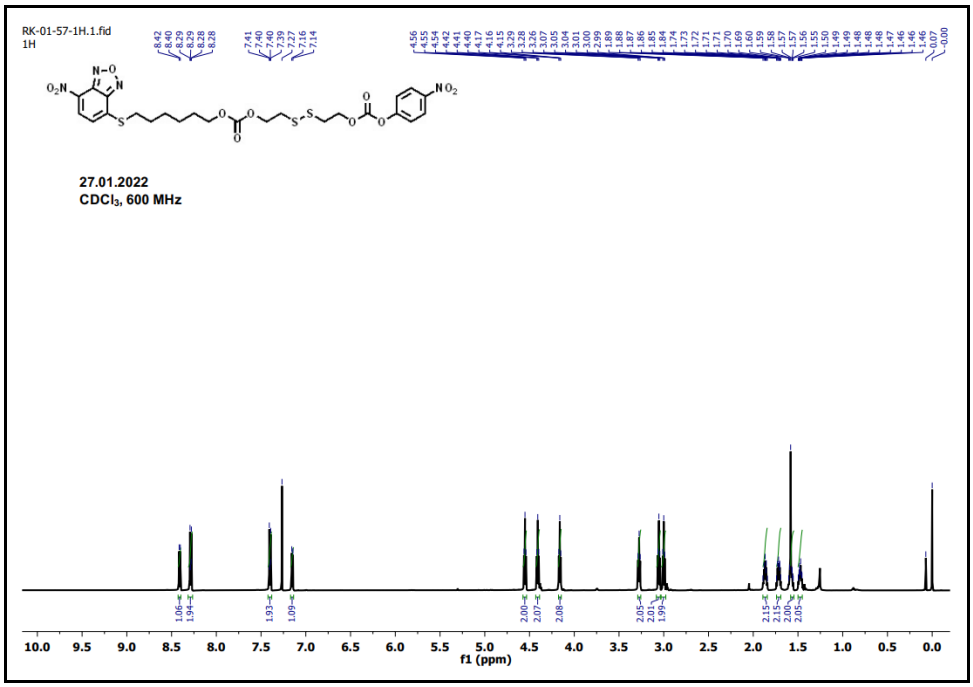


Figure S39. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **14**.

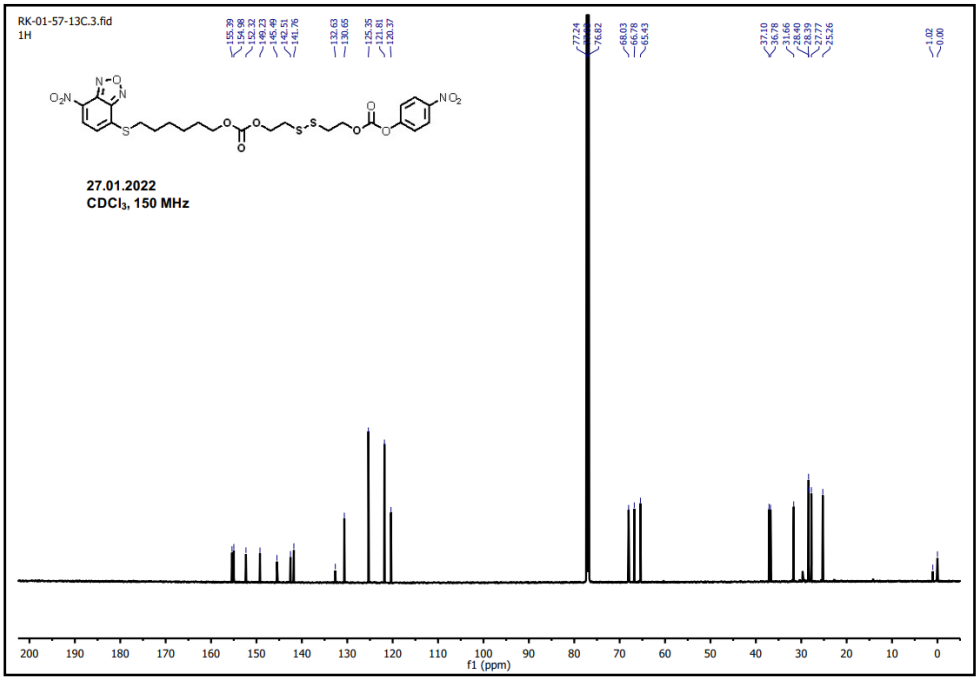


Figure S40. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **14**.

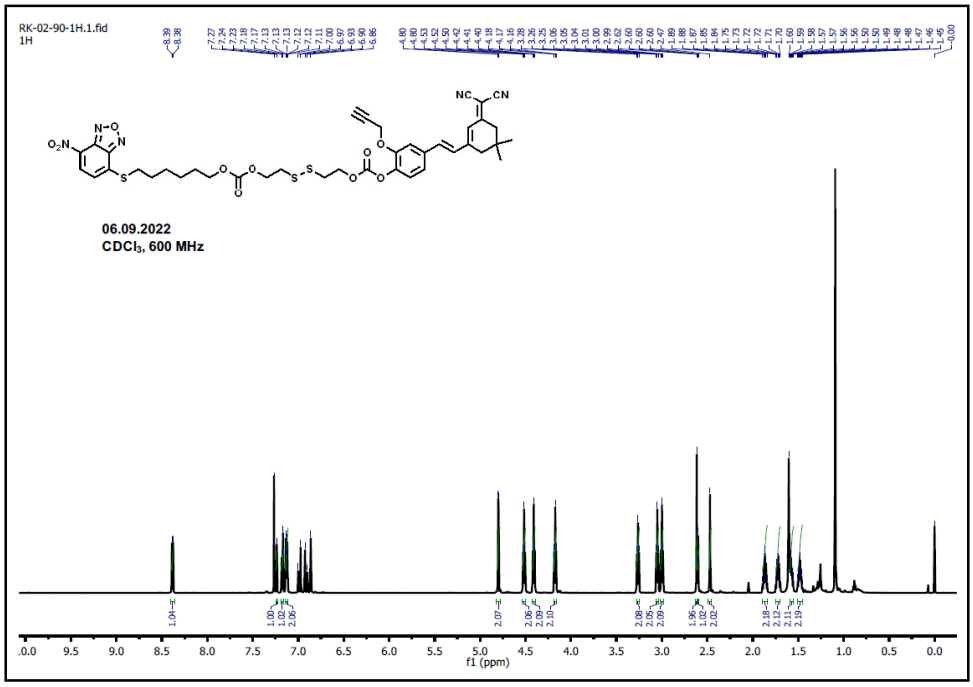


Figure S41. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **15**.

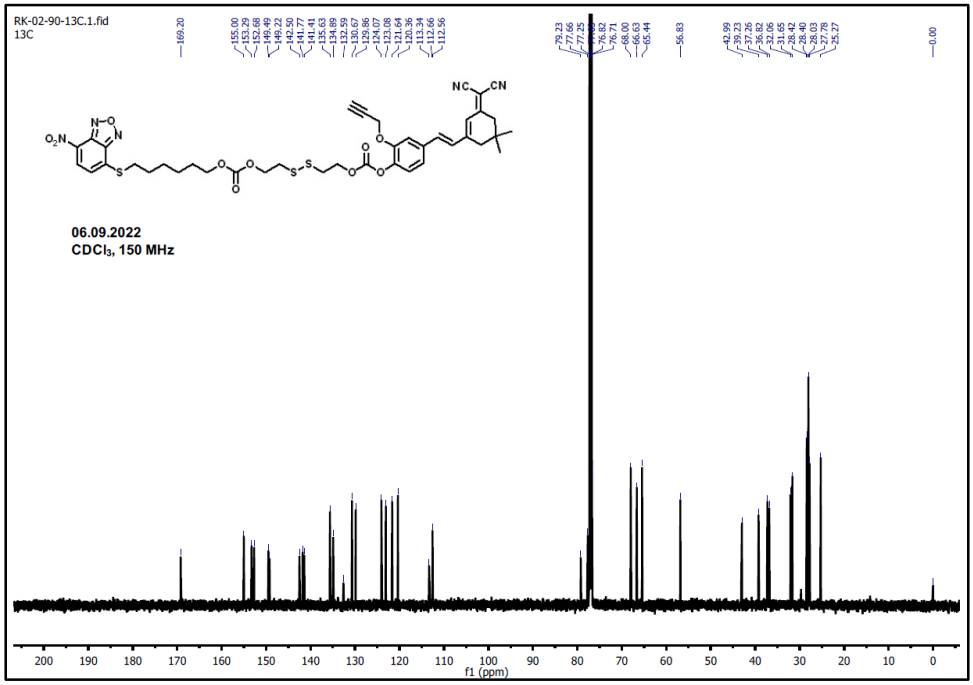


Figure S42. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **15**.

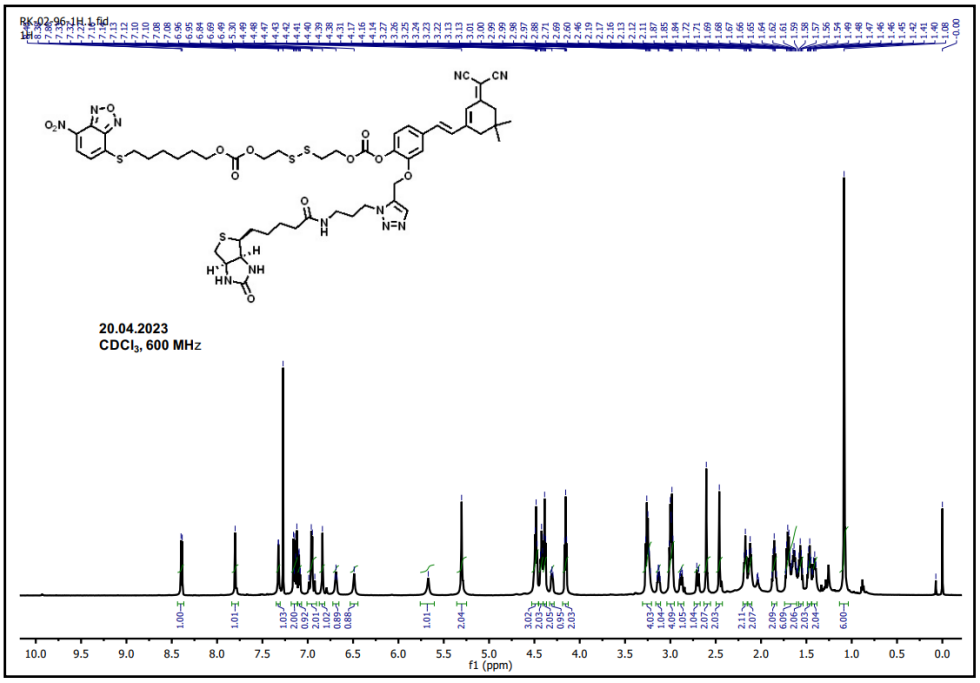


Figure S43. ^{1}H NMR ($CDCl_3$, 600 MHz) spectrum of compound **RK-296**.

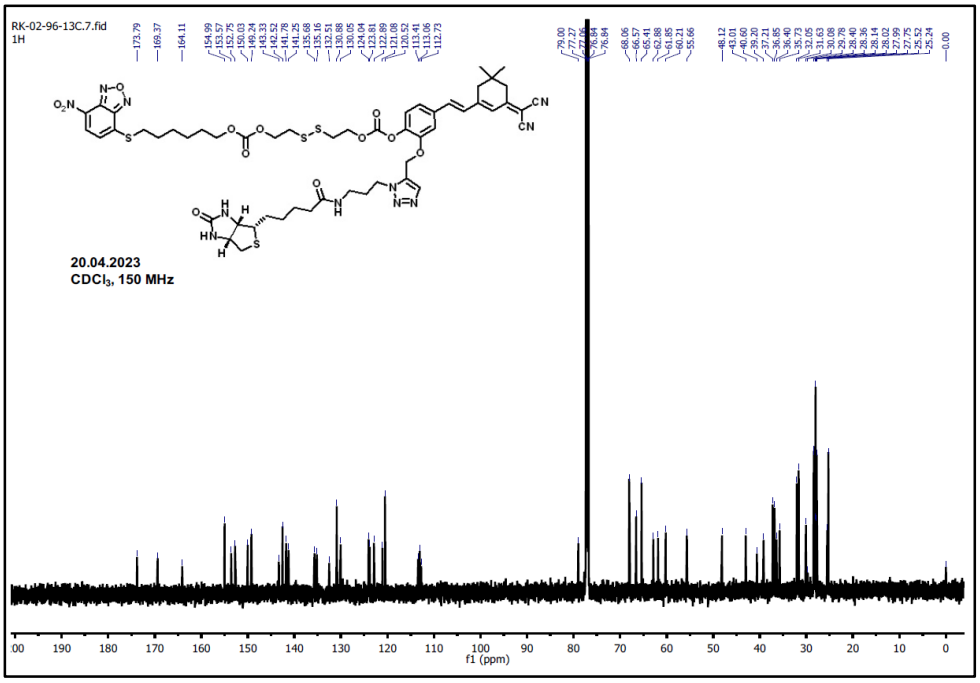


Figure S44. ^{13}C NMR ($CDCl_3$, 150 MHz) spectrum of compound **RK-296**.

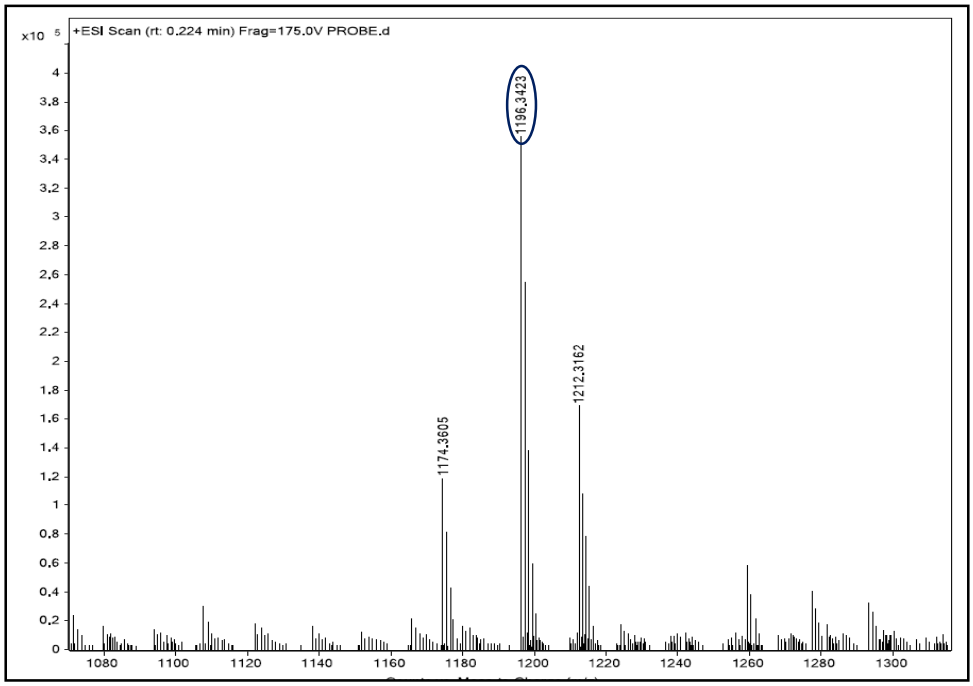


Figure S45. ESI-MS (+ve) spectrum of **RK-296**. ESI-MS: *m/z* calcd. for $C_{53}H_{63}N_{11}O_{12}S_4Na$ $[M+Na]^+$: 1196.3433; obs. $[M+Na]^+$: 1196.3423.

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