Supporting Information

Novel Acid-Activated Fluorophores Reveal a Dynamic Wave of Protons in the Intestine of *Caenorhabditis elegans*

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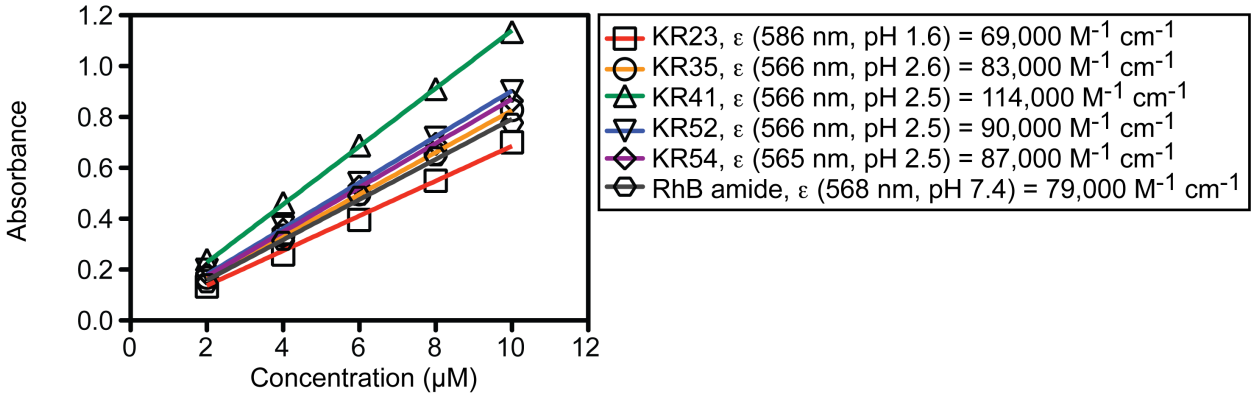
General experimental section. ^{1}H NMR (400 MHz) and ^{13}C NMR (125 MHz) spectra were acquired on Bruker DRX-400, Omega-500 or GN-500 instruments. Chemical shifts (d) are reported in ppm referenced to $CDCl_3$ (7.27 ppm for ^{1}H and 77.0 ppm for ^{13}C). Coupling constants (J_{HH}, Hz) are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dt = doublet of triplets, dt = doublet of quartets, dd = doublet of doublets), coupling constant, and integration. Infrared spectra (IR) were recorded with a Perkin-Elmer Spectrum 100 FT-IR spectrophotometer. Absorbance spectra were obtained using semimicro (1.5 mL) methacrylate cuvettes on a Aligent 8452A diode array spectrometer. Fluorescence spectra were acquired using semimicro (1.5 mL) methacrylate cuvettes and a Perkin-Elmer LS55 Fluorescence Spectrometer (10 nm excitation slit width). High Resolution mass spectra were obtained at the Mass Spectrometry Laboratory at the University of Kansas. Thin layer chromatography (TLC) was performed using EMD aluminum-backed (0.20 mm) silica plates (60 F-254), and flash chromatography used ICN silica gel (200-400 mesh). TLC plates were visualized by UV lamp or staining with ceric sulfate/molybdic acid. All non-aqueous reactions were carried out using flame- or oven-dried glassware under an atmosphere of dry argon or nitrogen. Tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), *N*,*N*-dimethylformamide (DMF), methanol (MeOH), ether (Et_2O), and triethylamine (TEA) were purified via filtration through two columns of activated basic alumina under an atmosphere of Ar using a solvent purification system from Pure Process Technology (GlassContour). Oregon Green 488 and Oregon Green dextran (70 KDa) were purchased from Life Technologies. Other commercial reagents were used as received unless otherwise noted.

Values for pKa were determined by non-linear least squares fitting of absorbance data (GraphPad Prism 5 software) obtained in aqueous solution containing bovine serum albumin (BSA, 1%), Triton X-100 (1%), and DMSO (1%). Molar extinction coefficients (ε) in acidic aqueous buffer (10 mM phosphate) containing Triton X-100 (1%) and DMSO (1%) were calculated from Beer's Law plots of absorbance l_{max} versus concentration as shown in Figure S1 (panel A). Linear least squares fitting of the data (including a zero intercept) was used to determine the slope (corresponding to ε). Values (M^{-1} cm^{-1}) were calculated as follows: Absorbance = ε [concentration (M)] L, where L = 1 cm. Relative quantum yields (Φ) in ethanol, containing 1% TFA for the acid-activated fluorophores, were determined by the method of Williams[1] as shown in Figure S1 (panel B). Fluorophores were excited at absorbance l_{max} minus 10 nm and the integrated fluorescence emission (from absorbance l_{max} plus 15 nm to 800 nm) was quantified (concentrations of 3.3 nM to 16.7 nM). Rhodamine 6G (Φ = 0.95 in ethanol) and rhodamine B (Φ = 0.68 in 94% ethanol) provided standards.[2-4] The integrated fluorescence emission at a given concentration was plotted against the maximum absorbance of the sample at that concentration determined by extrapolation based on absorbance measurements at higher concentrations. Linear least squares fitting of the data (including a zero intercept) was used to calculate the slope, which is proportional to the quantum yield. Quantum yields were calculated using the average of the values for the standards as follows: $\Phi_x = \Phi_{st}(Grad_x/Grad_{st})$, where Φ_{st} represents the quantum yield of the standard, Φ_x represents the quantum yield of the unknown, and *Grad* is the slope of the best linear fit. The supporting video (16.5 s) shows the PAP transition in unrestrained *C. elegans* during after feeding on KR35 (10 μM) for 30 min. Fluorescence

was rendered as a spectrum heat map, with red representing the most intense fluorescence (highest acidity) and black the least intense fluorescence (lowest acidity).

(A) Determination of extinction coefficients



(B) Determination of relative quantum yields

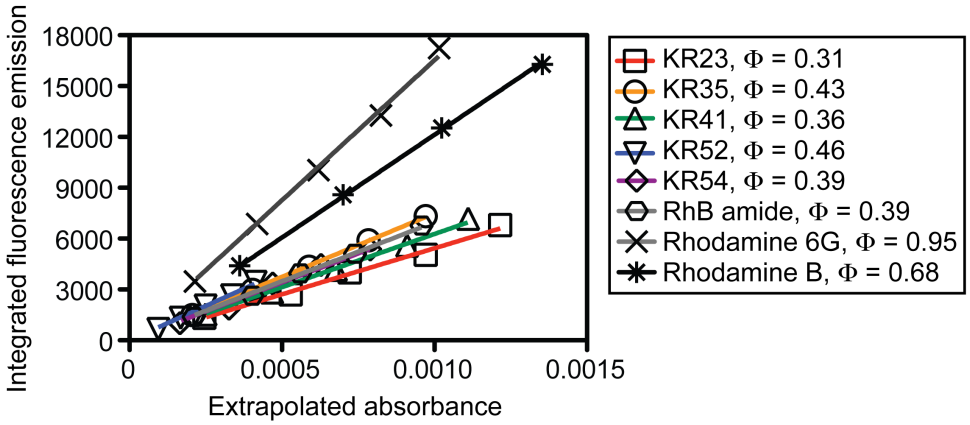


Figure S1. Panel A: Data and linear regression used to determine molar extinction coefficients in aqueous buffer (10 mM phosphate) containing 1% Triton X-100 and 1% DMSO at the pH values shown. Panel B: Data and linear regression used to determine quantum yields relative to known values for Rhodamine 6G and Rhodamine B in ethanol. The Kansas Red fluorophores in ethanol were protonated by addition of 1% trifluoroacetic acid.

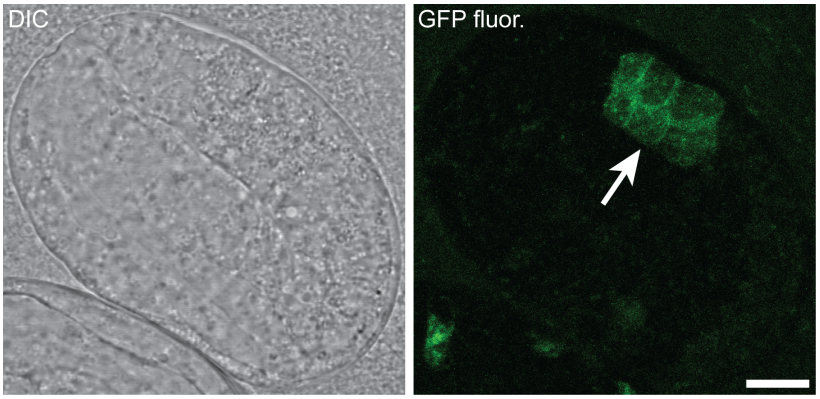


Figure S2. DIC and confocal images of PBO-4::GFP in a 2-fold embryo. Expression is strongest in posterior cells of the intestine (arrow). Scale bar = 10 microns.

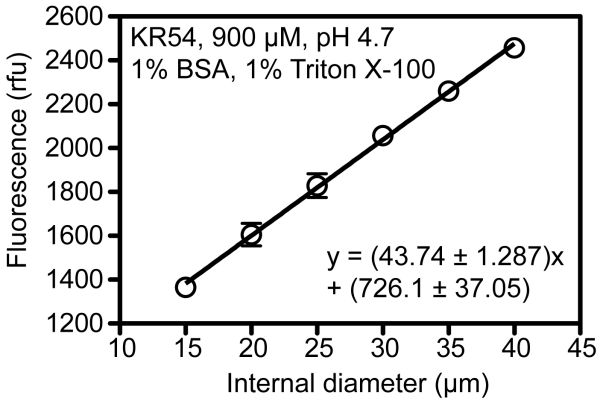


Figure S3. Fluorescence of KR54 in glass microneedles of varying internal diameter. Maximal fluorescence values were measured in triplicate with 5 µm linear ROIs oriented parallel to the needle.

Synthetic procedures and compound characterization data.

General Procedure A. A mixture of rhodamine B (1.00 g, 2.09 mmol), 1,2-dichloroethane (17 mL), and $POCl_3$ (1.03 mL, 10.9 mmol) was heated to 80 °C and stirred for 12 h. This mixture was concentrated under reduced pressure, and THF (20 mL) was added to dissolve the acid chloride. The corresponding primary amine (12.0 mmol) and TEA (1.0 mL, 7.13 mmol) dissolved in THF (5.00 mL) was slowly added. Over the course of 3 h, the resulting dark red mixture became lighter color and a precipitate was generated. The reaction mixture was concentrated under reduced pressure and filtered through a plug of silica (pretreated with 5% TEA in CH_2Cl_2) using 5% TEA in CH_2Cl_2 as the eluant. The filtrate was concentrated under reduced pressure and the oily residue was purified by column chromatography (using silica that had been pretreated with 1% TEA in hexanes) with a solvent gradient of 5–20 % EtOAc in hexanes containing 1% TEA. Evaporation of solvent under reduced pressure provided viscous oils that foamed under high vacuum.

General Procedure B. The lactam (0.50 mmol) in dry THF (15 mL) was treated with $LiAlH_4$ (0.57 g, 15.0 mmol). After gas evolution completely subsided, the mixture was heated to 50 °C and stirred for 3 h. The colorless reaction mixture was diluted with THF (50 mL), cooled in an ice bath, and quenched with wet Na_2SO_4. After gas evolution subsided, the resulting colorless slurry was filtered and the salts were washed with ether (2 x 75 mL). The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography (using silica that had been pretreated with 1% TEA in hexanes) with a solvent gradient of 5–20 % EtOAc in hexanes

containing 1% TEA. Evaporation of solvent under reduced pressure provided viscous oils that foamed under high vacuum.

General Procedure C. The lactam (0.10 mmol) in a dry round-bottom flask was treated with diisobutylaluminum hydride (1.0 M in cyclohexane, 2.0 mL, 2.0 mmol) at 4 °C. The mixture was stirred for 8 h at room temperature (22 °C). The reaction was diluted with ether (20 mL), washed three times with H_2O (3 x 5 mL), and the organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the products were purified by flash chromatography (eluent: 5% EtOAc, 2.5% TEA in hexanes) to afford amino analogues of rhodamine in the spirocyclic form as white solids.

3',6'-bis(diethylamino)-2-ethylspiro[isoindoline-1,9'-xanthen]-3-one, KR54-lactam. Using General Procedure A with ethylamine, **KR54-lactam** (905 mg, 96%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98–7.85 (m, 1H), 7.50–7.36 (m, 2H), 7.16–7.01 (m, 1H), 6.48 (d, *J* = 8.8 Hz, 2H), 6.41 (d, *J* = 2.6 Hz, 2H), 6.29 (dd, *J* = 8.8, 2.6 Hz, 2H), 3.36 (q, *J* = 7.1 Hz, 8H), 3.25–3.15 (m, 2H), 1.19 (t, *J* = 7.0 Hz, 12H), 0.84 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.8, 153.8, 153.3, 148.7, 132.1, 131.5, 129.0, 127.9, 123.6, 122.7, 108.0, 105.9, 97.7, 64.7, 44.4, 35.1, 13.8, 12.6; IR (film) ν_{max}: 2971, 2931, 1686, 1633, 1614, 1546, 1512, 1467, 1374, 1326, 1264, 1218, 1117, 787, 702 cm^{-1}; HRMS (ESI) *m/z* 470.2802 (M+H$^+$, $C_{30}H_{36}N_3O_2$ requires 470.2808).

3',6'-bis(diethylamino)-2-(2-fluoroethyl)spiro[isoindoline-1,9'-xanthen]-3-one, KR52-lactam. Using General Procedure A with 2-fluoroethylamine, **KR52-lactam** (762 mg, 76%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00–7.88 (m, 1H), 7.54–7.38 (m,

2H), 7.16–7.01 (m, 1H), 6.47 (d, *J* = 8.8 Hz, 2H), 6.40 (d, *J* = 2.6 Hz, 2H), 6.30 (dd, *J* = 8.8, 2.6 Hz, 2H), 4.11 (dt, *J* = 44.0, 6.7 Hz, 2H), 4.05 (t, *J* = 6.7 Hz, 1H), 3.49 (dt, *J* = 16.7, 6.7 Hz, 2H), 3.36 (q, *J* = 7.1 Hz, 8H), 1.19 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.4, 153.7, 153.2, 148.8, 132.6, 130.7, 128.8, 128.1, 123.8, 122.9, 108.1, 105.2, 97.7, 80.90, 79.6, 64.8, 44.4, 39.8, 39.6, 12.6; IR (film) ν_{max}: 2970, 1695, 1615, 1547, 1514, 1376, 1265, 1219, 1118, 788, 702 cm^{-1}; HRMS (ESI) *m/z* 510.2545 ($M+Na^+$, $C_{30}H_{34}FN_3O_2Na$ requires 510.2533).

3',6'-bis(diethylamino)-2-(2,2-difluoroethyl)spiro[isoindoline-1,9'-xanthen]-3-one, KR41-lactam. Using General Procedure A with 2,2-difluoroethylamine, **KR41-lactam** (852 mg, 85%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05–7.88 (m, 1H), 7.57–7.41 (m, 2H), 7.13 (d, *J* = 9.8 Hz, 1H), 6.46 (d, *J* = 11.1 Hz, 2H), 6.44–6.40 (m, 2H), 6.31 (dd, *J* = 9.0, 2.6 Hz, 2H), 5.36 (tt, *J* = 56.4, 4.9 Hz, 1H), 3.51 (td, *J* = 13.9, 4.9 Hz, 2H), 3.36 (q, *J* = 7.1 Hz, 8H), 1.19 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.6, 153.5, 153.2, 148.9, 132.9, 130.2, 128.8, 128.2, 123.9, 123.1, 115.2, 113.2, 111.3, 108.3, 104.7, 97.7, 65.0, 44.4, 42.8, 42.6, 42.3, 12.6; IR (film) ν_{max}: 2971, 2930, 1698, 1634, 1613, 1513, 1375, 1264, 1218, 1116, 1036, 786 cm^{-1}; HRMS (ESI) *m/z* 528.2438 ($M+Na^+$, $C_{30}H_{33}F_2N_3O_2Na$ requires 528.2439).

3',6'-bis(diethylamino)-2-(2,2,2-trifluoroethyl)spiro[isoindoline-1,9'-xanthen]-3-one, KR35-lactam. Using General Procedure A with 2,2,2-trifluoroethylamine, **KR35-lactam** (923 mg, 88%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05–7.90 (m, 1H), 7.58–7.41 (m, 2H), 7.19–7.05 (m, 1H), 6.41 (dd, *J* = 15.4, 8.8 Hz, 4H), 6.31 (dd, *J* = 9.0, 2.6 Hz, 2H), 3.75 (q, *J* = 9.4 Hz, 2H), 3.36 (q, *J* = 7.1 Hz, 8H), 1.19 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.3, 154.0, 153.3, 148.9, 133.2, 129.5, 129.0, 128.2,

125.0, 124.2, 123.2, 122.7, 108.3, 104.6, 97.6, 65.6, 44.4, 42.2, 41.9, 41.6, 41.3, 12.6; IR (film) ν_{max}: 2972, 2934, 1704, 1613, 1513, 1467, 1372, 1356, 1263, 1218, 1144, 1117, 1064, 820, 701 cm^{-1}; HRMS (ESI) *m/z* 524.2527 ($M+H^+$, $C_{30}H_{33}F_3N_3O_2$ requires 524.2525).

***N*$^{3'}$,*N*$^{3'}$,*N*$^{6'}$,*N*$^{6'}$,2-pentaethylspiro[isoindoline-1,9'-xanthene]-3',6'-diamine, KR54.** Using General Procedure B with **KR54-lactam**, **KR54** (226 mg, 94%) was isolated. ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.32 (m, 1H), 7.28–7.23 (m, 1H), 7.20 (td, *J* = 7.4, 1.3 Hz, 1H), 6.93 (d, *J* = 7.5 Hz, 1H), 6.67 (d, *J* = 8.7 Hz, 2H), 6.38 (d, *J* = 2.6 Hz, 2H), 6.33 (dd, *J* = 8.8, 2.6 Hz, 4H), 4.14 (s, 2H), 3.36 (q, *J* = 7.0 Hz, 8H), 2.27 (q, *J* = 7.2 Hz, 2H), 1.19 (t, *J* = 7.0 Hz, 12H), 0.97 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.2, 149.2, 147.8, 139.6, 130.4, 127.4, 126.7, 124.5, 121.8, 111.8, 107.3, 97.5, 55.4, 44.3, 42.4, 14.4, 12.7; IR (film) ν_{max}: 2968, 2930, 1634, 1615, 1509, 1466, 1263, 1219, 1115, 1078, 1019, 819, 755 cm^{-1}; HRMS (ESI) *m/z* 456.3020 ($M+H^+$, $C_{30}H_{38}N_3O$ requires 456.3015).

***N*$^{3'}$,*N*$^{3'}$,*N*$^{6'}$,*N*$^{6'}$-tetraethyl-2-(2-fluoroethyl)spiro[isoindoline-1,9'-xanthene]-3',6'-diamine, KR52.** Using General Procedure B with **KR52-lactam**, **KR52** (184 mg, 77%) was isolated. ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, *J* = 7.4 Hz, 1H), 7.30–7.24 (m, 1H), 7.19 (td, *J* = 7.4, 1.3 Hz, 1H), 6.91 (d, *J* = 7.5 Hz, 1H), 6.77–6.63 (m, 2H), 6.41–6.31 (m, 4H), 4.38 (tt, *J* = 44.2, 5.3 Hz, 2H), 4.39–4.11 (m, 2H), 3.42–3.33 (m, 8H), 2.61 (dt, *J* = 26.8, 5.3 Hz, 2H), 1.20–1.09 (m, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.0, 148.9, 147.9, 139.3, 130.4, 127.5, 126.8, 124.5, 121.8, 111.4, 107.5, 97.4, 85.19, 83.9, 68.5, 57.2, 48.6, 44.4, 12.7; IR (film) ν_{max}: 2969, 2930, 1633, 1613, 1545, 1509, 1466,

1356, 1263, 1219, 1154, 1018, 787, 756 cm^{-1}; HRMS (ESI) *m/z* 474.2909 ($M+H^+$, $C_{30}H_{37}FN_3O$ requires 474.2921).

2-(2,2-difluoroethyl)-*N*$^{3'}$,*N*$^{3'}$,*N*$^{6'}$,*N*$^{6'}$-tetraethylspiro[isoindoline-1,9'-xanthene]-3',6'-diamine, KR41. Using General Procedure B with **KR41-lactam**, **KR41** (225 mg, 92%) was isolated. ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 7.6 Hz, 1H), 7.29–7.23 (m, 1H), 7.19 (td, *J* = 7.4, 1.3 Hz, 1H), 6.94–6.84 (m, 1H), 6.73 (d, *J* = 8.6 Hz, 2H), 6.43–6.28 (m, 4H), 5.52 (tt, *J* = 56.0, 4.2 Hz, 1H), 4.38 (s, 2H), 3.37 (q, *J* = 7.1 Hz, 8H), 2.73 (td, *J* = 15.0, 4.6 Hz, 2H), 1.20 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 152.8, 148.9, 148.1, 138.6, 130.2, 127.6, 126.9, 124.5, 121.8, 119.3, 117.3, 115.4, 111.1, 107.7, 97.4, 68.8, 57.8, 51.4, 51.2, 51.0, 44.3, 12.7; IR (film) ν_{max}: 2970, 2871, 1613, 1509, 1261, 1217, 1157, 1059, 1018, 1004, 788 cm^{-1}; HRMS (ESI) *m/z* 492.2840 ($M+H^+$, $C_{30}H_{36}F_2N_3O$ requires 492.2826).

***N*$^{3'}$,*N*$^{3'}$,*N*$^{6'}$,*N*$^{6'}$-tetraethyl-2-(2,2,2-trifluoroethyl)spiro[isoindoline-1,9'-xanthene]-3',6'-diamine, KR35.** Using General Procedure B with **KR35-lactam**, **KR35** (230 mg, 90%) was isolated. ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, *J* = 7.5 Hz, 1H), 7.29–7.25 (m, 1H), 7.19 (td, *J* = 7.4, 1.3 Hz, 1H), 6.90 (d, *J* = 7.6 Hz, 1H), 6.77 (d, *J* = 8.5 Hz, 2H), 6.43–6.30 (m, 4H), 4.44 (s, 2H), 3.37 (q, *J* = 7.0 Hz, 8H), 2.94 (q, *J* = 9.6 Hz, 2H), 1.20 (t, *J* = 7.1 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.6, 147.8, 147.1, 136.9, 129.3, 128.4, 126.7, 126.2, 125.9, 123.9, 123.4, 121.7, 120.8, 109.7, 106.7, 96.3, 68.1, 56.7, 50.0, 49.7, 49.5, 49.2, 43.3, 11.6; IR (film) ν_{max}: 2971, 2932, 2872, 1613, 1509, 1356, 1323, 1268, 1138, 1114, 1040, 907, 788, 726 cm^{-1}; HRMS (ESI) *m/z* 510.2729 ($M+H^+$, $C_{30}H_{35}F_3N_3O$ requires 510.2732).

4,5,6,7-Tetrafluororhodamine B, TFRB. To a stirred solution of *N*, *N*-diethyl-3-aminophenol (650 mg, 3.9 mmol) in toluene (10 mL) was added tetrafluorophthalic anhydride (880 mg, 4.0 mmol). The reaction mixture was refluxed for 12 h. After cooling to room temperature, the solvent was removed under reduced pressure. Flash column chromatography (elution gradient: 4% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2) afforded **TFRB** (380 mg, 38%) as a dark purple solid. ^{1}H NMR (400 MHz, $CDCl_3$) 7.01 (d, *J* = 9.0 Hz, 2H), 6.59 (d, *J* = 9.1 Hz, 2H), 6.53 (d, *J* = 2.0 Hz, 2H), 3.44 (q, *J* = 7.0 Hz, 8H), 1.23 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.4, 154.3, 151.7, 142.9, 141.5, 141.0, 140.5, 128.8, 110.0, 107.4, 95.1, 44.2, 11.5; Note: Some C-F splitting was not fully resolved in the ^{13}C NMR spectra. IR (film) ν_{max}: 2973, 2925, 1770, 1634, 1590, 1511, 1469, 1417, 1396, 1338, 1275, 1247, 1182, 1133, 1076, 1012, 924, 827, 686 cm^{-1}; HRMS (ESI) m/z 515.1947 ($M+H^+$, $C_{28}H_{27}F_4N_2O_3$ requires 515.1958).

3',6'-bis(diethylamino)-4,5,6,7-tetrafluoro-2-(2,2,2-trifluoroethyl)spiro[isoindoline-1,9'-xanthen]-3-one, KR23-lactam. Using General Procedure A with **TFRB** and 2,2,2-trifluoroethylamine, **KR23-lactam** (200 mg, 89%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.52 (d, *J* = 8.8 Hz, 2H), 6.38 (d, *J* = 2.5 Hz, 2H), 6.33 (dd, *J* = 8.8 Hz, 2.5 Hz, 2H), 3.71 (q, *J* = 9.1 Hz, 2H), 3.45 (q, *J* = 7.1 Hz, 8H), 1.17 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 162.8, 152.0, 148.4, 143.1, 142.4, 140.7, 140.0, 134.3, 126.6, 107.3, 100.4, 96.9, 62.8, 43.4, 40.7, 11.5; Note: Some C-F splitting was not fully resolved in the ^{13}C NMR spectra. IR (film) ν_{max}: 2974, 1721, 1636, 1616, 1514, 1429, 1383, 1359, 1332, 1266, 1222, 1151, 1120, 1070, 990, 884, 824, 785, 668 cm^{-1}; HRMS (ESI) *m/z* 596.2099 ($M+H^+$, $C_{30}H_{29}F_7N_3O_2$ requires 596.2070).

***N*$^{3'}$,*N*$^{3'}$,*N*$^{6'}$,*N*$^{6'}$-tetraethyl-4,5,6,7-tetrafluoro-2-(2,2,2-trifluoroethyl)spiro[isoindoline-1,9'-xanthene]-3',6'-diamine, KR23.** Using General Procedure C with **KR23-lactam**, **KR23** (50 mg, 83%) was isolated. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.80 (d, *J* = 8.6 Hz, 2H), 6.36 (d, *J* = 2.6 Hz, 2H), 6.34 (dd, *J* = 8.8 Hz, 2.6 Hz, 2H), 4.54 (s, 2H), 3.35 (q, *J* = 7.0 Hz, 8H), 2.92 (q, *J* = 9.5 Hz, 2H), 1.18 (t, *J* = 7.0 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 151.3, 147.7, 141.2, 140.5, 139.2, 138.8, 130.2, 130.1, 128.2, 128.1, 128.0, 127.6, 125.8, 123.6, 121.3, 118.9, 107.1, 106.7, 96.6, 67.5, 53.5, 48.8, 43.3, 11.6; Note: Some C-F splitting was not fully resolved in the ^{13}C NMR spectra. IR (film) ν_{max}: 2929, 2973, 1699, 1635, 1615, 1508, 1271, 1220, 1147, 1118, 974, 823, 776 cm^{-1}; HRMS (ESI) *m/z* 582.2261 ($M+H^+$, $C_{30}H_{31}F_7N_3O$ requires 582.2277).

***N*-(6-(diethylamino)-9-(2-(morpholine-4-carbonyl)phenyl)-3H-xanthen-3-ylidene)-*N*-ethylethanaminium, Rhodamine B amide.** To morpholine (70 mg, 0.80 mmol) in DMF (3 mL) under argon was added HOBT (108 mg, 0.80 mmol), rhodamine B (192 mg, 0.40 mmol) and HBTU (303 mg, 0.80 mmol), and the mixture stirred at room temperature for 1 h. 4-Methylmorpholine (0.11 mL, 1.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 h. The reaction was diluted with ethyl acetate (30 mL), washed three times with H_2O, and the dark red organic layer was dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the products were purified by flash chromatography (2% to 10% MeOH in CH_2Cl_2) to provide the amide (150 mg, 74%) as a red amorphous solid.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71–7.61 (m, 2H), 7.55–7.48 (m, 1H), 7.37–7.29 (m, 1H), 7.25 (d, J = 9.4 Hz, 2H), 6.94 (dd, J = 9.6, 2.5 Hz, 2H), 6.76 (d, J = 2.4 Hz, 2H), 3.60 (qd, J = 7.3, 4.2 Hz, 8H),

3.48 (t, J = 4.6 Hz, 4H), 3.40 (dd, J = 11.8, 5.7 Hz, 4H), 1.32 (t, J = 7.1 Hz, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 12.56, 42.21, 46.07, 48.07, 96.21, 113.78, 114.13, 127.60, 130.05, 130.17, 130.23, 130.74, 132.16, 135.01, 155.65, 156.00, 157.75, 167.62; IR (film) l_{max} 2979, 1633, 1589, 1469, 1414, 1340, 1181, 1134, 1075, 1012, 841, 683 cm^{-1}; HRMS (ESI) *m/z* 512.2903 ($M+H^+$, $C_{32}H_{38}N_3O_3$ requires 512.2908).

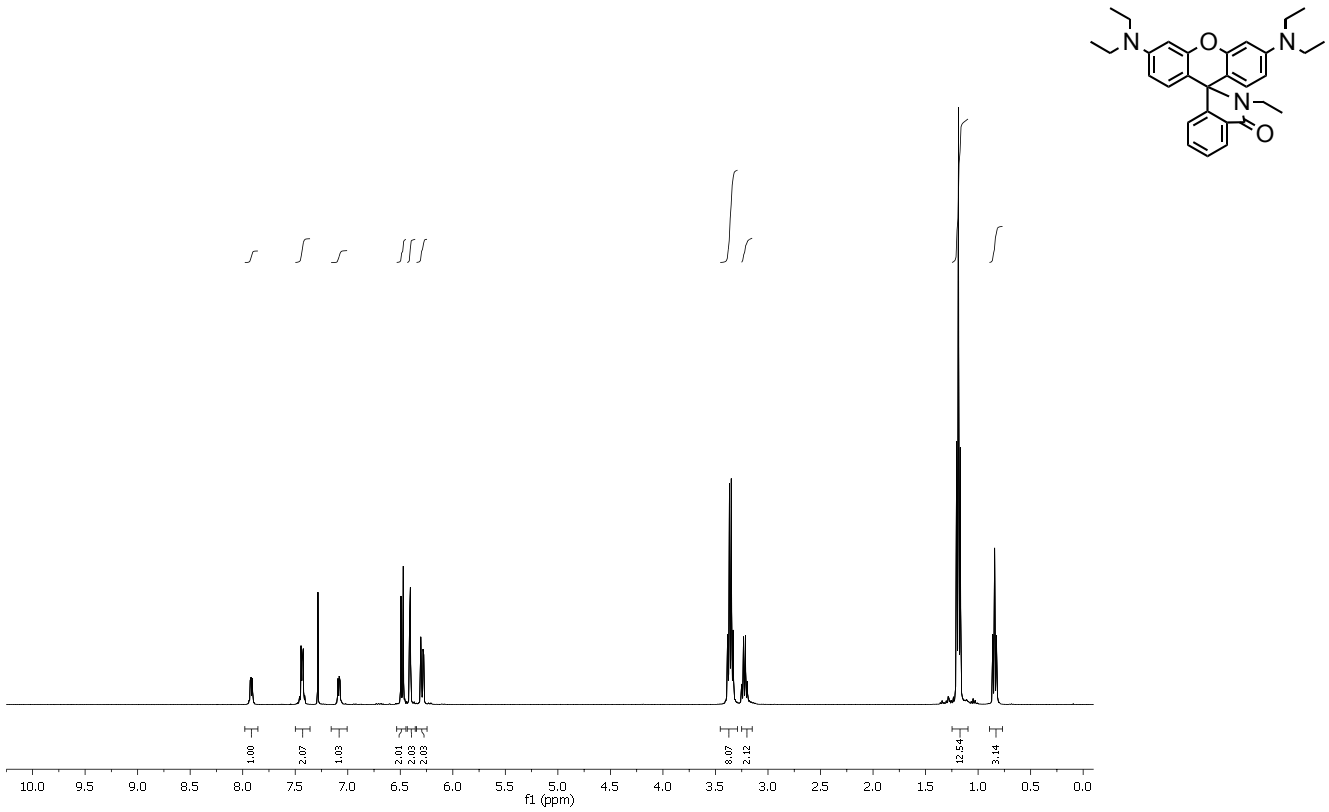


Figure S4. ^{1}H NMR (400 MHz) of a solution of **KR54-lactam** in $CDCl_3$.

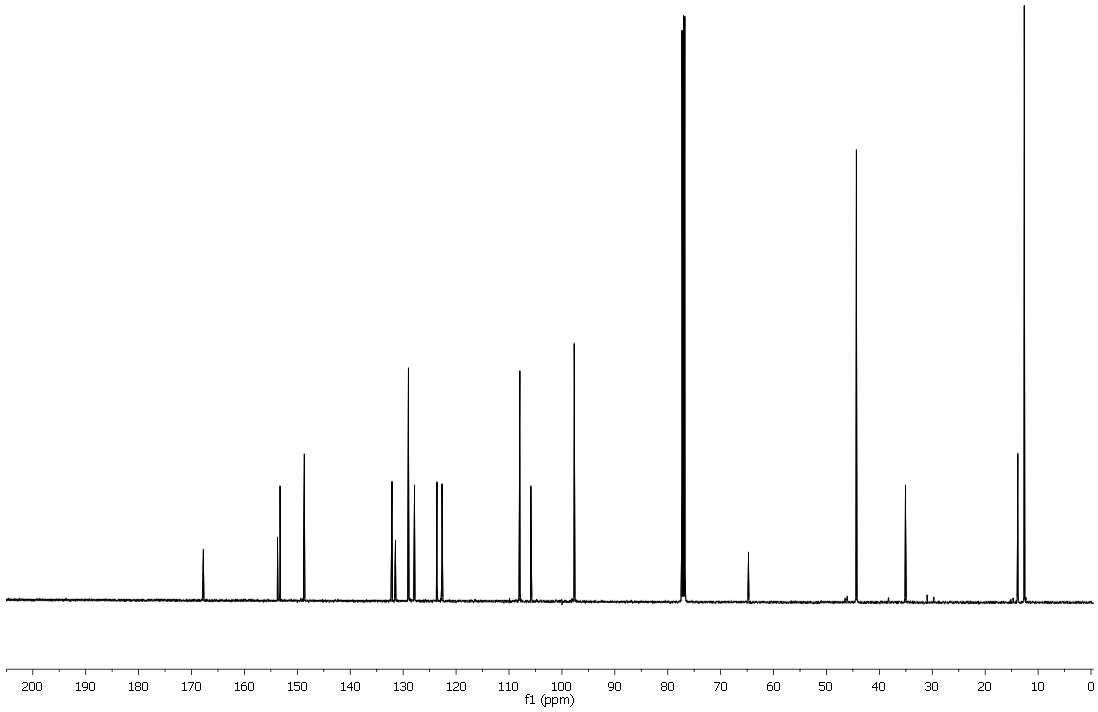


Figure S5. ^{13}C NMR (126 MHz) of a solution of **KR54-lactam** in $CDCl_3$.

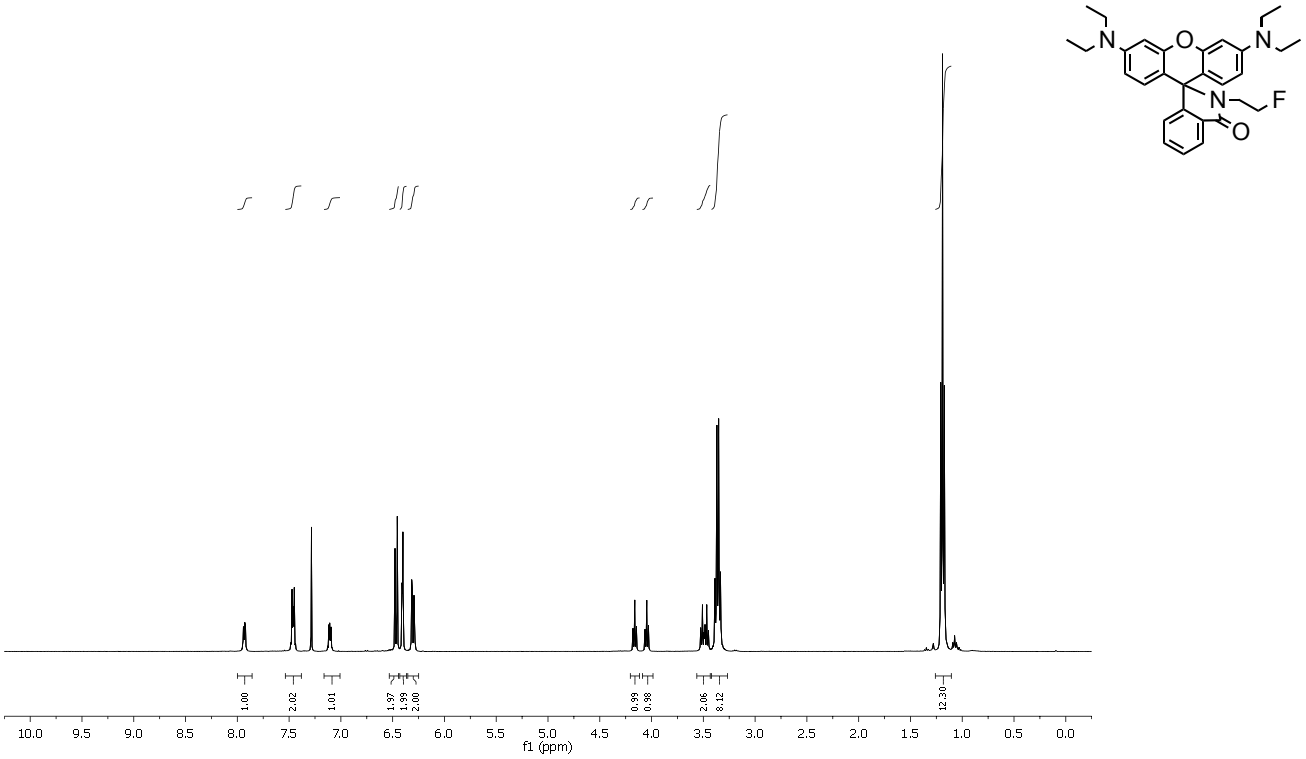


Figure S6. ^{1}H NMR (400 MHz) of a solution of **KR52-lactam** in $CDCl_3$.

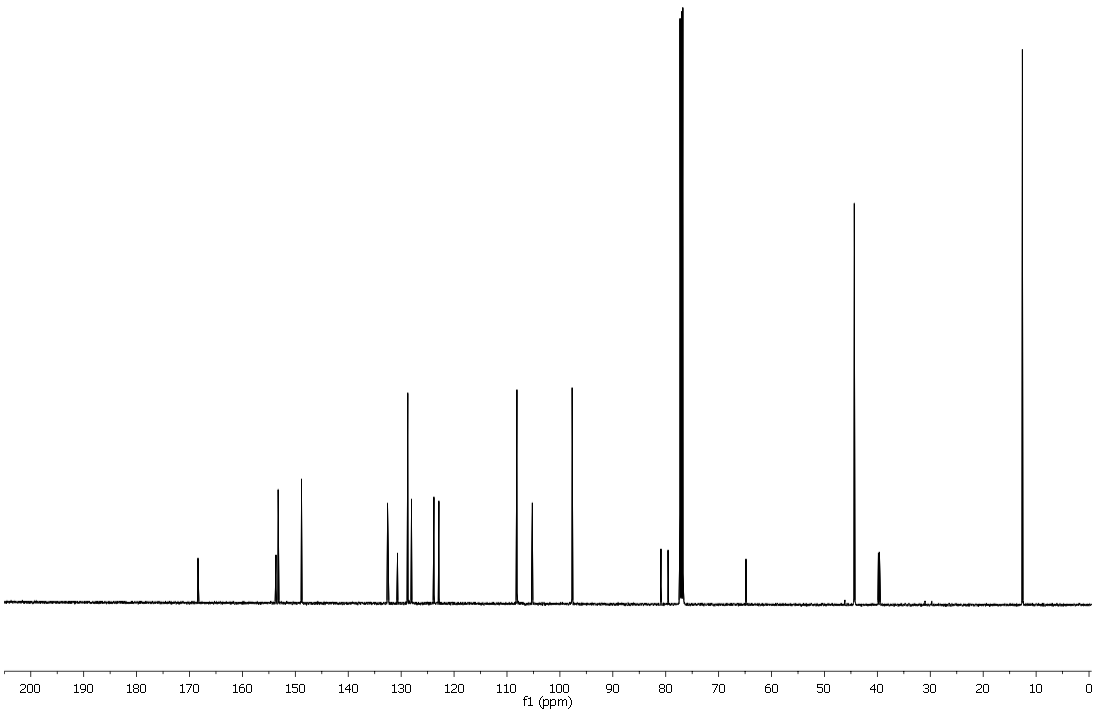


Figure S7. ^{13}C NMR (126 MHz) of a solution of **KR52-lactam** in $CDCl_3$.

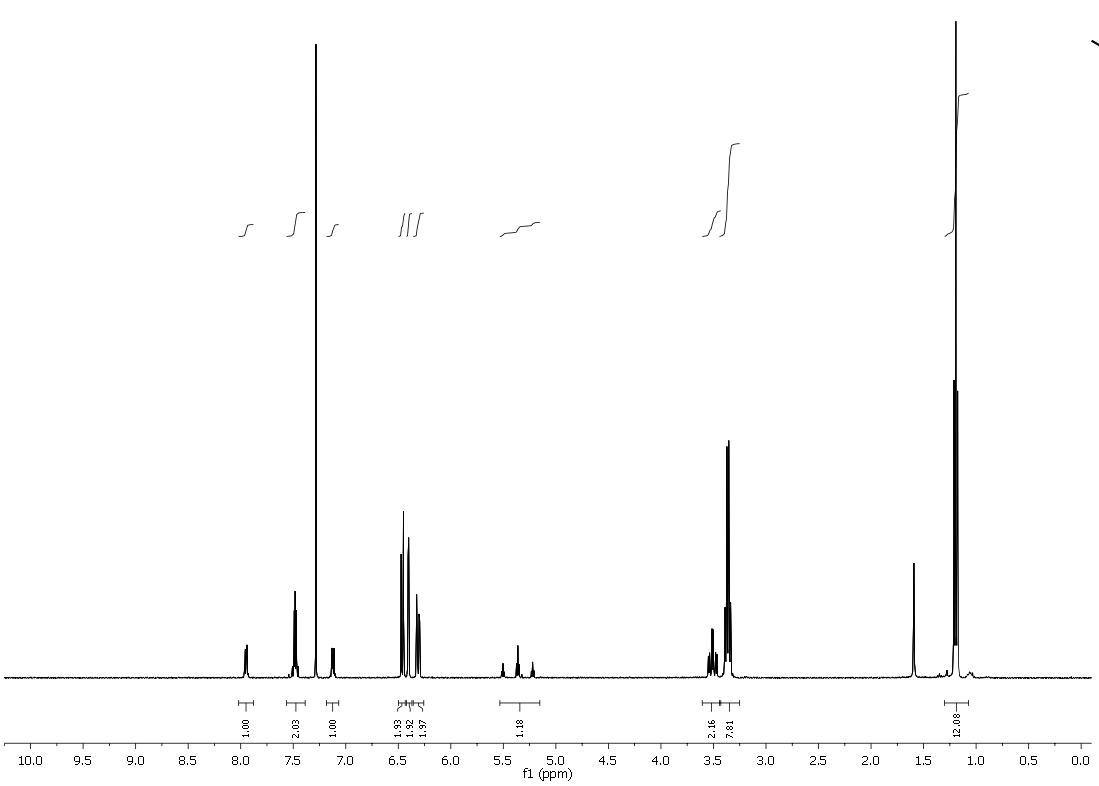


Figure S8. ^{1}H NMR (400 MHz) of a solution of **KR41-lactam** in $CDCl_3$.

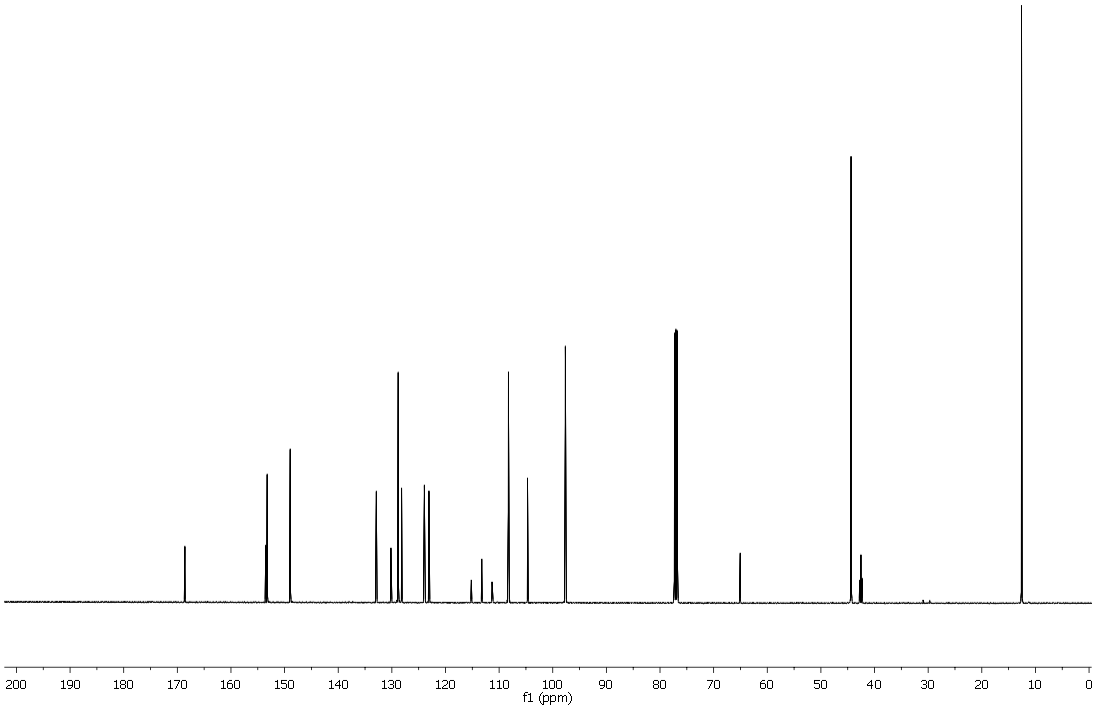


Figure S9. ^{13}C NMR (126 MHz) of a solution of **KR41-lactam** in $CDCl_3$.

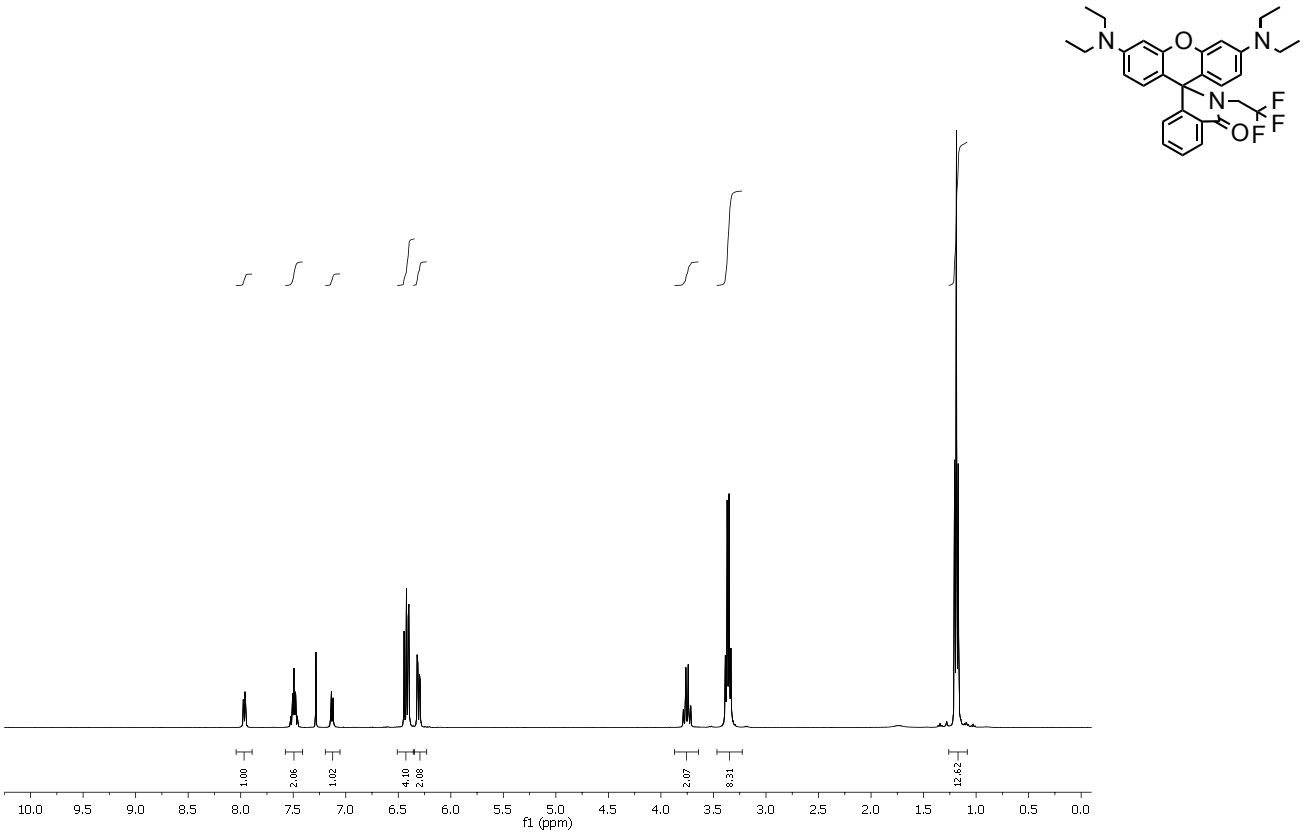


Figure S10. ^{1}H NMR (400 MHz) of a solution of **KR35-lactam** in $CDCl_3$.

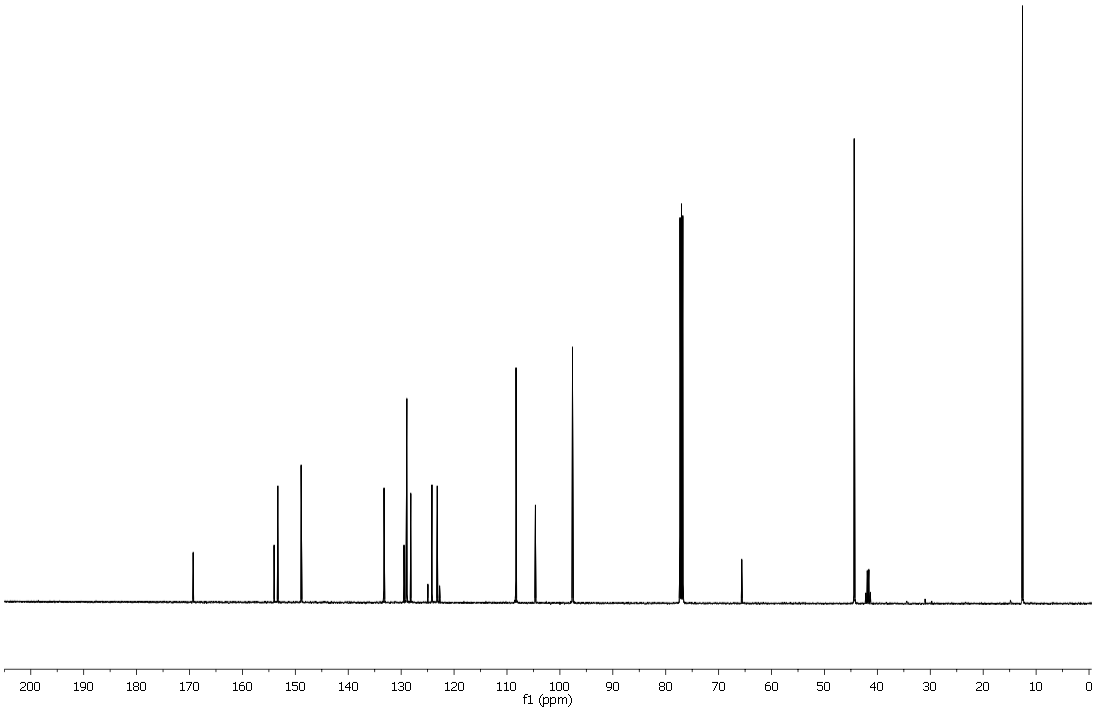


Figure S11. ^{13}C NMR (126 MHz) of a solution of **KR35-lactam** in $CDCl_3$.

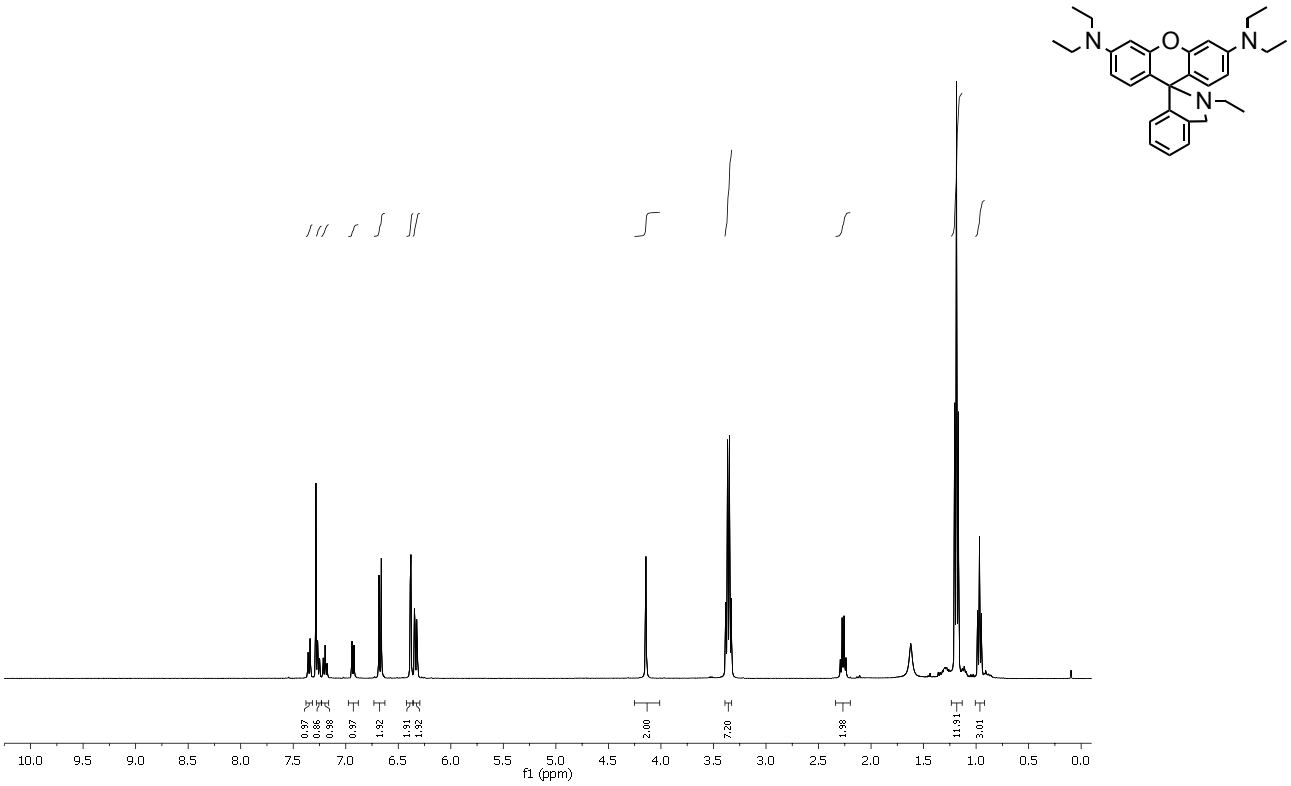


Figure S12. ^{1}H NMR (400 MHz) of a solution of **KR54** in $CDCl_3$.

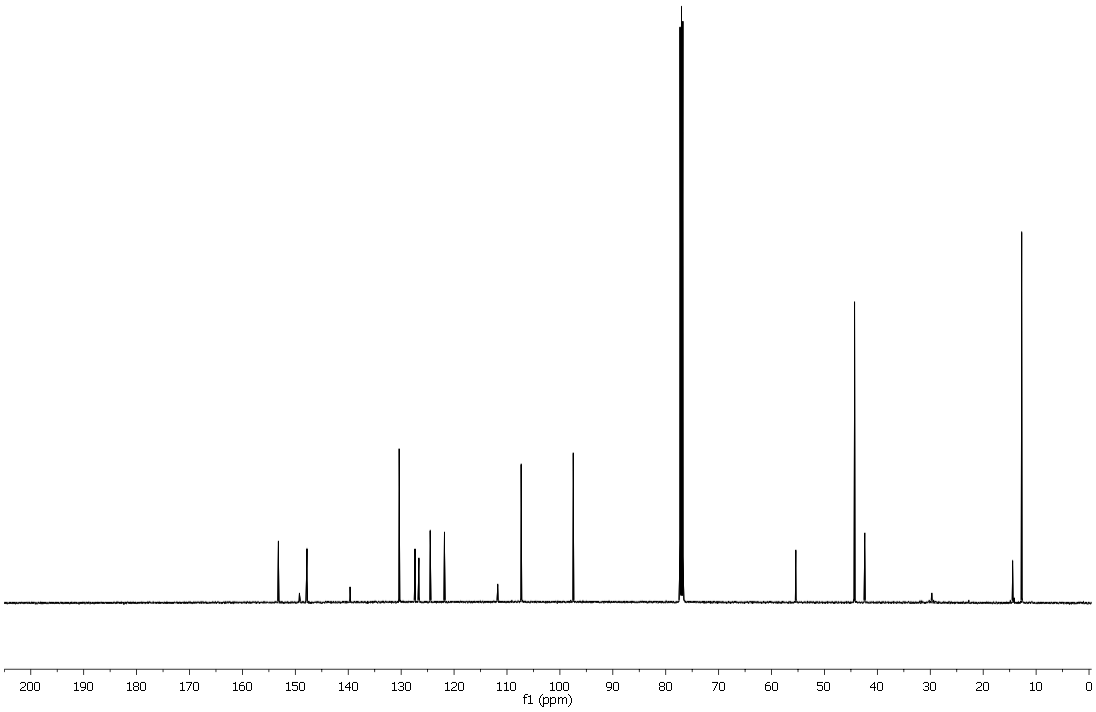


Figure S13. ^{13}C NMR (126 MHz) of a solution of **KR54** in $CDCl_3$.

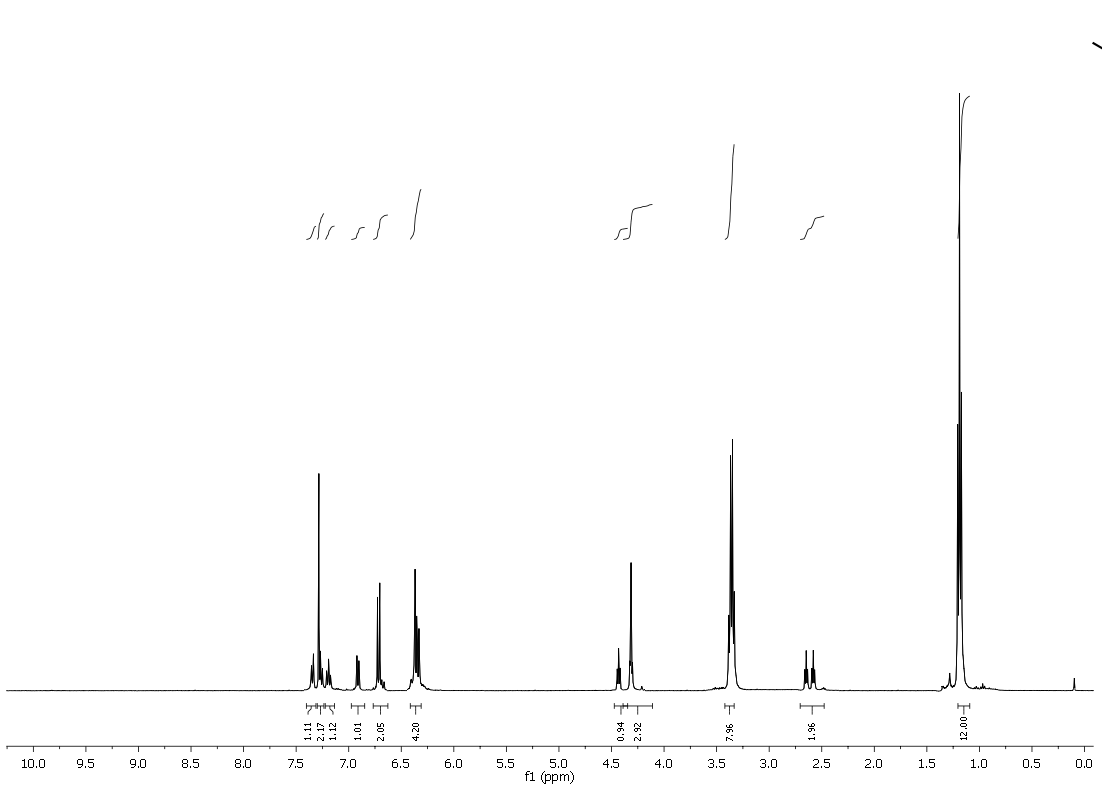


Figure S14. ^{1}H NMR (400 MHz) of a solution of **KR52** in $CDCl_3$.

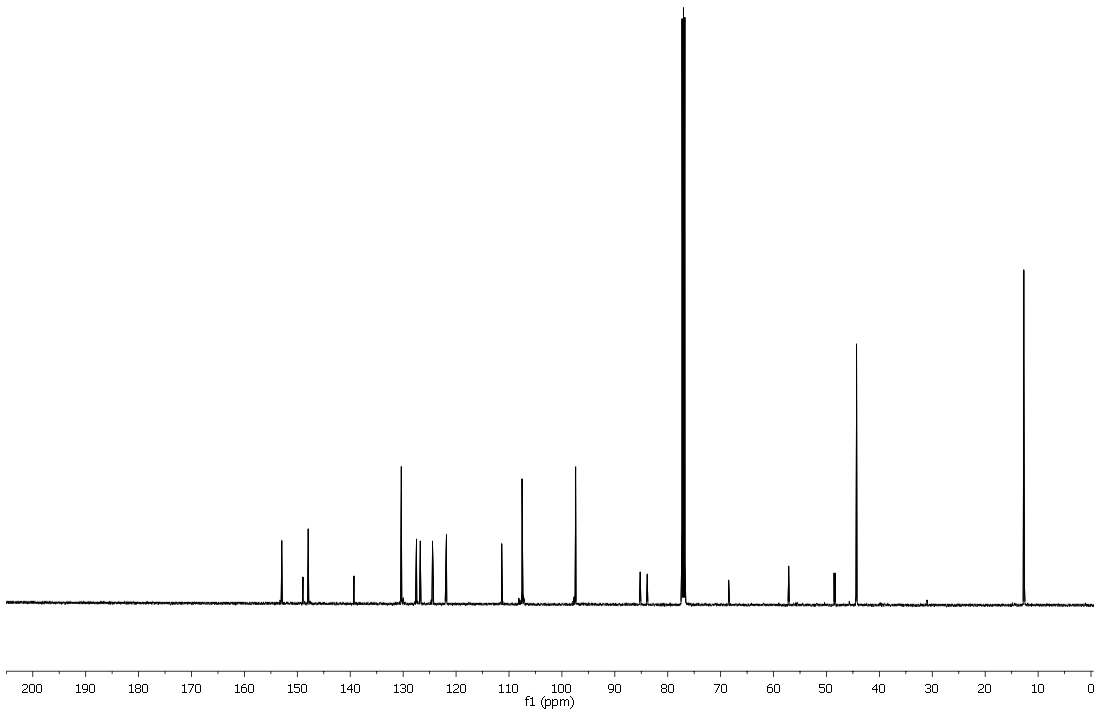


Figure S15. ^{13}C NMR (126 MHz) of a solution of **KR52** in $CDCl_3$.

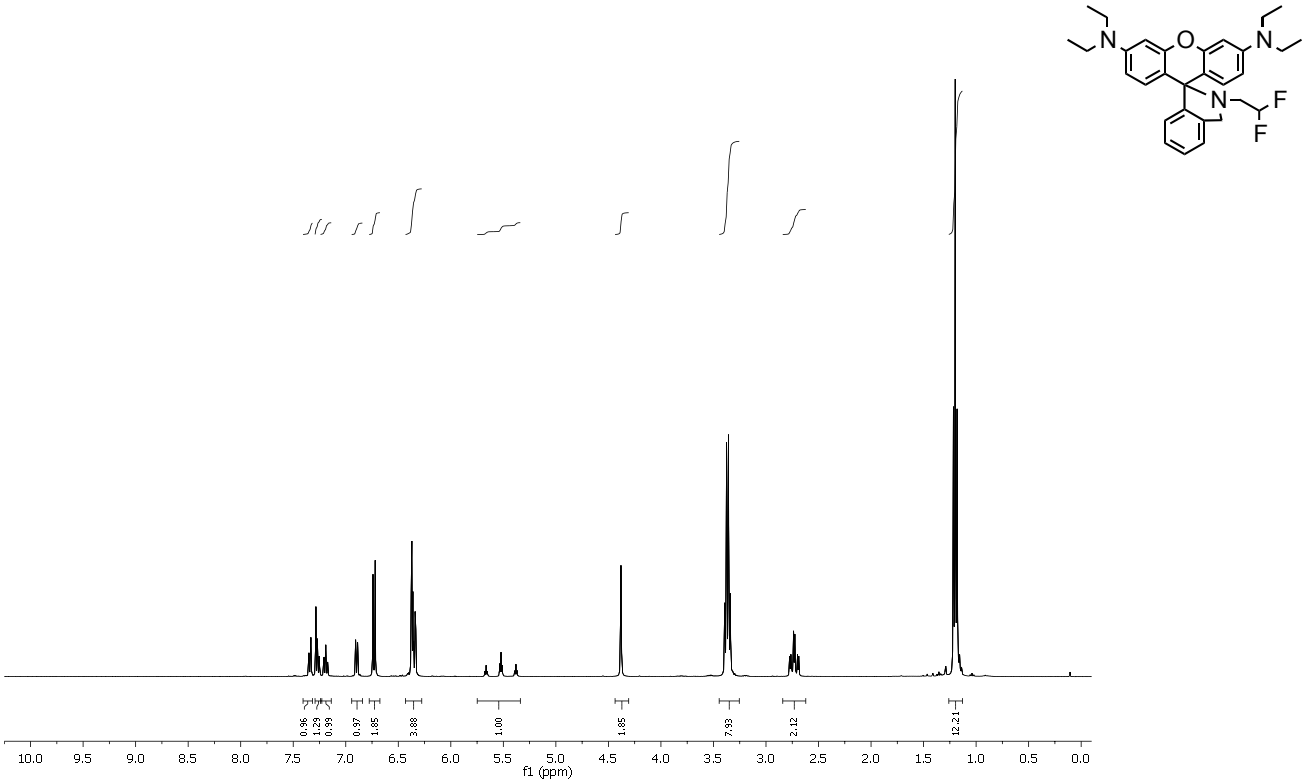


Figure S16. ^{1}H NMR (400 MHz) of a solution of **KR41** in $CDCl_3$.

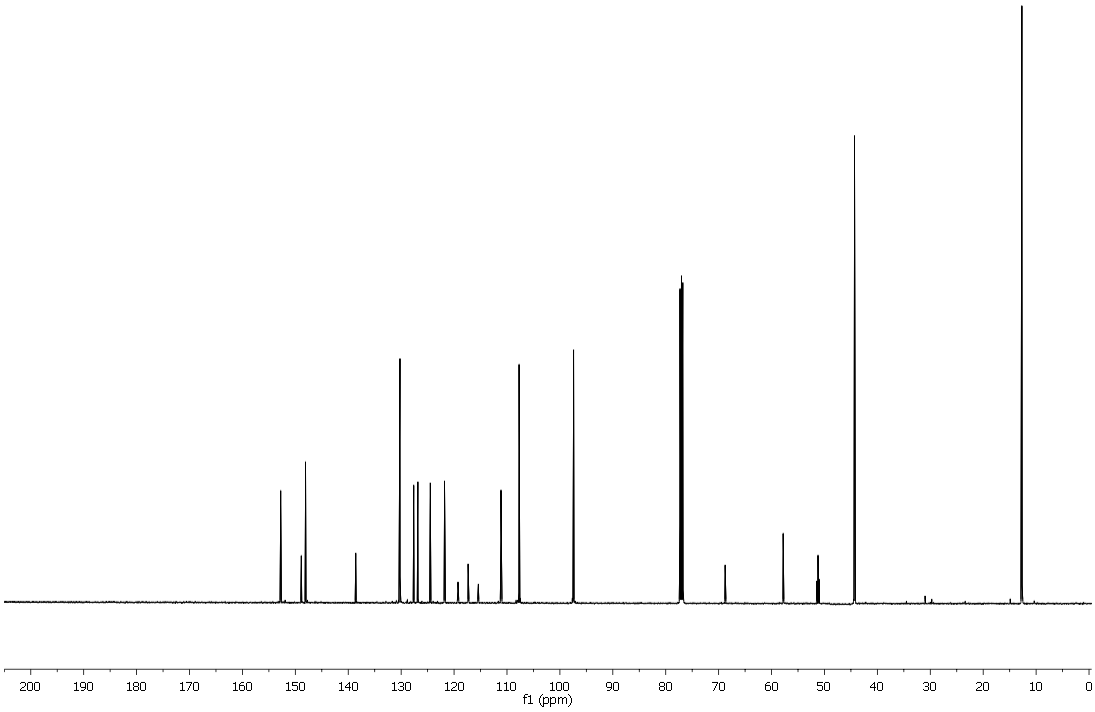


Figure S17. ^{13}C NMR (126 MHz) of a solution of **KR41** in $CDCl_3$.

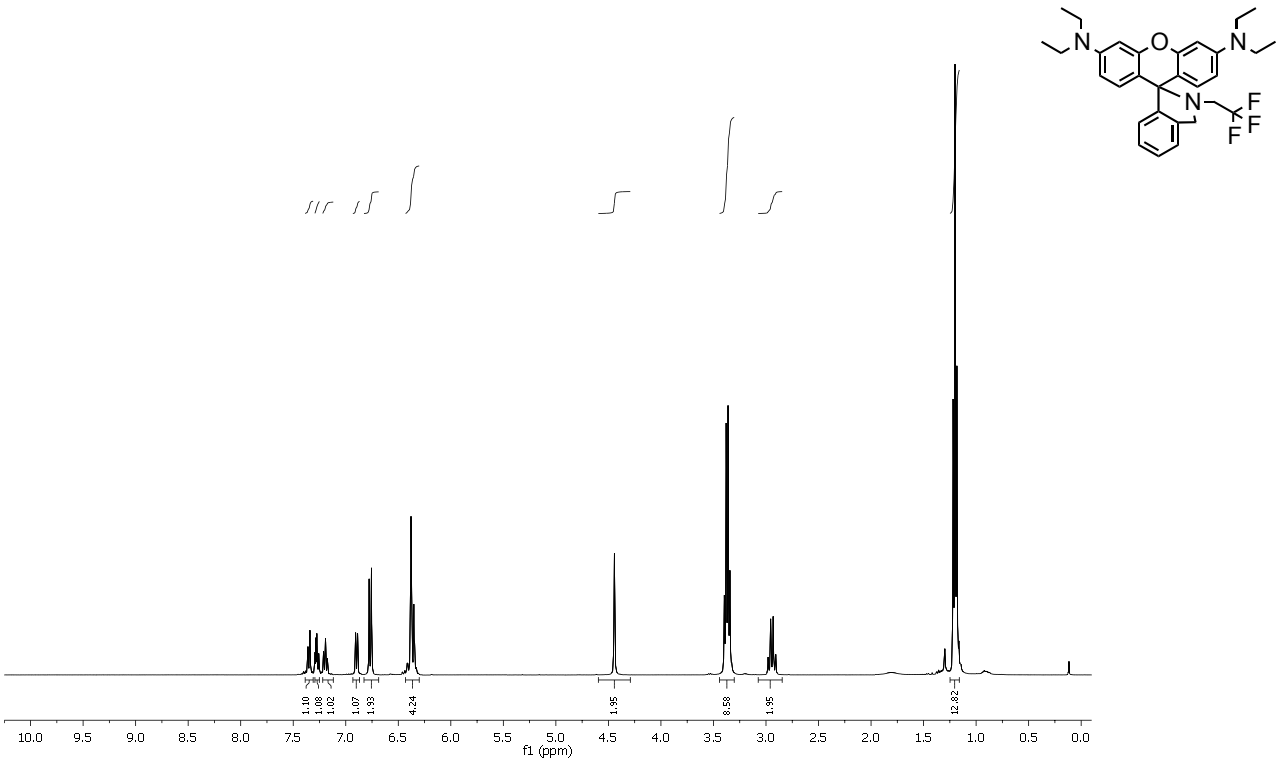


Figure S18. ^{1}H NMR (400 MHz) of a solution of **KR35** in $CDCl_3$.

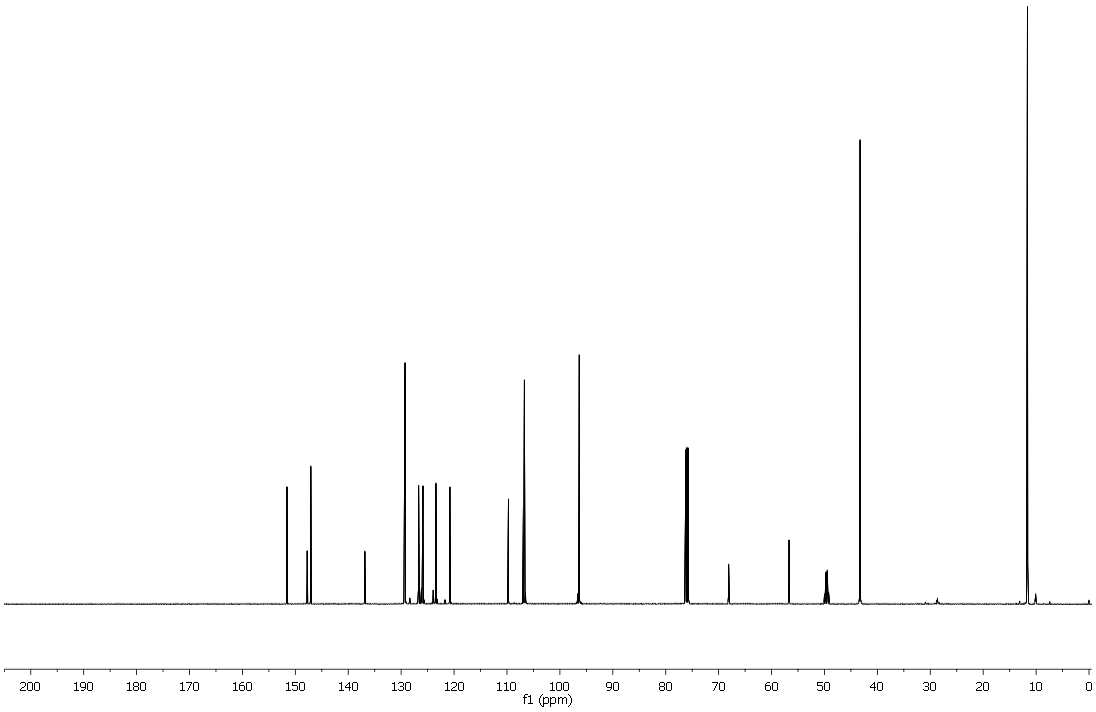


Figure S19. ^{13}C NMR (126 MHz) of a solution of **KR35** in $CDCl_3$

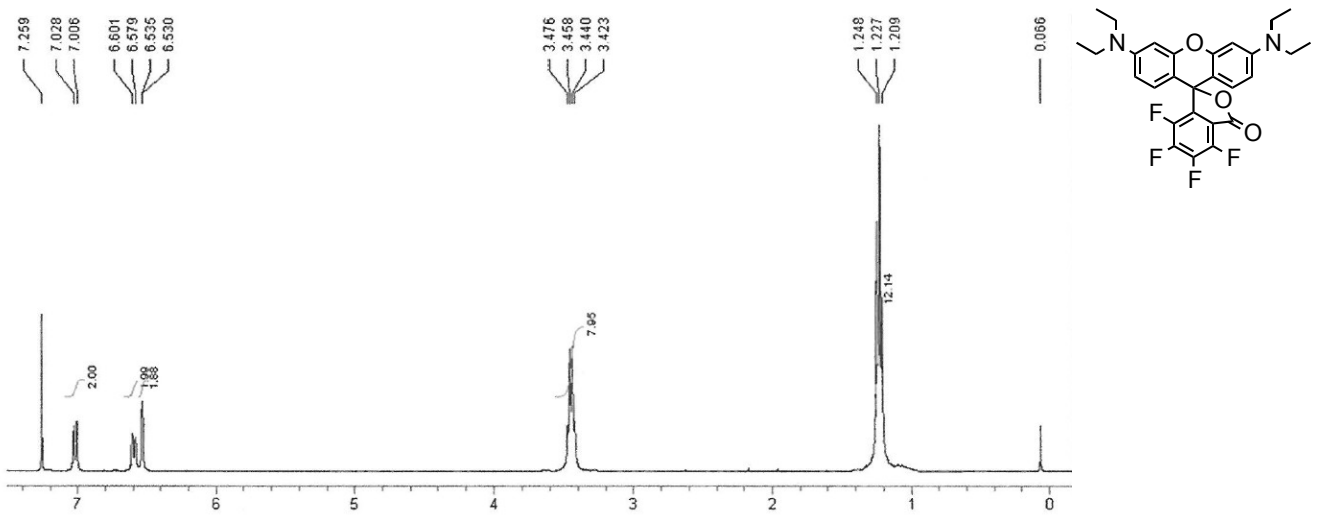


Figure S20. ^{1}H NMR (400 MHz) of a solution of **TFRB** in $CDCl_3$.

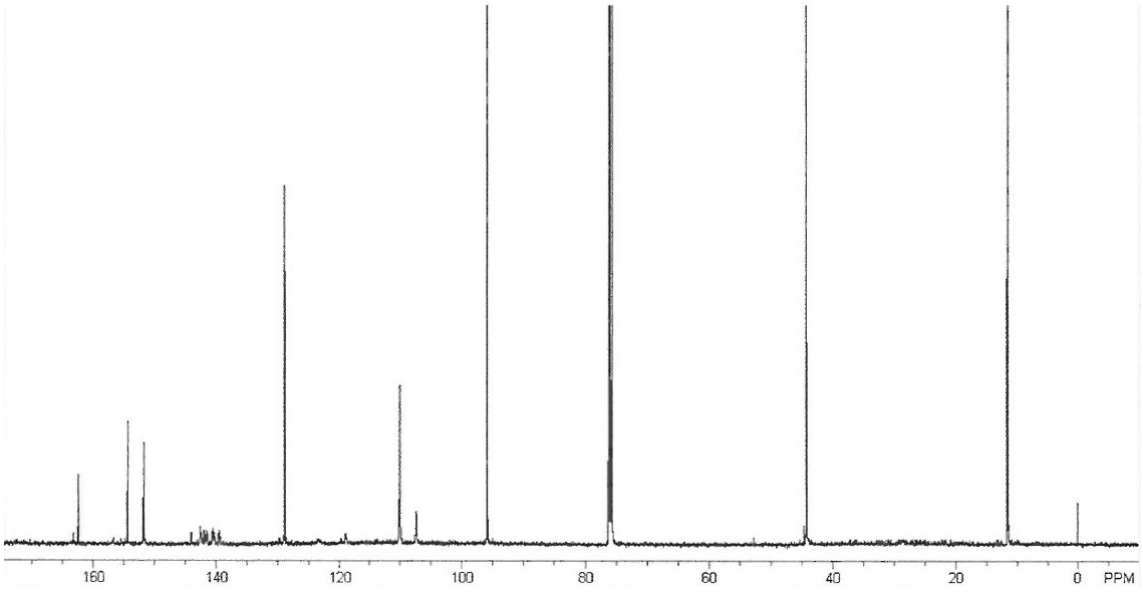


Figure S21. ^{13}C NMR (126 MHz) of a solution of **TFRB** in $CDCl_3$.

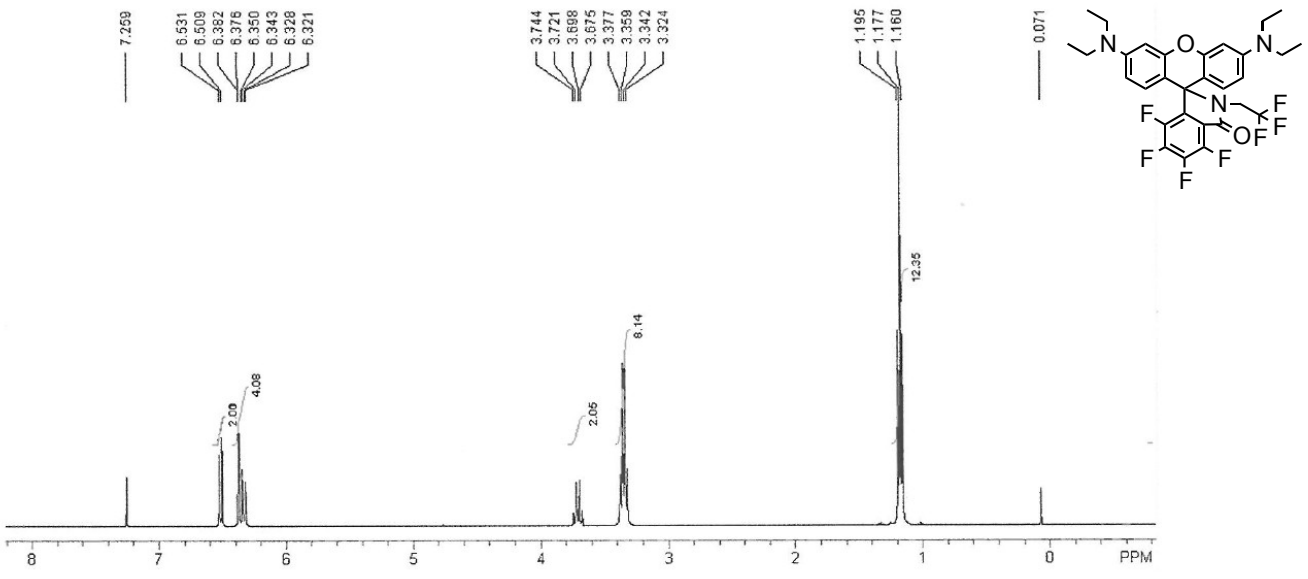


Figure S22. ^{1}H NMR (400 MHz) of a solution of **KR23-lactam** in $CDCl_3$.

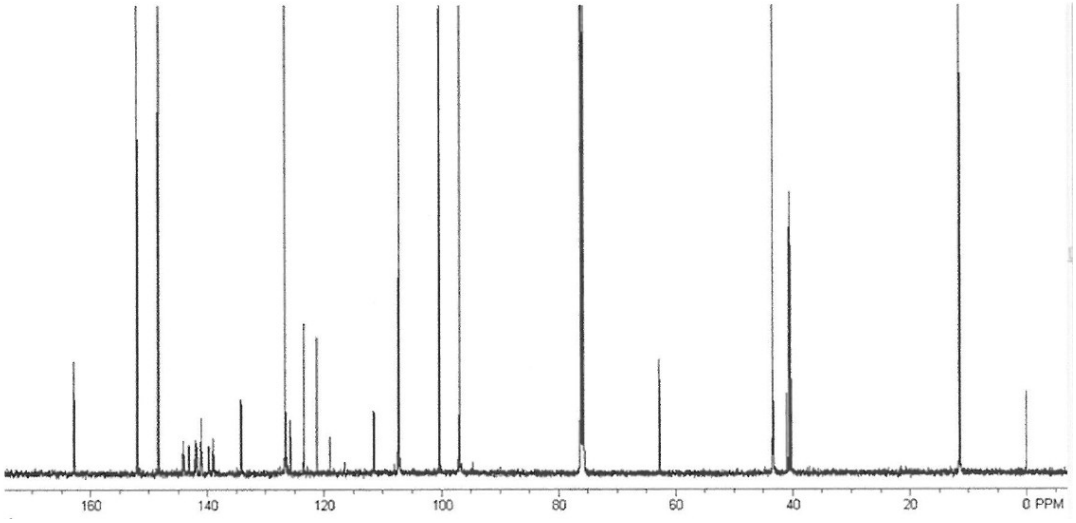


Figure S23. ^{13}C NMR (126 MHz) of a solution of **KR23-lactam** in $CDCl_3$.

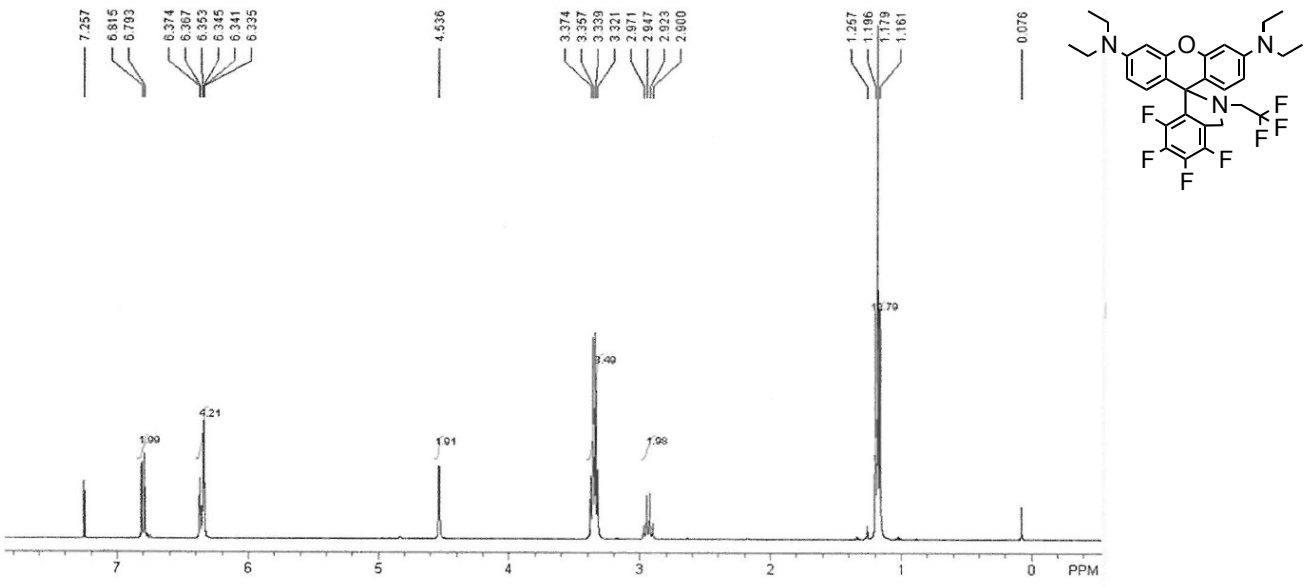


Figure S24. ^{1}H NMR (400 MHz) of a solution of **KR23** in $CDCl_3$.

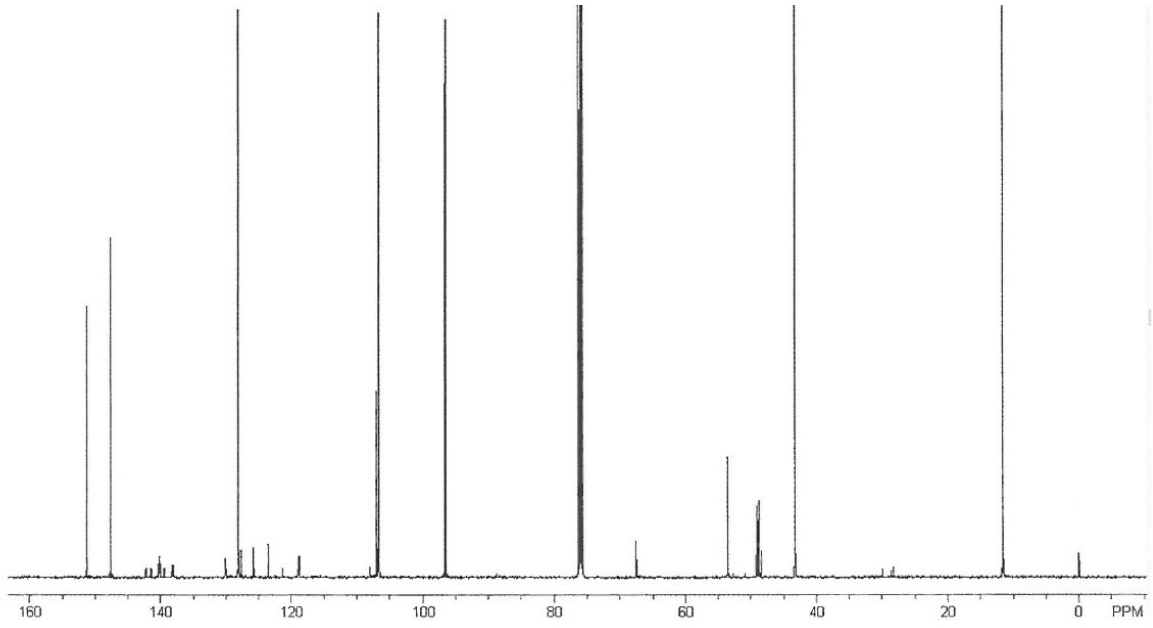


Figure S25. ^{13}C NMR (126 MHz) of a solution of **KR23** in $CDCl_3$.

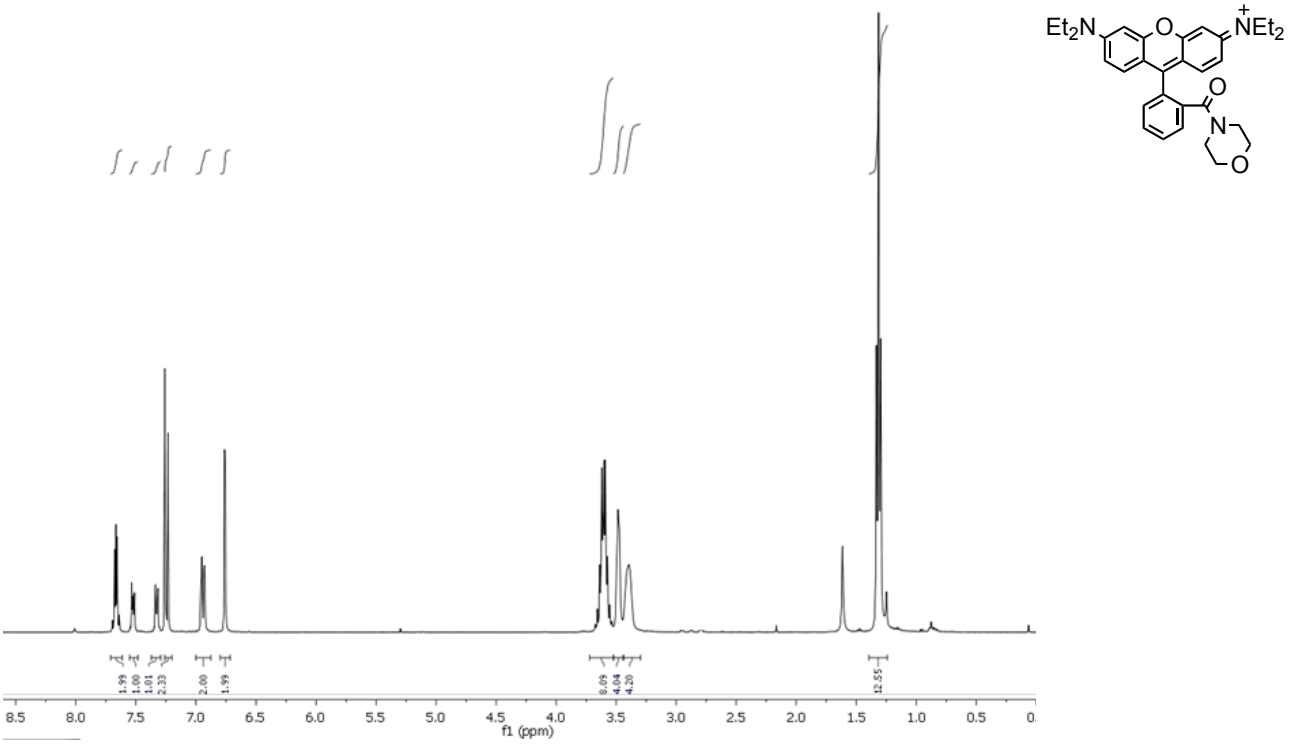


Figure S26. ^{1}H NMR (400 MHz) of a solution of **RhB amide** in $CDCl_3$.

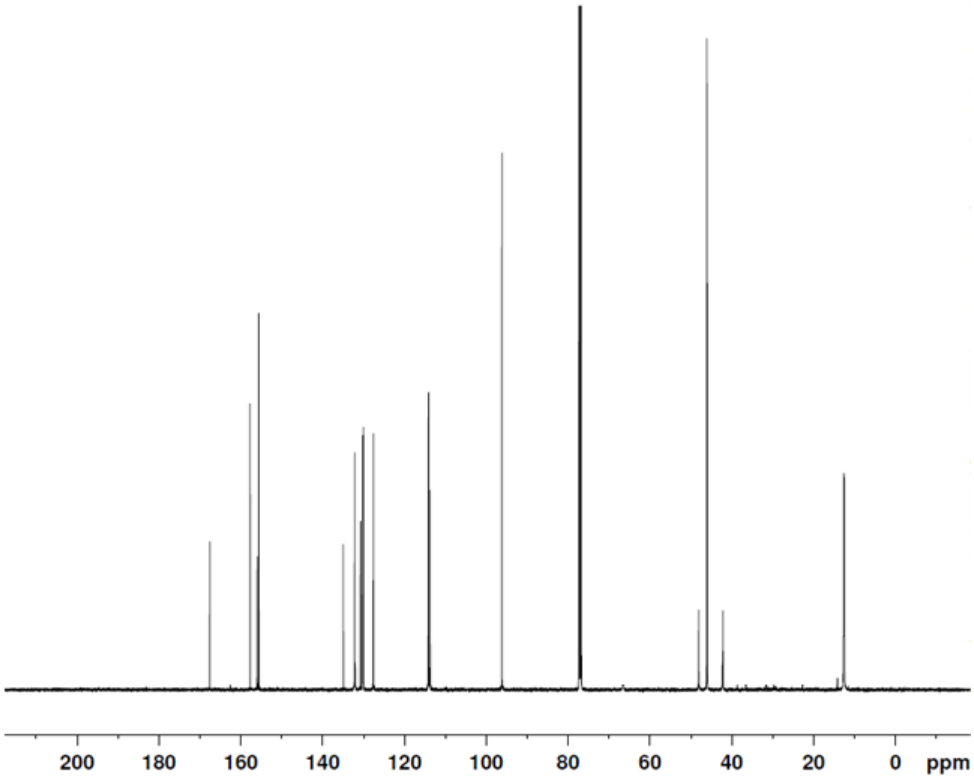


Figure S27. ^{13}C NMR (126 MHz) of a solution of **RhB amide** in $CDCl_3$.

References for the supporting information

(1) Williams, A. T., and Winfield, S. A. (1983) Relative Fluorescence Quantum Yields Using a Computer-controlled Luminescence Spectrometer. *Analyst 108*, 1067-1071.

(2) Velapoldi, R. A., and Tonnesen, H. H. (2004) Corrected Emission Spectra and Quantum Yields for a Series of Fluorescent Compounds in the Visible Spectral Region. *J. Fluorescence 14*, 465-472.

(3) Kubin, R. F., and Fletcher, A. N. (1982) Fluorescence Quantum Yields of Some Rhodamine Dyes. *J. Luminescence 27*, 455-462.

(4) Snare, M. J., Treloar, F. E., Ghiggino, K. P., and Thistlethwaite, P. J. (1982) The Photophysics of Rhodamine-B. *J. Photochem. 18*, 335-346.