Fluorescent Bisphosphonate and Carboxyphosphonate Probes: A Versatile Imaging Toolkit for Applications in Bone Biology and Biomedicine

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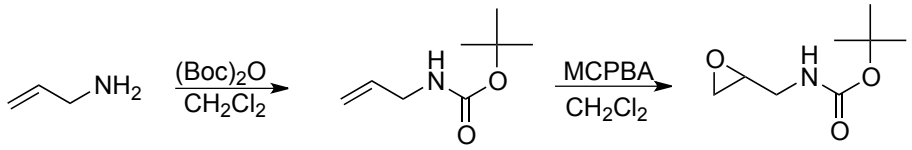


Scheme S1 Synthetic route of epoxide linker 5 (tert-butyl (oxiran-2-ylmethyl)carbamate)

The allylamine (2.3 mL, 30 mmol, 1.0 equiv) was dissolved in 10 mL dry CH_2Cl_2 and the solution was cooled in an ice bath (0 °C) by stirring for 20 min. To this cold solution, 6.54 g di-*tert*-butyl dicarbonate (30.0 mmol, 1.00 equiv) in 20 mL dry CH_2Cl_2 was added. The solution was brought to rt and stirred for 4 h. The reaction mixture was then diluted with additional CH_2Cl_2 (25 mL) and washed with 5% citric acid solution, followed by brine. The organic layer was dried over Na_2SO_4 and concentrated in vacuo, yielding 3.39 g (73%) of the *tert*-butyl allylcarbamate. ^{1}H NMR ($CDCl_3$): δ 5.85 - 5.71 (m, 1H), 5.21-5.04 (m, 2H), 4.70 (brs, 1H), 3.75 (br, 2H), 1.28 (s, 9H).

The *tert*-butyl allylcarbamate (1.0 g, 6.4 mmol, 1.0 equiv.) was dissolved in 50 mL dry CH_2Cl_2. The solution was cooled to 0 °C and kept cold during addition of 2.8 g of 3-chlorobenzenecarboperoxoic acid (MCPBA, commercially available as 77% pure; 12 mmol, 1.9 equiv.). The solution was then brought to rt and stirred overnight. The reaction mixture was then diluted with additional CH_2Cl_2 (80 mL). The solution was washed with 10% Na_2SO_3, followed by washing with saturated $NaHCO_3$ (3×), and finally by washing with water. The organic layer was dried over Na_2SO_4 and concentrated in vacuo, yielding crude epoxide **5**. According to the ^{1}H NMR spectrum, the yield was 88%. ^{1}H NMR ($CDCl_3$): δ 4.65 (brs, 1H), 3.53 - 3.42 (m, 1H), 3.21 - 3.09 (m, 1H), 3.07 – 2.98 (m, 1H), 2.72 (dd, J = 4.7, 4.0 Hz, 1H), 2.53 (dd, J = 4.7, 2.6 Hz, 1H), 1.38 (s, 9H).

Synthesis of drug-linker intermediates 4a-4c

General procedure: the parent drug (**1a-1c**) was dissolved in water and the pH adjusted to 5.7-6.2 with 1 M NaOH. Epoxide **5** was dissolved in minimal methanol (MeOH) and added to the water solution, causing a slight precipitation to occur. The precipitate disappeared on heating (40-50 °C) and as the reaction progressed. The reaction was monitored by ^{31}P NMR. After 90-95% of the desired product was obtained (^{31}P NMR), the solvent was removed in vacuo, and the resulting white powder washed with diethyl ether, filtered, and dried in a dessicator. Standard deprotection was performed with 1:1 trifluoroacetic acid (TFA):H_2O. After the reaction mixture was stirred for 3-4 h at rt, the solvent was removed in vacuo, and the resulting crystals washed with diethyl ether and MeOH to yield the drug-linker intermediates.

*Synthesis of 1-(3-amino-2-hydroxypropyl)-3-(2-hydroxy-2,2- diphosphonoethyl)pyridinium (**4a**):*

Synthesized according to the method described above with the monosodium salt of (1-hydroxy-2-pyridin-3-ylethane-1,1-diyl)bis(phosphonic acid), **1a** (288 mg, 0.94 mmol, 1.00 equiv.) in 4 mL water, and pH adjusted to 6.2 with 1 M NaOH, to which added in 164 mg of **5** (0.94 mmol, 1.00 equiv.) in minimal MeOH. The reaction mixture was stirred at 40 °C for 18.5 h, yielding 90% of **2a** by ^{31}P NMR. The solvent was removed in vacuo, and the residue was washed with diethyl ether, filtered, and dried in a desiccator. **2a**, a white solid, was then used without further purification. ^{1}H NMR (D_2O): δ 8.68 (s, 1H), 8.46 (d, J = 6.3 Hz, 1H), 8.42 (d, J = 8.1 Hz, 1H), 7.78 (dd, J = 8.2, 5.8 Hz, 1H), 4.67 – 4.62 (part. obscured by HDO, about 1H), 4.27 (dd**,** J = 13.6, 9.6 Hz, 1H), 4.13 – 3.92 (m, 1H), 3.41 – 3.10 (m, 4H), 1.31 (s, 9H). ^{31}P NMR (D_2O) δ 16.55 (d, J = 21.7s Hz, 1P), 16.33 (d, J = 21.9 Hz, 1P).

The entire sample of **2a** was dissolved in 50:50 water:TFA (v/v) and the solution was stirred at rt for 3 h; a quantitative yield of **4a** was achieved according to ^{1}H NMR. The solvent was then removed in

vacuo, and the resulting solids were washed with ether, filtered, and dried, yielding **4a** as white crystals, which were used without further purification. ^{1}H NMR (D_2O): δ 8.71 (s, 1H), 8.54 (d, *J* = 6.0 Hz, 1H), 8.44 (d, *J* = 8.1 Hz, 1H), 7.84 (dd, *J* = 8.1, 6.0 Hz, 1H), 4.74 (part. obscured by HDO, about 1H), 4.41 – 4.21 (m, 2H), 3.39 – 3.21 (m, 3H), 2.96 (dd, *J* = 13.0, 9.9 Hz, 1H). ^{31}P NMR (D_2O): δ 16.35 (d, *J* = 26.4 Hz, 1P), 16.04 (d, *J* = 27.7 Hz, 1P).

*Synthesis of 1-(3-amino-2-hydroxypropyl)-3-(2-carboxy-2-hydroxy-2- phosphonoethyl)pyridinium (**4b**):*

Synthesized according to the method described above with 2-hydroxy-2-phosphono-3-pyridin-3-ylpropanoic acid, **1b** (0.52 g, 2.10 mmol, 1.00 equiv.) in 10 mL water, and pH adjusted to 5.9 with 1 M NaOH, to which added in 0.45 g of **5** (2.57 mmol, 1.22 equiv.) in minimal MeOH. The reaction mixture was stirred at 50 °C for 6 h and then stirred at rt overnight, yielding 90% of **2b** (^{31}P NMR). The solvent was removed in vacuo, and the residue was washed with diethyl ether, filtered, and dried in a desiccator, leaving **2b**, which was then used without further purification. ^{1}H NMR (D_2O): δ 8.53 - 8.49 (brd, 1H), 8.47 (d, *J* = 6.0 Hz, 1H), 8.29 - 8.24 (m, 1H), 7.78 (dd, *J* = 8.3 Hz, 6.2 Hz, 1H), 4.64 – 4.58 (brd, 1H), 4.27 – 4.19 (m, 1H), 4.00 – 3.91 (m, 1H), 3.49 – 3.43 (m, 1H), 3.23 – 3.00 (m, 3H), 1.27 (s, 9H). ^{31}P NMR (D_2O): δ 14.97 (s, 1P).

The entire sample of **2b** was dissolved in 50:50 water:TFA (v/v). After stirring at rt for 4 h, a 100% yield of **4b** was obtained according to ^{1}H NMR. The solvent was then removed in vacuo, and the residue was washed with diethyl ether, filtered, and dried, yielding **4b** (a diastereoisomeric mixture) as white crystals, which were used without further purification. ^{1}H NMR (D_2O): δ 8.68 – 8.64 (m, 1H), 8.63 – 8.59 (m, 1H), 8.42 – 8.39 (m, 1H), 7.91 (dd, *J* = 8.0 Hz, 6.4 Hz, 1H), 4.78 – 4.72 (m, 1H), 4.46 – 4.33 (m, 1H), 4.24 – 4.14 (m, 1H), 3.59 – 3.49 (m, 1H), 3.33 – 3.21 (m, 2H), 2.95 (ddd, *J* = 13.3, 10.0, 3.6 Hz, 1H). ^{31}P NMR (D_2O): δ 12.73 – 12.51 (m, 1P).

*Synthesis of 1-(3-amino-2-hydroxypropyl)-3-(2,2-diphosphonoethyl)pyridinium (**4c**):*

Synthesized according to the method described above with (2-pyridin-3-ylethane-1,1-diyl)bis(phosphonic acid), **1c** (38.0 mg, 0.14 mmol, 1.00 equiv.) in 1 mL water and the pH adjusted to 5.7 with 1 M NaOH, to which added in 25.5 mg of **5** (0.15 mmol, 1.07 equiv.) in minimal MeOH. The reaction mixture was stirred at 40 °C overnight, and monitored by ^{31}P NMR. After 19 h, 80% of **2c** yielded. Thus, an additional 5.30 mg (0.03 mmol, 0.21 equiv.) of **5** in MeOH was added to the reaction mixture. After 42 h, 90% of the desired product was obtained. The solvent was removed in vacuo, and the resulting white powder was washed with diethyl ether, filtered, and dried, giving **2c**, which was used without further purification. ^{1}H NMR (D_2O): δ 8.69 (s, 1H), 8.49 (d, *J* = 6.1 Hz, 1H), 8.42 (d, *J* = 8.3 Hz, 1H), 7.84 (dd, *J* = 8.1 Hz, 6.1 Hz, 1H), 4.66 - 4.61 (m, 1H), 4.27 (dd, *J* = 13.5 Hz, 9.6 Hz, 1H), 4.00 – 3.94 (m, 1H), 3.30 – 3.10 (m, 4H), 2.15 (tt, *J* = 21.0 Hz, 7.2 Hz, 1H), 1.26 (s, 9H). ^{31}P NMR (D_2O): δ 17.25 (s, 2P).

The entire sample of **2c** was dissolved in 50:50 water:TFA (v/v). After stirring at rt for 4 h, a 100% yield of **4c** was obtained according to ^{1}H NMR. The solvent was removed in vacuo, and the residue was washed with diethyl ether and methanol, filtered, and dried, yielding **4c** as white crystals, which was then used without further purification. ^{1}H NMR (D_2O): δ 8.73 (s, 1H), 8.54 (d, *J* = 6.1 Hz, 1H), 8.45 (d, *J* = 8.2 Hz, 1H), 7.89 (dd, *J* = 8.1 Hz, 6.1 Hz, 1H), 4.76 – 4.70 (m, 1H), 4.37 (dd, *J* = 13.4 Hz, 9.3 Hz, 1H), 4.26 (t, *J* = 9.6 Hz, 1H), 3.37 – 3.13 (m, 3H), 2.98 (dd, *J* = 13.1, 9.8 Hz, 1H), 2.28 (tt, *J* = 21.4, 7.2 Hz, 1H). ^{31}P NMR (D_2O): δ 17.35 (s, 2P).

Preparation of 7a-7f

The following HPLC methods were used to purify **7a**-**7f**:

Method A: Dynamax C18 (21.4 mm x 25 cm, 5 μm, 100 Å pore size) column, flow rate 8.0 mL/min, UV detection at 260 nm, gradient as follows: linearly increasing from 10% MeOH 0.1 M TEAAc (pH

5.0 – 5.5) or TEAC (pH 7.0 – 7.8, buffer A) to 40% of 75% MeOH 0.1 M TEAAc (pH 5.0 – 5.5) or TEAC (pH 7.0 – 7.8, buffer B) in 12 min, then increasing to 70% of buffer B from 12 - 100 min;

Method B: Dynamax C18 (21.4 mm x 25 cm, 5 μm, 100 Å pore size) column, flow rate 8.0 mL/min, UV detection at 260 nm, isocratic elution with 20% MeOH 0.1 M TEAC (pH 7.0 – 7.8, buffer A) for 12 min, linearly increasing to 100% of 70% MeOH 0.1 M TEAC (pH 7.0 – 7.8, buffer B) from 12 - 22 min;

Method C: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 µm, 80 Å pore size), flow rate 6.0 mL/min, UV-vis detection at 260 nm and 568 nm, isocratic elution of 20% MeOH in 0.1 M TEAC (pH 7.0 – 7.8, buffer A) for 5 min, linearly increasing to 100% of 75% MeOH in 0.1 M TEAC (pH 7.0 – 7.8, buffer B) in 1 min;

Method D: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 µm, 80 Å pore size), flow rate 4.0 mL/min, UV-vis detection at 260 nm and 568 nm, isocratic elution of 20% MeOH in 0.1 M TEAAc (pH 5.0 – 5.5, buffer A) for 5 min, linearly increasing to 100% of 75% MeOH in 0.1 M TEAAc (pH 5.0 – 5.5, buffer B) in 1 min;

Method E: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 µm, 80 Å pore size), flow rate 4 mL/min, UV-vis detection at 260 nm and 568 nm, isocratic elution of 20 % MeOH in 0.1 M TEAC (pH 7.0 - 7.8, buffer A) for 5 min, linearly increasing to 100% of 70% MeOH in 0.1 M TEAC (pH 7.0 - 7.8, buffer B) in 1 min;

Method F: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 µm, 80 Å pore size), flow rate 4.0 mL/min, UV-vis detection at 260 nm and 576 nm, isocratic elution of 20% MeOH in 0.1 M TEAAc buffer (pH 5.0 - 5.5, buffer A) for 5 min, linearly increasing to 100% of 70% MeOH in 0.1 M TEAAc buffer (pH 5.0 - 5.5 buffer B) in 5 min;

Method G: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 µm, 80 Å pore size), flow rate 4.0

mL/min, UV-vis detection at 260 nm and 576 nm, isocratic elution of 10% MeOH in 0.1 M TEAC buffer (pH 7.0 - 7.8, buffer A) for 5 min, linearly increasing to 100% of 70% MeOH in 0.1 M TEAC buffer (pH 7.0 - 7.8, buffer B) in 5 min;

Method H: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 μm, 80 Å pore size), flow rate 4.0 mL/min, UV-vis detection at 260 nm (**7a6, 7b4**) or 230 nm (**7d3**) and 598 nm, isocratic elution of 20% MeOH in 0.1 M TEAAc buffer (pH 5.0 – 5.5, buffer A) for 5 min, linearly increasing to 40% of 70% MeOH in 0.1 M TEAAc buffer (pH 5.0 – 5.5, buffer B) in 20 min;

Method I: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 μm, 80 Å pore size), flow rate 4.0 mL/min, UV-vis detection at 230 nm (**7d1 - 7d2**) or 280 nm (**7e1, 7e2, 7f1, 7f2**) and 492 nm, gradient as follows: linearly increasing from 10% MeOH in 0.1 M TEAC (pH 7.0 – 7.8, buffer A) to 40% of 75% MeOH in 0.1 M TEAC (pH 7.0 – 7.8, buffer B) in 25 min, then increasing to 70% of buffer B from 25 - 100 min;

Method J: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 μm, 80 Å pore size), flow rate 4.0 mL/min, UV-vis detection at 230 nm and 598 nm, isocratic elution of 20% MeOH in 0.1 M TEAAc buffer (pH 5.0 – 5.5, buffer A) for 7 min, linearly increasing to 100% of 70% MeOH in 0.1 M TEAAc buffer (pH 5.0 – 5.5, buffer B) from 7 – 25 min;

Method K: Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 μm, 80 Å pore size), flow rate 4.0 mL/min, UV-vis detection 230 nm and 598 nm, isocratic elution of 20% MeOH in 0.1 M TEAAc buffer (pH 5.0 – 5.5, buffer A) for 5 min, linearly increasing to 40% of 70% MeOH in 0.1 M TEAAc buffer (pH 5.0 – 5.5, buffer B) from 5 – 15 min; maintained at 40% of buffer B from 15 – 30min, finally increase to 100% of buffer B from 30 – 35 min.

5(6)-FAM-RIS (**7a1**)**, 5-FAM-RIS** (**7a2**): 1-(3-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-3-(2-hydroxy-2,2-diphosphonoethyl)pyridin-1-ium; **6-FAM-RIS** (**7a3**):

1-(3-(4-carboxy-3-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-3-(2-hydroxy-2,2-diphosphonoethyl)pyridin-1-ium):

Synthesized according to the method above from 86.5 mg of **4a** (as TFA^-, Na^+ salt, 0.18 mmol, 1.7 equiv.) in 2 mL of H_2O and pH adjusted to 8.3 with Na_2CO_3 (s), to which 50.0 mg of 5(6)-FAM, SE (0.11 mmol, 1.00 equiv.) in 600 μL anhydrous DMF was added; the pH of reaction solution was further adjusted to pH 8.3 to dissolve precipitates, and the reaction mixture was then stirred at rt for overnight. After TLC purification (100% MeOH as eluent), the product was purified by HPLC according to Method A with TEAAc buffers. Peaks eluting from 25-75 min were collected. During evaporation of the buffer solution, product precipitated from the solution. Consequently, a second HPLC purification was performed according to Method A but eluting with TEAC buffers (pH 7.5). Obtained 29.0 mg, 46.8% yield (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.76 – 8.62 (m, 1H), 8.54 – 8.48 (m, 1H), 8.42 (dt, J = 17.6, 7.8 Hz, 1H), 8.04 (s 0.6 H), 7.92 – 7.64 (m, 2H), 7.45 (s, 0.4 H), 7.13 (s, 1H), 6.93 (ddd, J = 9.1, 4.5, 1.9 Hz, 2H), 6.45 – 6.38 (m, 4H), 4.82 – 4.70 (m, 1H), 4.44 – 4.28 (m, 1H), 4.28 – 4.12 (m, 1H), 3.65 – 3.55 (m, 1H), 3.56 – 3.44 (m, 1H), 3.44 – 3.19 (m, 2H). ^{31}P NMR (D_2O): δ 16.36 (s, 2P).

HPLC Separation of 5- and 6-FAM-RIS (**7a2** and **7a3**): Synthesized according to method described for **7a1**. Under HPLC conditions described as Method A, 6-FAMRIS and 5-FAMRIS elute at very different retention times, 27 and 44 min (the retention time has ±1.5 min difference between different runs), respectively. Each isomer was collected separately and then concentrated in vacuo to remove buffer. Compound **7a2** and **7a3** were also directly synthesized from 5-FAM, SE and 6-FAM, SE according to the method described above. Detailed NMR descriptions of **7a2** and **7a3** can be found in ref. 3.

5(6)-FAM-RISPC (**7b1**, also known as **5(6)-FAM-3-PEHPC**; including **5-FAM-RISPC**: 3-(2-carboxy-2-hydroxy-2-phosphonoethyl)-1-(3-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-

2-hydroxypropyl)pyridin-1-ium; **6-FAM-RISPC**: 3-(2-carboxy-2-hydroxy-2-phosphonoethyl)-1-(3-(4-carboxy-3-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)pyridin-1-ium):

Synthesized according to the method described above from 94.8 mg of intermediate **4b** (0.21 mmol, 3.5 equiv.) in 1 mL of water and pH adjusted to 8.3 with Na_2CO_3 (s), to which added in 30 mg of 5(6)-FAM, SE (0.06 mmol, 1.0 equiv.) in 200 μL anhydrous DMF. The pH of the reaction solution was further adjusted to pH 8.4 to dissolve precipitates, and the reaction mixture was then stirred at rt for overnight. After TLC purification (100% MeOH as eluent), the mixture was purified by HPLC according to Method B. Peaks eluting at 21-25 min were collected together as **7b1**. Obtained 23.2 mg, 53.9% yield (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.66 – 8.44 (m, 2H), 8.29 (brd, 1H), 8.05 (s, 0.6 H), 7.89 – 7.68 (m, 2H), 7.45 (s, 0.4 H), 7.13 (d, J = 8.0 Hz, 1H), 6.93 (d, J = 9.2 Hz, 2H), 6.50 – 6.35 (m, 4H), 4.43 – 4.25 (m, 2H), 3.78 – 3.55 (m, 1H), 3.54 – 3.41 (m, 2H), 3.41 – 3.23 (m, 1H), 2.87 (part. obscured by triethylamine, about 1H). ^{31}P NMR (D_2O): δ 15.15 (brs, 1P). HRMS (positive ion MALDI): calcd 679.1324 *m/z*; found $[M]^+$= 679.1321 *m/z*.

5(6)-FAM-dRIS (**7c1**, including **5-FAM-dRIS**: 1-(3-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-3-(2,2-diphosphonoethyl)pyridin-1-ium; **6-FAM-dRIS**: 1-(3-(4-carboxy-3-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-3-(2,2-diphosphonoethyl)pyridin-1-ium):

Synthesized according to the method described above from 53 mg of intermediate **4c** (0.1 mmol, 2.5 equiv.) in 1 mL HPLC water and pH adjusted to 8.3 with Na_2CO_3 (s), to which added in 18.0 mg of 5(6)-FAM, SE (0.04 mmol, 1.00 equiv.) in 100 μL anhydrous DMF. The pH of reaction solution was further adjusted to pH 8.4 to dissolve precipitates, and the reaction mixture was then stirred at rt for overnight. After TLC purification, the mixture was purified according to Method B. Peaks eluting from 27-45 min were collected as **7c1**. Obtained 9.4 mg, 36% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.73

– 8.69 (m, 1H), 8.50 (d, *J* = 6.1 Hz, 1H), 8.47 – 8.36 (m, 1H), 8.06 (d, *J* = 1.9 Hz, 0.6 H), 7.94 – 7.68 (m, 2H), 7.53 (s, 0.4 H), 7.23 (d, *J* = 8.0 Hz, 1H), 6.99 (dd, *J* = 9.7, 2.4 Hz, 2H), 6.50 – 6.40 (m, 4H), 4.82 – 4.72 (m, 1H), 4.42 – 4.29 (m, 1H), 4.29 – 4.09 (m, 1H), 3.62 (dd, *J* = 14.1, 4.5 Hz, 1H), 3.23 – 3.11 (obscured by solvent peak, about 1H), 3.58 – 3.32 (m, 2H), 2.14 – 1.87 (m, 1H). ^{31}P NMR (D_2O): δ 17.17 (brs). HRMS (positive ion MALDI): calcd 699.1139 *m/z*; found $[M]^+$= 699.1137 *m/z*.

5(6)-RhR-RIS (**7a4**, 1-{3-[6-({4-[6-(diethylamino)-3-(diethylimino)-3H-xanthen-9-yl]-3-sulfobenzene}sulfonamido)hexanamido]-2-hydroxypropyl}-3-(2-hydroxy-2,2-diphosphonoethyl)pyridinium):

Synthesized according to the method described above from 11.2 mg of compound **4a** (0.032 mmol, 4.9 equiv.) in 0.5 mL H_2O and pH adjusted to 9.0 with Na_2CO_3 (s), to which 5 mg of RhR-X, SE (0.0065 mmol, 1 equiv.) in 250 μL DMF was added. After TLC purification, the mixture was purified by HPLC according to Method C. Peak eluting between 12.8 – 18 minutes (the retention time has ±1.0 min difference between different runs) were collected. Obtained 0.2 mg, 3% yield (as a triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.66 (s, 1H), 8.49 – 8.31 (m, 3H), 8.09 (s, 1H), 7.70 (s, 1H), 7.59 – 7.33 (m, 1H), 7.01 – 6.57 (m, 7H), 4.22 – 3.89 (m, 3H), 3.55 – 3.23 (m, obscured by solvent peak and TEA peak, around 12H), 3.02 – 2.96 (m, obscured by TEA peak, around 3H), 2.19 – 2.01 (m, 2H), 1.47 – 1.24 (m, obscured by TEA peak, around 5H), 1.10 (obscured by TEA peak, about 12H). ^{31}P NMR (D_2O): δ 16.76 (s, 2P). HRMS (positive ion MALDI): calcd 1011.2913 *m/z*, found $[M-H]^+$ = 1010.2866 *m/z*.

5(6)-RhR-RISPC (**7b2**, 3-(2-carboxy-2-hydroxy-2-phosphonoethyl)-1-{3-[6-({4-[6-(diethylamino)-3-(diethylimino)-3H-xanthen-9-yl]-3-sulfobenzene}sulfonamido)hexanamido]-2-hydroxypropyl}pyridinium, also known as **5(6)-RhR-3-PEHPC**):

Synthesized according to the method described above from 10.9 mg of compound **4b** (0.04 mmol, 3 equiv.) in 0.5 mL of H_2O and pH adjusted to 8.3 with Na_2CO_3 (s), to which 5 mg of 5(6)-RhR-X, SE in 500

μL DMF was added. After TLC purification, the solution was then purified by HPLC according to Method D. Peak eluting at 13 min (the retention time has ±1.0 min difference between different runs) was collected as **7b2**. Obtained 2.1 mg, 33% yield (triethylammonium bicarbonate salt). ^{1}H NMR (400 MHz, D_2O): δ 8.51 (s, 1H), 8.43 (d, *J* = 10.1 Hz, 2H), 8.29 (s, 1H), 8.09 (d, *J* = 8.0 Hz, 1H), 7.83 – 7.66 (m, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 6.87 – 6.70 (m, 4H), 6.65 (s, 2H), 4.56 – 4.43 (m, 1H), 4.16 (d, *J* = 14.4 Hz, 1H), 3.95 (s, 1H), 3.48 (dd, *J* = 23.6, 8.0 Hz, 8H), 3.28 – 3.12 (m, obscured by solvent, about 4H), 2.93 (td, *J* = 17.6, 16.8, 8.9 Hz, 3H), 2.10 (t, *J* = 7.6 Hz, 2H), 1.47 – 1.21 (m, 5H), 1.09 (obscured by TEA peak, about 12H). ^{31}P NMR (D_2O): 15.2 (s). HRMS (positive ion MALDI): calcd 975.3148 *m/z*, found $[M-H]^+$ = 974.3118 *m/z*.

5(6)-RhR-dRIS (**7c2**, 1-{3-[6-({4-[6-(diethylamino)-3-(diethylimino)-3H-xanthen-9-yl]-3-sulfobenzene}sulfonamido)hexanamido]-2-hydroxypropyl}-3-(2,2-diphosphonoethyl)pyridin-1-ium):

Synthesized according to the method described above from 9.4 mg of compound **4c** (0.02 mmol, 3.3 equiv.) in 0.6 mL H_2O and pH adjusted to 8.3 with Na_2CO_3 (s), to which 5 mg of 5(6)-RhR-X, SE (0.0065 mmol, 1 equiv.) in 0.45 mL of DMF was added. Precipitation was observed. The reaction mixture was stirred for 2 h, then evaporated to dryness. The resulting solids were extracted with acetone (3 x 1 mL, in order to remove partially unconjugated dye. The remaining precipitate was dissolved in ~2 mL H_2O and purified by TLC as described above, followed by HPLC purification (Method E). A broad peak eluting between 13 – 17.3 min (the retention time has ±1.0 min difference between different runs) was collected. Obtained 2.75 mg, 42.5% yield (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.67 (d, *J* = 2.2 Hz, 1H), 8.50 – 8.36 (m, 3H), 8.10 (t, *J* = 6.9 Hz, 1H), 7.79 (dd, *J* = 8.4, 6.4 Hz, 1H), 7.42 (t, *J* = 8.6 Hz, 1H), 6.86 – 6.68 (m, 4H), 6.68 – 6.57 (m, 2H), 4.62 – 4.50 (m, 1H), 4.19 (dd, *J* = 13.3, 9.7 Hz, 1H), 4.10 – 3.94 (m, 1H), 3.45 (p, *J* = 7.0 Hz, 8H), 3.34 – 3.13 (m, 4H), 2.97 (q, *J* = 6.7 Hz, 3H), 2.36 – 2.01 (m, 3H), 1.53 – 1.23 (m, 5H), 1.10 (td, *J* = 7.0, 3.2 Hz, 12H). ^{31}P NMR (D_2O): 17.29 (s). HRMS (positive ion

MALDI): calcd 995.2695 *m/z*, found $[M\text{-}H]^+$ = 994.2872 *m/z*.

5(6)-ROX-RIS (**7a5**, including **5-ROX-RIS**: 16-[2-carboxy-4-({2-hydroxy-3-[4-(2-hydroxy-2,2-diphosphonoethyl)pyridin-1-ium-1-yl]propyl}carbamoyl)phenyl]-3-oxa-9λ^5,23-diazaheptacyclo[17.7.1.1^{5,9}.0^{2,17}.0^{4,15}.0^{23,27}.0^{13,28}]octacosa-1(27),2(17),4,9(28),13,15,18-heptaen-9-ylium):

Synthesized according to the method described above from 23.6 mg of compound **4a** (0.047 mmol, 3 equiv.) in 0.8 mL of $H_2O/NaHCO_3$ (pH 9.0), to which 10 mg of 5(6)-ROX, SE (0.016 mmol, 1 equiv.) in 200 μL anhydrous DMF was added, and the solution stirred overnight. The solvent was concentrated under vacuo, and the resulting purple residue was dissolved in 20% MeOH in 0.1 M TEAAc buffer (pH 5.3) and purified by HPLC (Method F). Peaks eluting at 17.0 min (the retention time has ±1.0 min difference between different runs) were collected as **7a5**. Obtained 7.4 mg, 54.0% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.74 (s, 1H), 8.55 (d, *J* = 6.0 Hz, 1H), 8.43 (d, *J* = 8.1 Hz, 1H), 8.07 (s, 1H), 7.81 (t, *J* = 7.2 Hz, 1H), 7.63 (d, *J* = 7.6 Hz, 1H), 6.77 (d, *J* = 7.9 Hz, 1H), 6.52 (s, 2H), 4.35 – 4.21 (m, 2H), 3.57 – 3.48 (m, 2H), 3.37 – 3.16 (m, 13H), 2.70 – 2.62 (m, 2H), 2.47 – 2.27 (m, 5H), 1.79 – 1.53 (m, 7H). ^{31}P NMR (D_2O): 16.36 (s). HRMS (positive ion MALDI): calcd 873.2660 *m/z*, found $[M\text{-}H]^+$ = 873.2647 *m/z*.

5(6)-ROX-RISPC (**7b3**, also known as **5(6)-ROX-3-PEHPC**, **5-ROX-RISPC**: 16-[2-carboxy-4-({3-[4-(2-carboxy-2-hydroxy-2-phosphonoethyl)pyridin-1-ium-1-yl]-2-hydroxypropyl}carbamoyl)phenyl]-3-oxa-9λ^5,23-diazaheptacyclo[17.7.1.1^{5,9}.0^{2,17}.0^{4,15}.0^{23,27}.0^{13,28}]octacosa-1(27),2(17),4,9(28),13,15,18-heptaen-9-ylium):

Synthesized according to the method described above from 54.3 mg of **4b** (0.119 mmol, 3 equiv.) in 1.6 mL of $H_2O/NaHCO_3$ (pH 9.0) and 25 mg of 5(6)-ROX, SE (0.04 mmol, 1 equiv.) in 1 mL anhydrous DMF, and the solution was stirred overnight. The solvent was concentrated under vacuo, and the resulting

purple residue was dissolved in 10% MeOH in 0.1 M TEAC buffer (pH 7.0) and purified by HPLC (Method G). Peaks eluting at 21.9 min (the retention time has ±1.0 min difference between different runs) were collected as **7b3**. Obtained 9.4 mg, 35.0% yield (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.63 (s, 1H), 8.54 (d, *J* = 6.2 Hz, 1H), 8.31 (s, 1H), 8.03 (d, *J* = 1.9 Hz, 1H), 7.86 – 7.78 (m, 1H), 7.75 (d, *J* = 8.0 Hz, 1H), 7.00 (s, 1H), 6.59 (s, 2H), 4.45 – 4.33 (m, 1H), 4.31 – 4.18 (m, 1H), 3.67 – 3.48 (m, 2H), 3.36 – 3.14 (m, 13H), 2.83 – 2.72 (m, 2H), 2.51 – 2.35 (m, 5H), 1.81 – 1.62 (m, 7H). ^{31}P NMR (D_2O): 14.34 (s). HRMS (negative ion MALDI): calcd 835.2750 *m/z*, found $[M-3H]^-$ = 835.2733 *m/z*.

AF647-RIS (**7a6**, 2-(5-(3-(6-((2-hydroxy-3-(3-(2-hydroxy-2,2-diphosphonoethyl)pyridin-1-ium-1-yl)propyl)amino)-6-oxohexyl)-3-methyl-5-sulfo-1-(3-sulfopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)-3*H*-indol-1-ium):

Synthesized according to the method described above from 25.9 mg of compound **4a** (0.05 mmol, 10 equiv.) in 1 mL of $H_2O/NaHCO_3$ (pH 8.3) and 5 mg of AF647, SE (0.005 mmol, 1 equiv.) in 250 μL anhydrous DMF, and the solution was stirred at rt overnight. The solvent was concentrated under vacuo, and the resulting blue residue was dissolved in 20% MeOH in 0.1 M TEAAc buffer (pH 5.3) and purified by HPLC (Method H). Peaks eluting at 19.8 min (the retention time has ±1.0 min difference between different runs) were collected as **7a6**. Obtained 4.8 mg, 76.7% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.61 (s, 1H), 8.45 (d, *J* = 6.2 Hz, 1H), 8.39 (d, *J* = 8.1 Hz, 1H), 8.00 (t, *J* = 13 Hz, 2H), 7.80 – 7.67 (m, 5H), 7.31 – 7.20 (m, 2H), 6.55 (t, *J* = 12.6 Hz, 1H), 6.37 – 6.23 (m, 2H), 4.70 – 4.60 (obscured by HDO, about 1H), 4.14 – 3.98 (m, 4H), 2.92 – 2.80 (m, 6H), 2.14 – 2.10 (m, 5H), 1.95 – 1.92 (m, 2H), 1.57 – 1.53 (m, 9H), 1.48 – 1.45 (m, 1H), 1.29 – 1.27 (m, 2H), 1.00 – 0.95 (m, 3H), 0.82 – 0.79 (m, 3H), 0.45 – 0.43 (m, 2H). ^{31}P NMR (D_2O): δ 16.50 (d, *J* = 26.9 Hz, 1P), 16.30 (d, *J* = 29.0 Hz, 1P). HRMS (positive ion MALDI): calcd 1198.2410 *m/z*, found $[M-H]^+$ = 1197.2358 *m/z*.

AF647-RISPC (**7b4**, 2-(5-(3-(6-((3-(3-(2-carboxy-2-hydroxy-2-phosphonoethyl)pyridin-1-ium-1-

yl)-2-hydroxypropyl)amino)-6-oxohexyl)-3-methyl-5-sulfo-1-(3-sulfopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)-3*H*-indol-1-ium**,** also known as **AF647-3-PEHPC**):

Synthesized according to the method described above from 22.5 mg of compound **4b** (0.05 mmol, 10 equiv.) in 1 mL of H_2O and pH adjusted to 8.3 with Na_2CO_3 (s), to which 5 mg of AF647, SE (0.005 mmol, 1 equiv.) in 300 μL anhydrous DMF was added. The solution was stirred at rt overnight. The solvent was concentrated under vacuo, and the resulting blue residue was dissolved in 20% MeOH in 0.1 M TEAAc buffer (pH 5.3) and purified by HPLC (Method H). Peaks eluting at 18.8 min (the retention time has ±1.0 min difference between different runs) were collected as **7b4**. Obtained 5.3 mg, 87.2% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.47 (m, 2H), 8.25 (d, *J* = 7.7 Hz, 1H), 7.94 (t, *J* = 13.1 Hz, 2H), 7.81 – 7.73 (m, 1H), 7.73 – 7.63 (m, 4H), 7.26 (t, *J* = 8.0 Hz, 2H), 6.52 (t, *J* = 12.4 Hz, 1H), 6.25 (dd, *J* = 13.6, 9.8 Hz, 2H), 4.70 – 4.60 (obscured by HDO, about 1H), 4.21 – 4.11 (m, 4H), 3.42 – 3.40 (m, 1H), 2.91 – 2.83 (m, 5H), 2.20 – 2.01 (m, 6H), 1.95 – 1.92 (m, 2H), 1.51 – 1.50 (m, 9H), 1.25 – 1.20 (obscured by triethylamine peak, about 4H), 0.99 – 0.95 (obscured by triethylamine peak, 4H), 0.69 – 0.42 (m, about 2H). ^{31}P NMR (D_2O): δ 15.21 (s). HRMS (positive ion MALDI): calcd 1162.2645 *m/z*, found $[M-H]^+$ = 1161.2572 *m/z*.

5-FAM-ZOL (**7d1**, 3-(3-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-1-(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium**) and 6-FAM-ZOL (7d2**, 3-(3-(4-carboxy-3-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-1-(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium):

Synthesized according to the method described above from 60.2 mg of compound **4d** (as TEA^+ salt (4 equiv. of TEA), 0.08 mmol, 2.7 equiv.) in 0.5 mL of H_2O and pH adjusted to 8.4 with Na_2CO_3 (s), to which 15.2 mg of 5(6)-FAM, SE (0.03 mmol, 1 equiv.) in 100 μL anhydrous DMF was added. The pH was adjusted to 8.3 to dissolve precipitates and the solution stirred at rt overnight. After TLC purification, the

product was purified by HPLC (Method I). **6-FAM-ZOL** (**7d2**) and **5-FAM-ZOL** (**7d1**) were eluted at very different retention times, 20 and 30 min (the retention time has ±1.5 min difference between different runs), respectively. Each isomer was collected separately and then concentrated in vacuo to remove buffer. Compound **7d1** and **7d2** could also be directly synthesized from 5-FAM, SE and 6-FAM, SE according to the method described above. Detailed NMR descriptions given below correspond to the HPLC-separated products. Total amount of **7d1** and **7d2** is 15.4 mg, 68.3% yield. **5-FAM-ZOL** (**7d1**, triethylammonium bicarbonate salt): obtained 9.2 mg (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.74 (s, 1H), 8.11 – 8.03 (m, 1H), 7.84 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.45 (t, *J* = 1.7 Hz, 1H), 7.34 (t, *J* = 1.8 Hz, 1H), 7.21 (d, *J* = 7.9 Hz, 1H), 6.99 (d, *J* = 9.0 Hz, 2H), 6.50 – 6.43 (m, 4H), 4.57 – 4.44 (m, 2H), 4.36 (d, *J* = 12 Hz, 1H), 4.22 – 4.03 (m, 2H), 3.57 (dd, *J* = 14.0, 4.5 Hz, 1H), 3.43 (dd, *J* = 14.0, 6.7 Hz, 1H). ^{31}P NMR (D_2O): δ 14.02 (s). HRMS (positive ion MALDI): calcd 704.1041 *m/z*, found M^+ = 704.1013 *m/z*. **6-FAM-ZOL** (**7d2**, triethylammonium bicarbonate salt): obtained 6.2 mg (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.70 (s, 1H), 7.90 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.78 (d, *J* = 8.1, 1H), 7.57 (d, *J* = 1.7 Hz, 1H), 7.43 (t, *J* = 1.7 Hz, 1H), 7.30 (t, *J* = 1.8 Hz, 1H), 7.03 (d, *J* = 8.8 Hz, 2H), 6.56 – 6.42 (m, 4H), 4.56 – 4.42 (m, 2H), 4.32 (d, *J* = 12.5 Hz, 1H), 4.17 – 3.99 (m, 2H), 3.51 (dd, *J* = 14.1, 4.2 Hz, 1H), 3.40 – 3.33 (m, 1H). ^{31}P NMR (D_2O): δ 14.03 (s). HRMS (positive ion MALDI): calcd 704.1041 *m/z*, found M^+ = 704.1027 *m/z*.

AF647-ZOL (**7d3**, 2-(5-(3-(6-((2-hydroxy-3-(1-(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium-3-yl)propyl)amino)-6-oxohexyl)-3-methyl-5-sulfo-1-(3-sulfopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-5-sulfo-1-(3-sulfopropyl)-3*H*-indol-1-ium):

Synthesized according to the method described above from 18.9 mg of compound **4d** (0.05 mmol, 5 equiv.) in 500 μL of H_2O and pH adjusted to 8.4 with Na_2CO_3 (s), to which 10 mg of AF647, SE (0.0105 mmol, 1 equiv.) in 300 μL anhydrous DMF was added. The solution was stirred at rt overnight and then was concentrated under vacuo, and the resulting blue residue was dissolved in 20% MeOH in 0.1 M

TEAAc buffer (pH 5.3) and purified by HPLC (Method H). Peaks eluting at 16.5 min were collected as **7d3** (the retention time has ±1.5 min difference between different runs). Obtained 6.6 mg, 53.1% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.63 (s, 1H), 7.99 (t, *J* = 13.2 Hz, 2H), 7.78 – 7.65 (m, 4H), 7.39 (s, 1H), 7.34 – 7.20 (m, 3H), 6.55 (t, *J* = 12.5 Hz, 1H), 6.29 (dd, *J* = 13.6, 9.7 Hz, 2H), 4.52 – 4.48 (m, 2H), 4.26 – 4.07 (m, 5H), 3.98 – 3.81 (m, 2H), 2.95 – 2.85 (m, 5H), 2.13 – 2.08 (m, 6H), 1.93 – 1.89 (m, 2H), 1.58 – 1.54 (m, 9H), 1.34 – 1.21 (m, 3H), 1.04 – 0.94 (m, 2H), 0.81 – 0.66 (m, 1H), 0.52 – 0.36 (m, 1H). ^{31}P NMR (D_2O): δ 13.52 (s). HRMS (positive ion MALDI): calcd 1186.2290 *m/z*, found $[M-H]^+$ = 1186.2337 *m/z*.

800CW-ZOL (**7d4**, (*E*)-2-((*E*)-2-(3-((*E*)-2-(1-(6-((2-hydroxy-3-(1-(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium-3-yl)propyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-2-yl)vinyl)-2-(4-sulfophenoxy)cyclohex-2-en-1-ylidene)ethylidene)-3,3-dimethyl-1-(4-sulfonatobutyl)indoline-5-sulfonate, sodium salt):

Synthesized according to the method described above from 7.4 mg of compound **4d** (0.021 mmol, 5.3 equiv.) in 1 mL of H_2O and pH adjusted to 8.4 with Na_2CO_3 (s), to which 5 mg of IRDye 800CW, SE (0.004 mmol, 1 equiv.) in 100 μL anhydrous DMF was added. The solution was stirred at 4 ˚C overnight and was then concentrated under vacuo, and the resulting greenish black residue was dissolved in 20% MeOH in 0.1 M TEAAc buffer (pH 5.3) and purified by HPLC (Method J). Peaks eluting at 23.5 min were collected as **7d4** (the retention time has ±1.5 min difference between different runs). Obtained 4.8 mg, 83.2% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.65 (s, 1H), 7.67 (d, *J* = 8.6 Hz, 2H), 7.63 – 7.50 (m, 6H), 7.39 (s, 1H), 7.28 (t, *J* = 1.8 Hz, 1H), 7.14 – 6.96 (m, 4H), 5.99 – 5.84 (dd, *J* = 14.2, 9.4 Hz, 2H), 4.56 – 4.46 (m, 2H), 4.18 (d, *J* = 12.6 Hz, 1H), 4.02 – 3.66 (m, 6H), 3.15 – 3.09 (m, 2H), 2.81 – 2.73 (m, 3H), 2.45 (brd, 5H), 2.09 – 2.06 (m, 2H), 1.83 (obscured by solvent peak, around 12H), 1.80 – 1.59 (obscured by solvent peak, around 6H), 1.53 – 1.38 (m, 4H). ^{31}P NMR (D_2O): δ 13.65 (s). HRMS

(positive ion MALDI): calcd 1330.2865 *m/z*, found $[M-H]^+$ = 1330.2885 *m/z*.

Sulfo-Cy5-ZOL (**7d5**, 1-(6-((2-hydroxy-3-(1-(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium-3-yl)propyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethyl-5-sulfonatoindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium-5-sulfonate, sodium salt):

Synthesized according to the method described above from 22.71 mg of compound **4d** (0.066 mmol, 5.1 equiv.) in 0.95 mL of H_2O and pH adjusted to 8.34 with Na_2CO_3 (s), to which 10 mg of Sulfo-Cy5, SE (0.013 mmol, 1 equiv.) in 450 μL anhydrous DMF was added. Precipitates could be seen. The solution was stirred at rt overnight and then was concentrated under vacuo, and the resulting blue residue was dissolved in 20% MeOH in 0.1 M TEAAc buffer (pH 5.3) and purified by HPLC (Method K). Peaks eluting at 18 min were collected as **7d5** (the retention time has ±1.5 min difference between different runs). Obtained 5.3 mg, 41.2% yield (triethylammonium acetate salt). ^{1}H NMR (D_2O): δ 8.65 (s, 1H), 7.81 (td, *J* = 13.1, 4.3 Hz, 2H), 7.74 – 7.56 (m, 4H), 7.39 (s, 1H), 7.26 (s, 1H), 7.15 (dd, *J* = 8.4, 2.2 Hz, 2H), 6.32 (t, *J* = 12.5 Hz, 1H), 6.00 (dd, *J* = 19.6, 13.7 Hz, 2H), 4.57 – 4.44 (m, 2H), 4.17 (d, *J* = 13.0 Hz, 1H), 3.92 (m, 4H), 3.42 (s, 3H), 2.16 – 2.09 (m, 2H), 1.68 – 1.61 (m, 2H), 1.53 – 1.41 (m, 15H), 1.26 – 1.17 (obscured by triethylamine peak, around 3H). ^{31}P NMR (D_2O): δ 13.51 (s). MS (negative ion ESI): calcd 483.6 *m/z*, found $[M-4H]^{2-}$ = 484.0 *m/z*.

5-FAM-MIN (**7e1**, 1-(3-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-3-(2-hydroxy-2,2-diphosphonoethyl)imidazo[1,2-*a*]pyridin-1-ium**) and 6-FAM-MIN (7e2**, 1-(3-(4-carboxy-3-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)-3-(2-hydroxy-2,2-diphosphonoethyl)imidazo[1,2-*a*]pyridin-1-ium):

Synthesized according to the method described above from 22.4 mg of compound **4e** (0.057 mmol, 2.5 equiv.) in 1 mL of H_2O and pH adjusted to 8.58 with Na_2CO_3 (s), to which 10.8 mg of 5(6)-FAM, SE (0.023 mmol, 1 equiv.) in 300 μL anhydrous DMF was added. The pH was adjusted to 8.4 to dissolve

precipitates and the solution was stirred at rt overnight. After TLC purification, the product was purified by HPLC (Method I). **6-FAM-MIN** (**7e2**) and **5-FAM-MIN** (**7e1**) were eluted at very different retention times, 21.5 and 31.5 min (the retention time has ±3 min difference between different runs), respectively. Each isomer was collected separately and then concentrated in vacuo to remove buffer. Compound **7e1** and **7e2** could also be directly synthesized from 5-FAM, SE and 6-FAM, SE according to the method described above. Detailed NMR descriptions given below correspond to the HPLC-separated products. Total amount of **7e1** and **7e2** is 11.6 mg, 67.2% yield. **5-FAM-MIN** (**7e1**, triethylammonium bicarbonate salt): obtained 6.3 mg (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.77 (d, *J* = 7.0 Hz, 1H), 8.09 (d, *J* = 1.6 Hz, 1H), 7.94 – 7.67 (m, 4H), 7.31 (td, *J* = 6.4, 1.6 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 7.06 (s, 1H), 7.03 (s, 1H), 6.55 (dq, *J* = 5.0, 2.3 Hz, 4H), 4.57 – 4.46 (m, 1H), 4.42 – 4.21 (m, 2H), 3.72 – 3.42 (m, 4H). ^{31}P NMR (D_2O): δ 16.52 (s). HRMS (positive ion MALDI): calcd 754.1198 *m/z*, found M^+ = 754.1178 *m/z*. **6-FAM-MIN** (**7e2**, triethylammonium bicarbonate salt): obtained 5.3 mg (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.75 (d, *J* = 7.0 Hz, 1H), 7.89 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.82 – 7.67 (m, 4H), 7.55 (d, *J* = 1.6 Hz, 1H), 7.27 (td, *J* = 6.7, 1.6 Hz, 1H), 7.07 (s, 1H), 7.05 (s, 1H), 6.63 – 6.51 (m, 4H), 4.50 – 4.42 (m, 1H), 4.34 – 4.19 (m, 2H), 3.63 – 3.48 (m, 3H), 3.42 (dd, *J* = 14.1, 6.9 Hz, 1H). ^{31}P NMR (D_2O): δ 16.52 (s). HRMS (positive ion MALDI): calcd 754.1198 *m/z*, found M^+ = 754.1187 *m/z*.

5-FAM-MINPC (**7f1**, also known as **5-FAM-3-IPEHPC**, 3-(2-carboxy-2-hydroxy-2-phosphonoethyl)-1-(3-(3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)imidazo[1,2-*a*]pyridin-1-ium**) and 6-FAM-MINPC** (**7f2**, also known as **6-FAM-3-IPEHPC**, 3-(2-carboxy-2-hydroxy-2-phosphonoethyl)-1-(3-(4-carboxy-3-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzamido)-2-hydroxypropyl)imidazo[1,2-*a*]pyridin-1-ium):

Synthesized according to the method described above from 20 mg of compound **4f** (0.056 mmol, 2.5 equiv.) in 1 mL of H_2O and pH adjusted to 8.57 with Na_2CO_3 (s), to which 10.5 mg of 5(6)-FAM, SE

(0.022 mmol, 1 equiv.) in 300 μL anhydrous DMF was added. The pH was adjusted to 8.4 to dissolve precipitates and the solution was stirred at rt overnight. After TLC purification, the product was purified by HPLC (Method I). **6-FAM-MINPC** (**7f2**) and **5-FAM-MINPC** (**7f1**) were eluted at very different retention times, 19 and 28 min (the retention time has ±1.5 min difference between different runs), respectively. Each isomer was collected separately and then concentrated in vacuo to remove buffer. Compound **7f1** and **7f2** could also be directly synthesized from 5-FAM, SE and 6-FAM, SE according to the method described above. Detailed NMR descriptions given below correspond to the HPLC-separated products. Total amount of **7f1** and **7f2** was 11.7 mg, 73.2% yield. **5-FAM-MINPC** (**7f1**, triethylammonium bicarbonate salt): obtained 6.3 mg (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.69 (d, *J* = 6.9 Hz, 1H), 8.08 (dq, *J* = 1.6, 0.8 Hz, 1H), 7.88 – 7.75 (m, 3H), 7.68 (d, *J* = 4.4 Hz, 1H), 7.33 (ddd, *J* = 6.9, 5.8, 2.1 Hz, 1H), 7.27 (dt, *J* = 8.0, 0.7 Hz, 1H), 7.01 (dt, *J* = 9.2, 0.9 Hz, 2H), 6.53 – 6.46 (m, 3H), 4.50 (dt, *J* = 14.6, 2.9 Hz, 1H), 4.32 (dd, *J* = 14.6, 9.0 Hz, 1H), 4.26 (dq, *J* = 9.2, 5.1, 4.1 Hz, 1H), 3.72 (dd, *J* = 15.9, 3.2 Hz, 1H), 3.62 (ddd, *J* = 14.1, 4.8, 1.9 Hz, 1H), 3.48 (ddd, *J* = 14.0, 7.0, 4.3 Hz, 1H), 3.40 (dd, *J* = 15.7, 7.4 Hz, 1H). ^{31}P NMR (D_2O): δ 14.82 (s). HRMS (positive ion MALDI): calcd 718.1433 *m/z*, found M^+ = 718.1399 *m/z*. **6-FAM-MINPC** (**7f2**, triethylammonium bicarbonate salt): obtained 5.4 mg (triethylammonium bicarbonate salt). ^{1}H NMR (D_2O): δ 8.64 (d, *J* = 7.0 Hz, 1H), 7.85 (ddd, *J* = 8.1, 1.8, 0.9 Hz, 1H), 7.78 – 7.62 (m, 4H), 7.50 (dt, *J* = 1.7, 0.8 Hz, 1H), 7.28 (td, *J* = 6.8, 1.6 Hz, 1H), 7.04 – 6.95 (m, 2H), 6.53 – 6.45 (m, 4H), 4.46 – 4.37 (m, 1H), 4.29 – 4.12 (m, 2H), 3.68 (dd, *J* = 15.7, 3.6 Hz, 1H), 3.52 (dd, *J* = 14.0, 4.0 Hz, 1H), 3.43 – 3.33 (m, 2H). ^{31}P NMR (D_2O): δ 15.03 (s). HRMS (positive ion MALDI): calcd 718.1433 *m/z*, found M^+ = 718.1416 *m/z*.

Investigation of reaction of pyridine with epichlorohydrin (6)

Pyridine : epichlorohydrin (1 : 5 ratio of equiv.) in D_2O:

Pyridine (16 μL, 0.2 mmol, 1 equiv.) was dissolved in 4 mL of D_2O and pH was brought to 6.2 using

methylenebisphosphonic acid (s). To this solution epichlorohydrin (79 μL, 1 mmol, 5 equiv.) was added. The reaction progress was monitored by ^{1}H NMR. In 4 h no more epichlorohydrin remained in the reaction mixture. The formation of two products was observed with the ratio of **15a** : **15b** = 1 : 2, with about 20% of unreacted pyridine remaining (**Figure S6**).

Pyridine : epichlorohydrin (1 : 1 ratio of equivalent) in D_2O:

Pyridine (16 μL, 0.2 mmol, 1 equiv.) was dissolved in 4 mL of D_2O and pH was brought to 6.2 using methylenebisphosphonic acid (s). To this solution epichlorohydrin (16 μL, 0.2 mmol, 1 equiv.) was added. The reaction progress was monitored by ^{1}H NMR. In 4 h no more epichlorohydrin remained in the reaction mixture. The formation of two products was observed with the ratio of **15a** : **15b** = 6 : 1, with about 54% of unreacted pyridine remaining (**Figure S7**).

Pyridine : epichlorohydrin (1 : 5 ratio of equivalent) in MeCN:

Pyridine (16 μL, 0.2 mmol, 1 equiv.) was dissolved in 4 mL of acetonitrile. To this solution epichlorohydrin (79 μL, 1 mmol, 5 equiv.) was added. In 4 h all volatiles were evaporated and ^{1}H NMR of the residue was taken. Compound B was observed as the only product. ^{1}H NMR (400 MHz, D_2O): δ 8.92 (d, *J* = 5.8 Hz, 2H, ring), 8.66 (t, *J* = 7.6 Hz, 1H, ring), 8.20 – 8.08 (m, 2H, ring), 5.19 (dd, *J* = 14.5, 2.2 Hz, 1H, C*H*-N), 4.60 (dd, *J* = 14.5, 7.4 Hz, 1H, C*H*-N), 3.70 (m, 1H, C*H*OCH2), 3.13 (t, *J* = 4.0 Hz, 1H, CHOC*H*2), 2.83 (dd, *J* = 4.0, 2.8 Hz, 1H, CHOC*H*2). ESI-MS (positive mode): m/z 136.0 [M^+].

^{1}H NMR reported by Demberelnyamba: ^{1}H NMR (500 mHz, D_2O): 8.92-8.85 (m, 2H, ring), 8.66 - 8.59 (m, 1H, ring), 8.16 - 8.10 (m, 2H, ring), 5.11 - 4.66 (m, 1H, C<u>H</u>OCH_2), 3.80–3.63 (m, 2H, CHOC<u>H_2</u>), 1.2–1.17 (d, 2H, C<u>H_2</u>–N). FAB–MS (MeOH matrix): m/z 135.9 [100%, GlPyþ].

Quantitative measurement of BP-HAP interaction by using Langmuir adsorption isotherms

1 mM phosphate-buffered saline (PBS) with 0.15 M NaCl (pH 6.8) was prepared freshly. Stock

solutions of 5(6)-ROX-RIS, 5(6)-ROX-RISPC, AF647-RIS and AF647-RISPC were made by dissolving the compounds in 1mM PBS to yield a 10 mM solution. Hydroxyapatite (Macro-Prep® Ceramic hydroxyapatite Type II 20 μM 100 g) was obtained from Bio-Rad Laboratories, Inc. Hercules, CA.

To measure and compare the bone mineral affinities of fluorescent BP/PCs, adsorption isotherm studies were carried out under identical experimental conditions. Accurately weighed HAP powder (1.4-1.6 mg) was suspended in 4 mL clear vial containing the appropriate volume of 1 mM PBS with 0.15 M NaCl (pH 6.8) for 3 hours. After premixing, 10 mM fluorescent BP stock solutions were added, resulting in concentrations of the fluorescent BP/PC additives ranging as 25, 50, 100, 200 and 300 μM. Equilibrium with the HAP was performed by rotating the vials end-over-end on a shaker at rt for 16 hours. Each sample was prepared in triplicate. Subsequent to the equilibrium period, the vials were centrifuged at 10,000 rpm for 5 min to separate the solids and the supernatant. 0.3 mL of the supernatant was collected and the equilibrium solution concentration was measured by using Nanodrop UV spectrometer. For the calibration series, fluorescent BP/PC standards were prepared by serial dilution from the stock solution with the same isotherm buffer to give the range from 0 to 400 μM. Calibration curves were constructed using standard solutions of the target fluorescent BP.

The amount of fluorescent BP/PC bound to the HAP (μmol/m^2) was calculated by comparing the end point concentration of fluorescent BP/PC detected after equilibrium to the initial fluorescent BP/PC additive concentration (μM) using the following equation:

Fluorescent BP/PC HAP surface concentration = (Initial fluorescent BP/PC concentration – end point concentration) / HAP surface area of the sample, where HAP surface area of the sample was 6700 m^2/L in our case. A plot of fluorescent BP/PC HAP surface concentration versus BP end point concentration provided the adsorption isotherm.

To describe the equilibrium binding of fluorescent BP/PC to HAP as a function of increasing

fluorescent BP/PC concentration, the experimental data were fitted to a saturation binding equation: Y (specific binding) = Bmax * X/(K_d +X) by using a non-linear curve-fitting algorithm, implemented in the Prism program (Graphpad, USA). Where X is the concentration of the fluorescent BP/PC, Y is the specific binding, and Bmax is the maximum number of binding sites, expressed in the same units as the Y-axis. K_d is the equilibrium dissociation constant, expressed in the same units as the X-axis (concentration). When the drug concentration equals K_d, half of the binding sites are occupied at equilibrium.

References:

(1) Kashemirov, B. A., Bala, J. L., Chen, X., Ebetino, F. H., Xia, Z., Russell, R. G. G., Coxon, F. P., Roelofs, A. J., Rogers, M. J., and McKenna, C. E. (2008) Fluorescently labeled risedronate and related analogues: "magic linker" synthesis. *Bioconjug. Chem. 19*, 2308-2310.

(2) Rocheblave, L., Bihel, F., De Michelis, C., Priem, G., Courcambeck, J., Bonnet, B., Chermann, J. C., and Kraus, J. L. (2002) Synthesis and antiviral activity of new anti-HIV amprenavir bioisosteres. *J. Med. Chem. 45*, 3321-3324.

Supplemental figures (Figure S1 – S9)

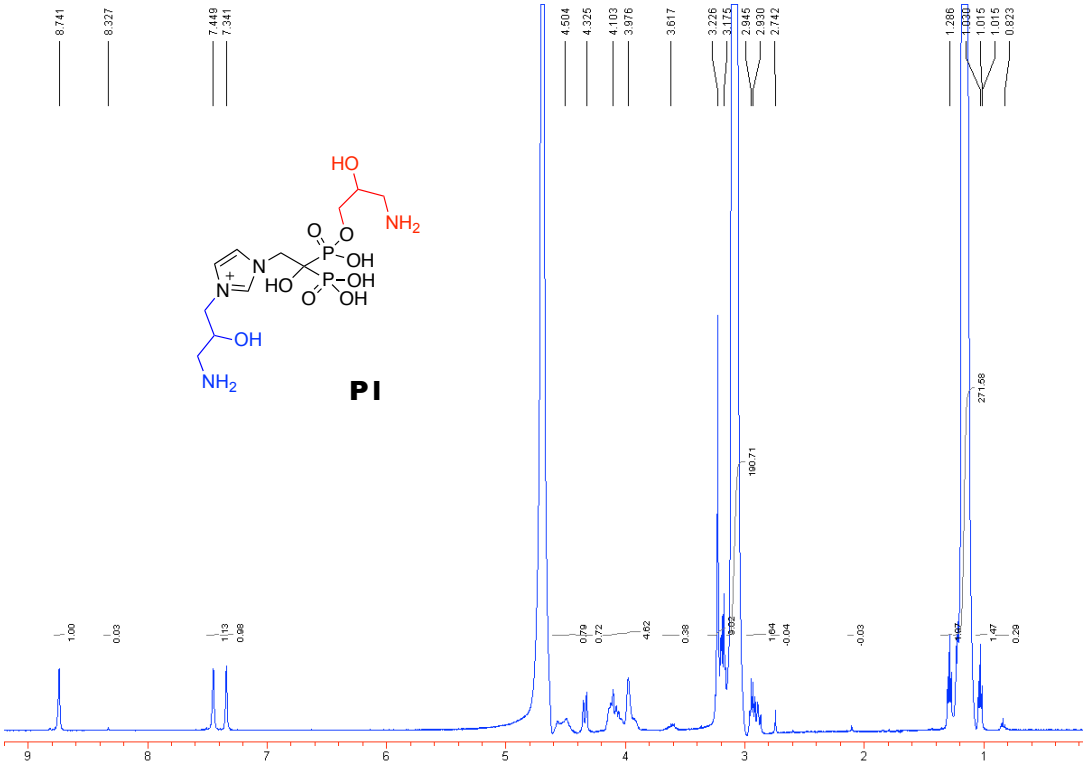


Figure S1. ^{1}H NMR (D_2O) spectrum of ZOL-N,O-dilinker P1.

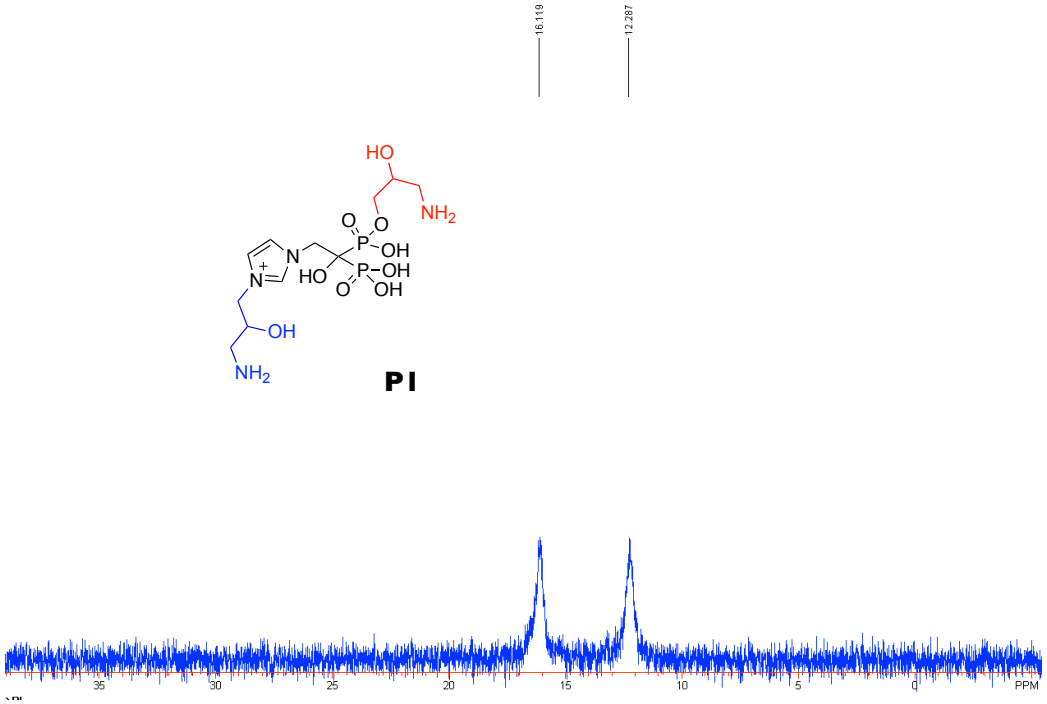


Figure S2. ^{31}P NMR (D_2O) spectrum of ZOL-N,O-dilinker P1.

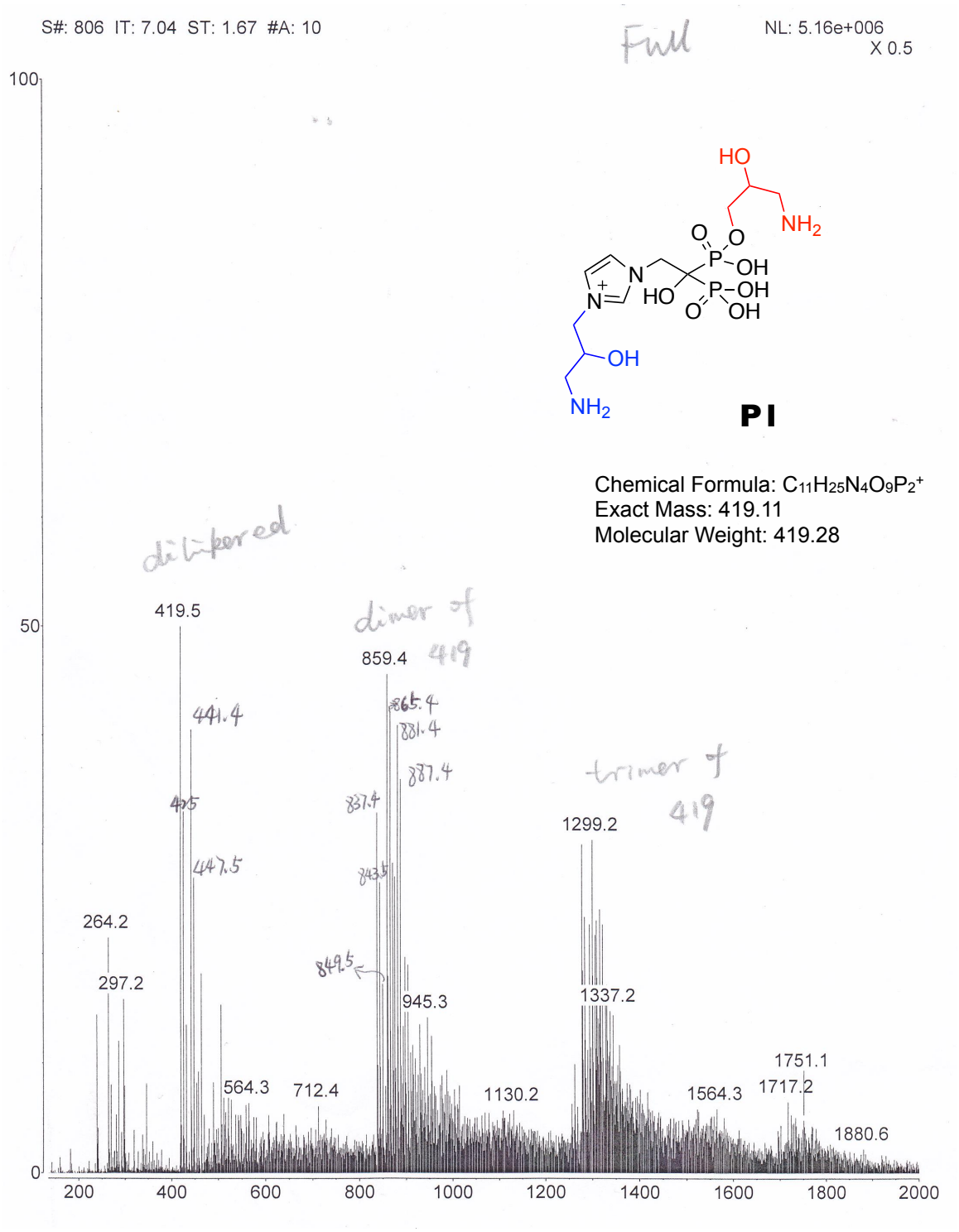


Figure S3. Mass spectrum (+) of ZOL-N,O-dilinker P1.

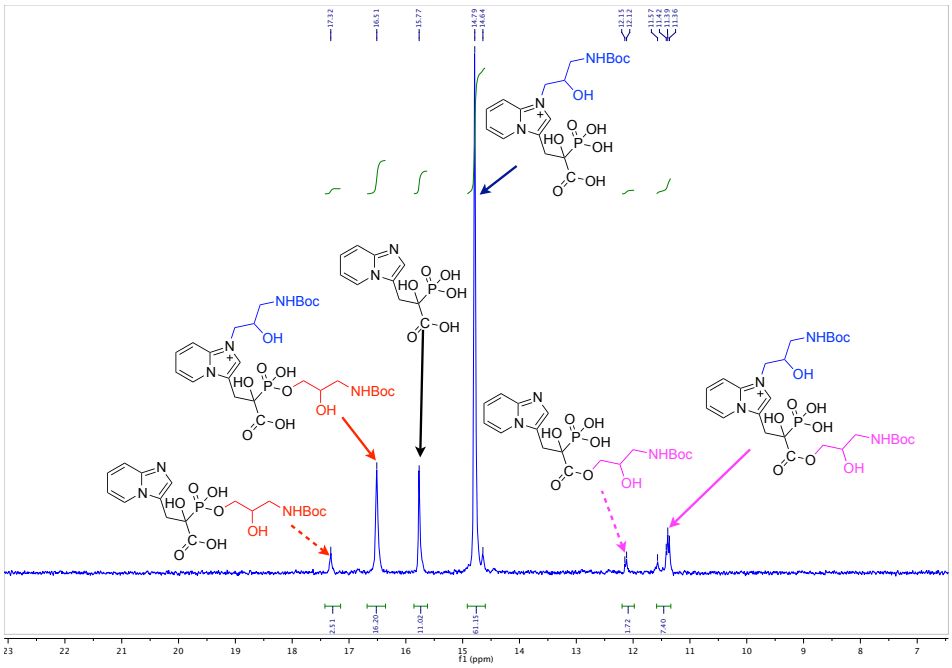


Figure S4. ^{31}P NMR of reaction mixture of MinPC (1f) and 5 via Route A.
(**1f** : **5** (equiv.) = 1 : 3, in D_2O, pH 7.5, heating at 45 °C for 48 h; structures of O-alkylation products are proposed and not investigated further).

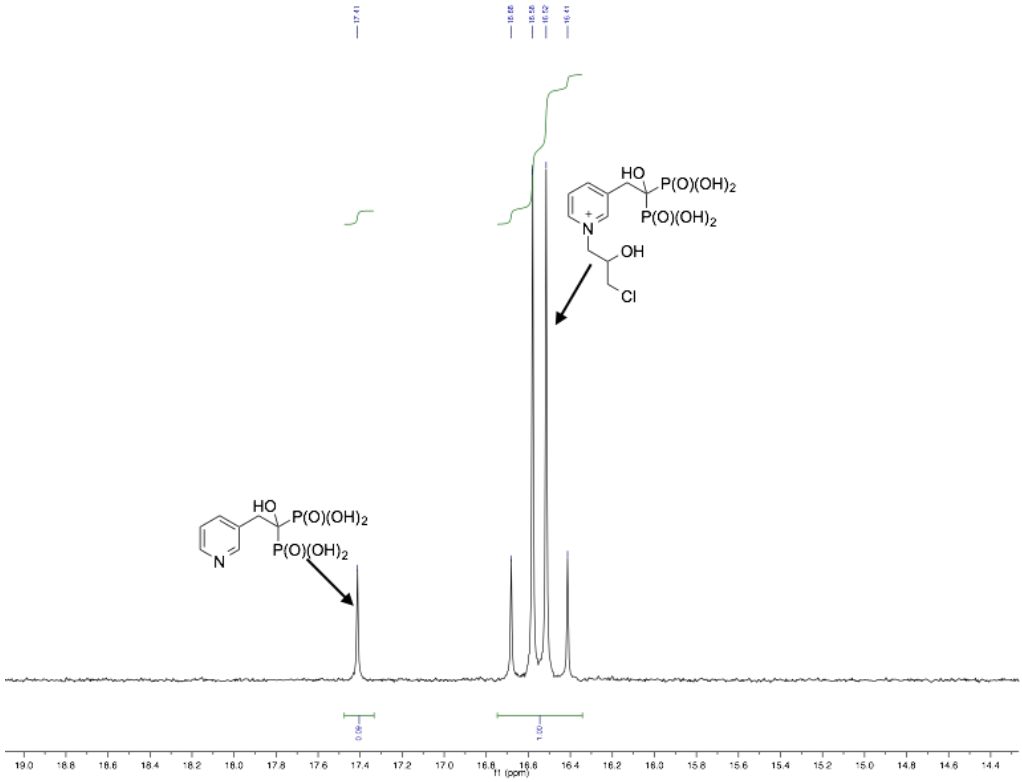


Figure S5. ^{31}P NMR of reaction mixture of RIS (1a) and 6 via Route B.
(**1a** : **6** (equiv.) = 1 : 5, in D_2O, pH 6.0, rt for 4 hrs).

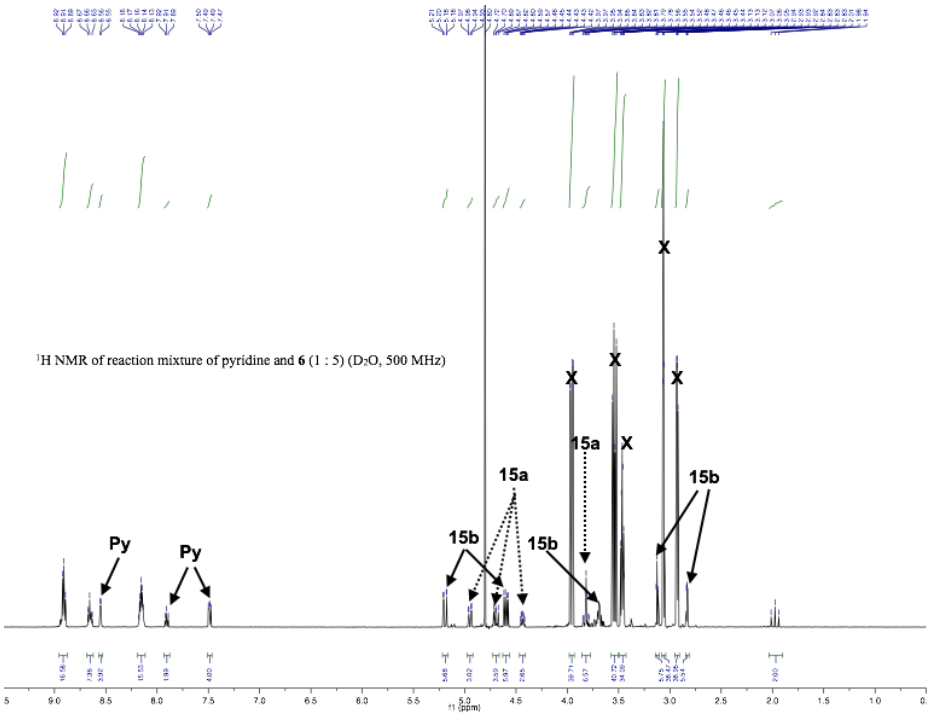


Figure S6. ^{1}H NMR of reaction mixture of pyridine (py) and **6** (**py** : **6** (equiv.) = 1 : 5, in D_2O), **15a** and **15b** are as shown in Scheme 2; **X** represents glycidol.

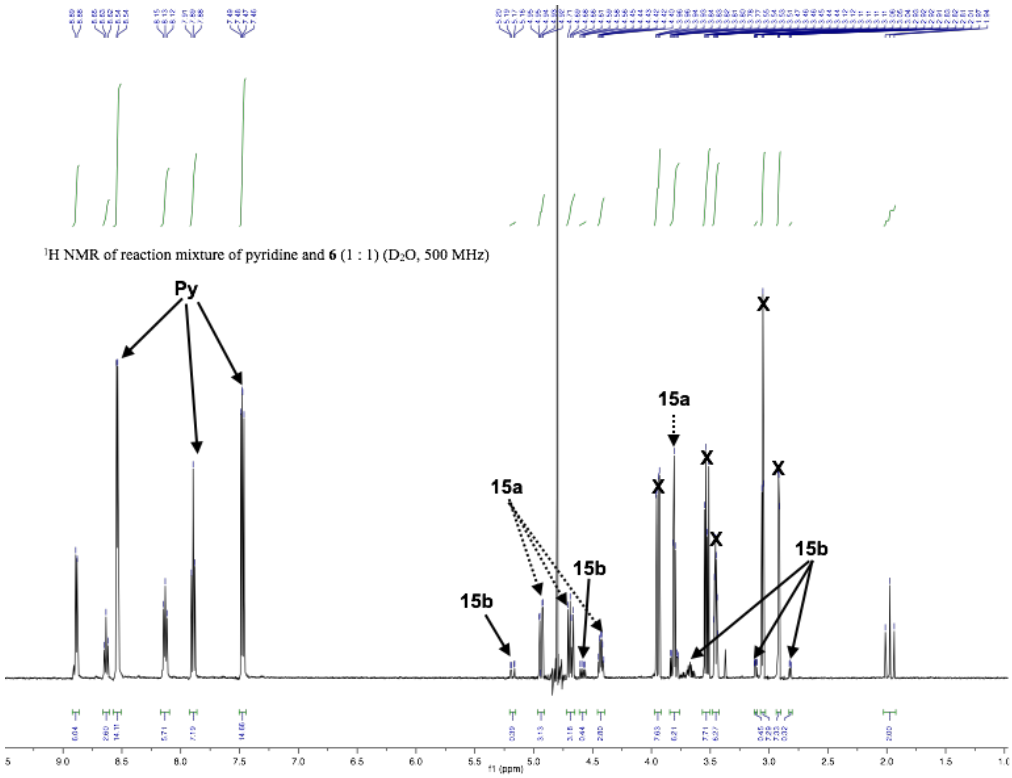


Figure S7. ^{1}H NMR of reaction mixture of pyridine (py) and **6** (**py** : **6** (equiv.) = 1 : 1, in D_2O), **A** and **B** are as shown in Scheme 2; **X** represents glycidol.

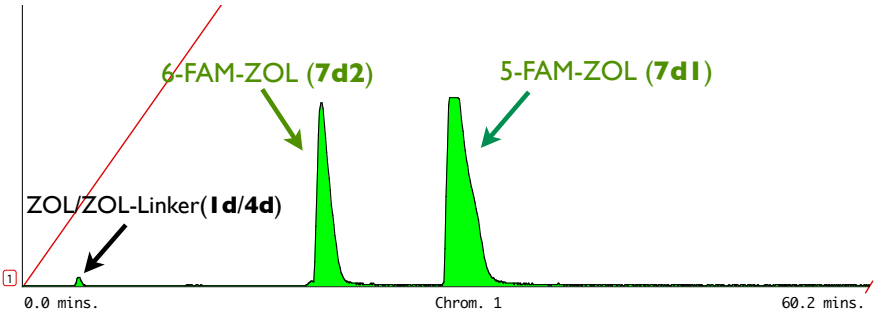


Figure S8. Preparative reverse phase HPLC separation of 5(6)-FAM-ZOL mixture through a semi-preparative C18 column.

(Beckman Ultrasphere ODS C18 (250 x 10 mm, 5 μm, 80 Å pore size), eluted with A: 0.1 M TEAC, 10% MeOH, pH 7.0, B: 0.1 M TEAC, 75% MeOH, pH 7.8 using a gradient that was increased from 0-40% of buffer B over 25 min, and then increased to 70% of buffer B from 25-100 min using a 4 mL/min flow rate; UV-vis detection set at 230 nm and then 492 nm after ZOL/ZOL-linker (**1d/4d**) was eluted.

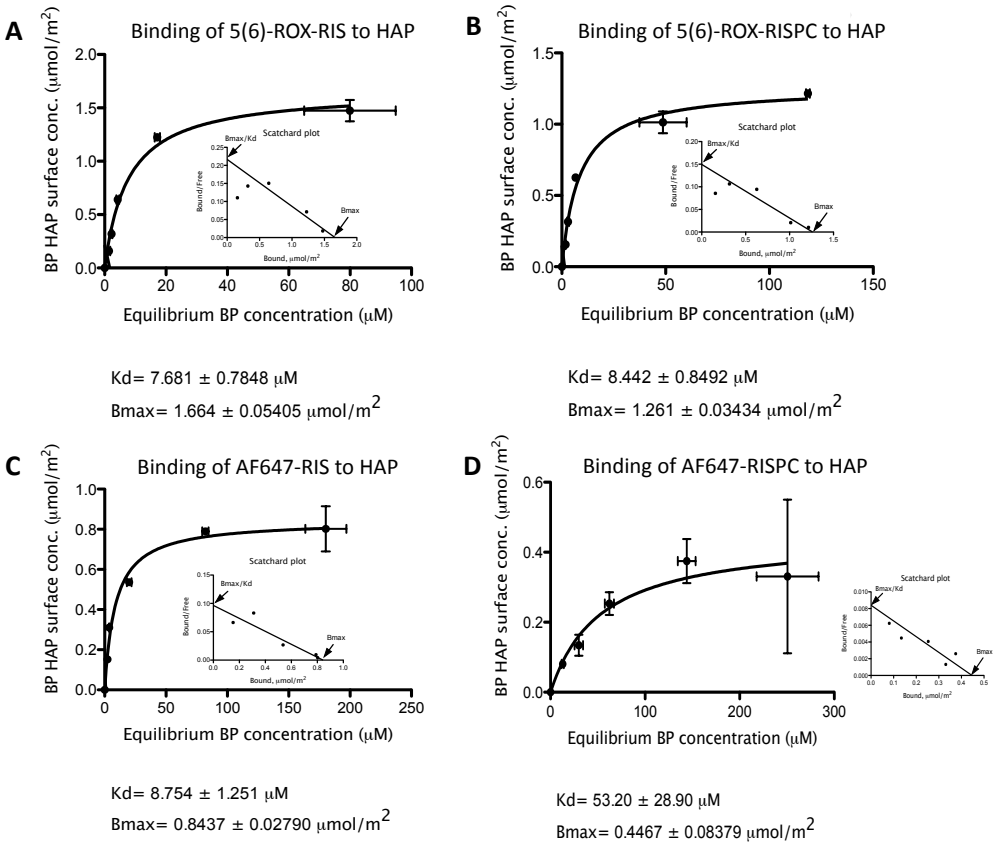


Figure S9. Adsorption isotherms for the binding of four fluorescent BP/PC probes.

A: 5(6)-ROX-RIS, **B**: 5(6)-ROX-RISPC, **C**: AF647-RIS, **D**: AF647-RISPC to HAP at pH 6.8 with Scatchard plots of the same data as inset, data are mean ± SD (n=3).

Appendix (spectra):

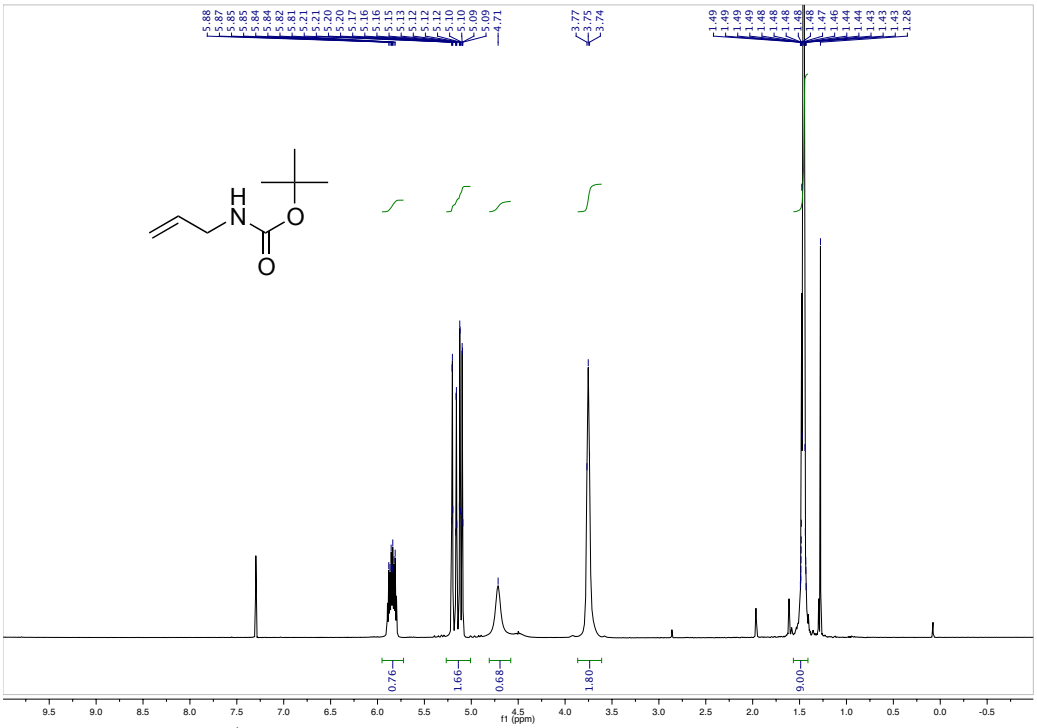


Figure A1. ^{1}H NMR ($CDCl_3$) spectrum of tert-butyl-allylcarbamate.

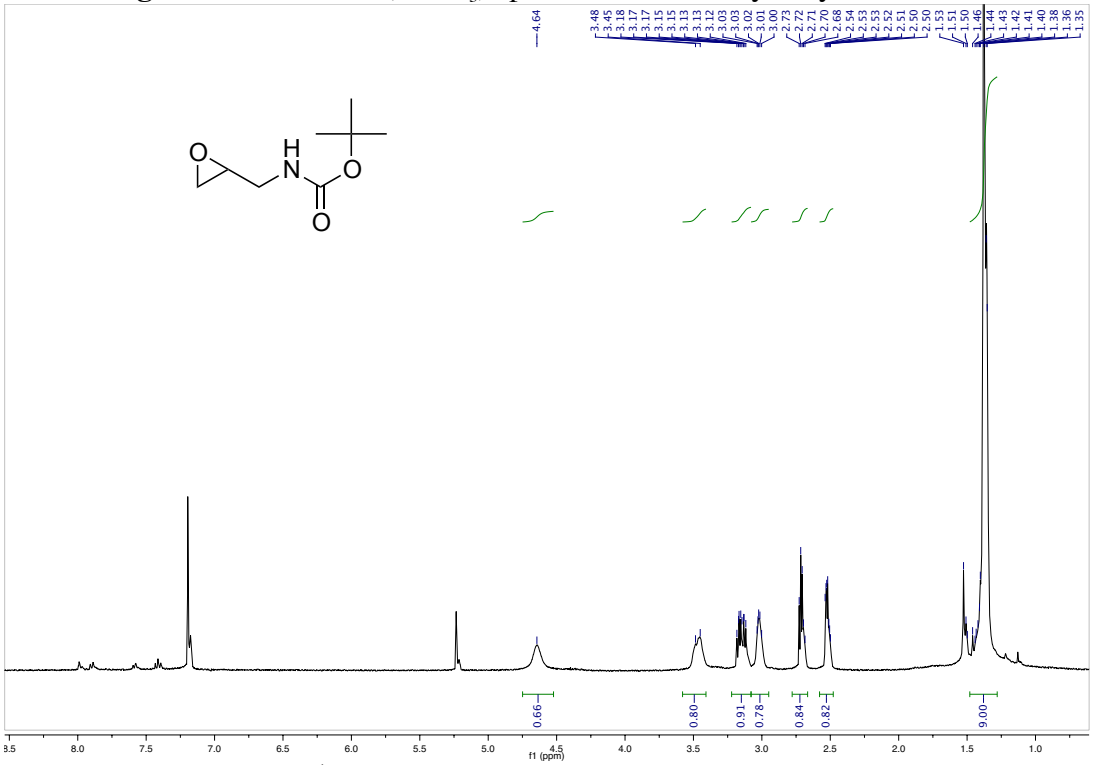


Figure A2. ^{1}H NMR ($CDCl_3$) spectrum of epoxide linker **5**.

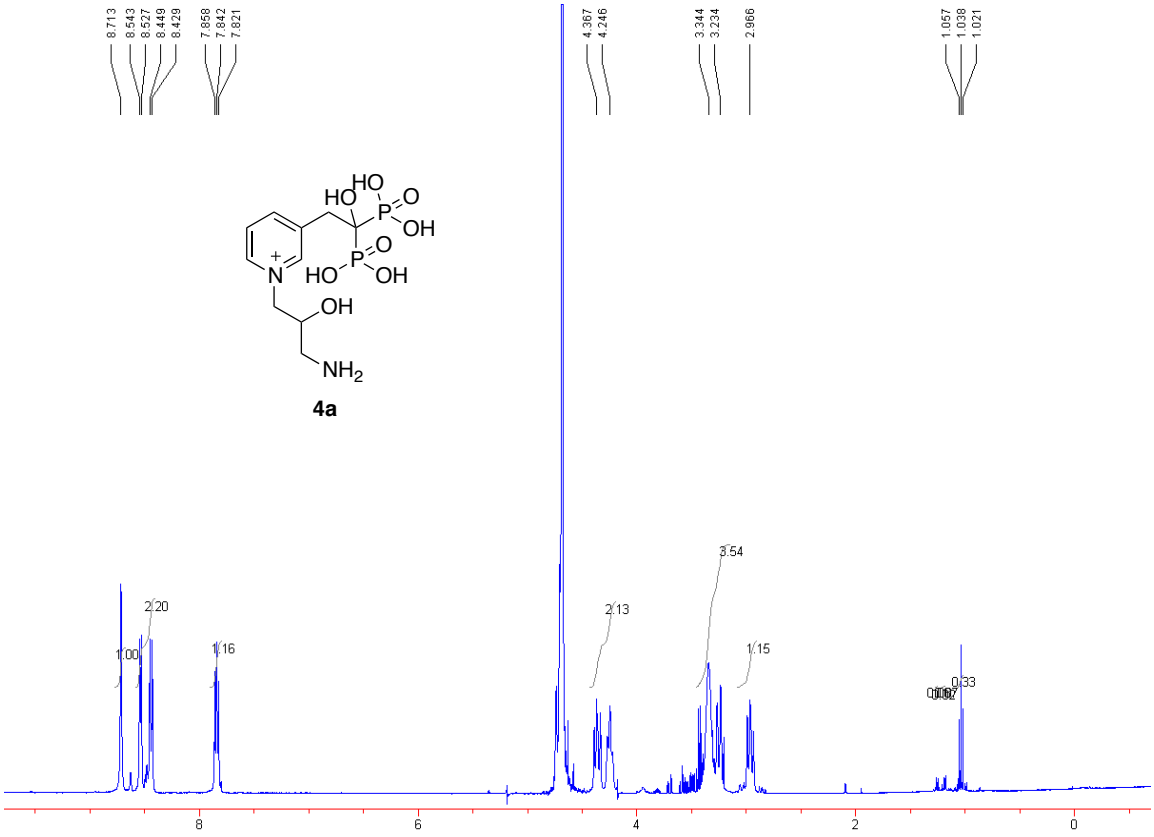


Figure A3. ^{1}H NMR (D_2O) spectrum of RIS-linker **4a**.

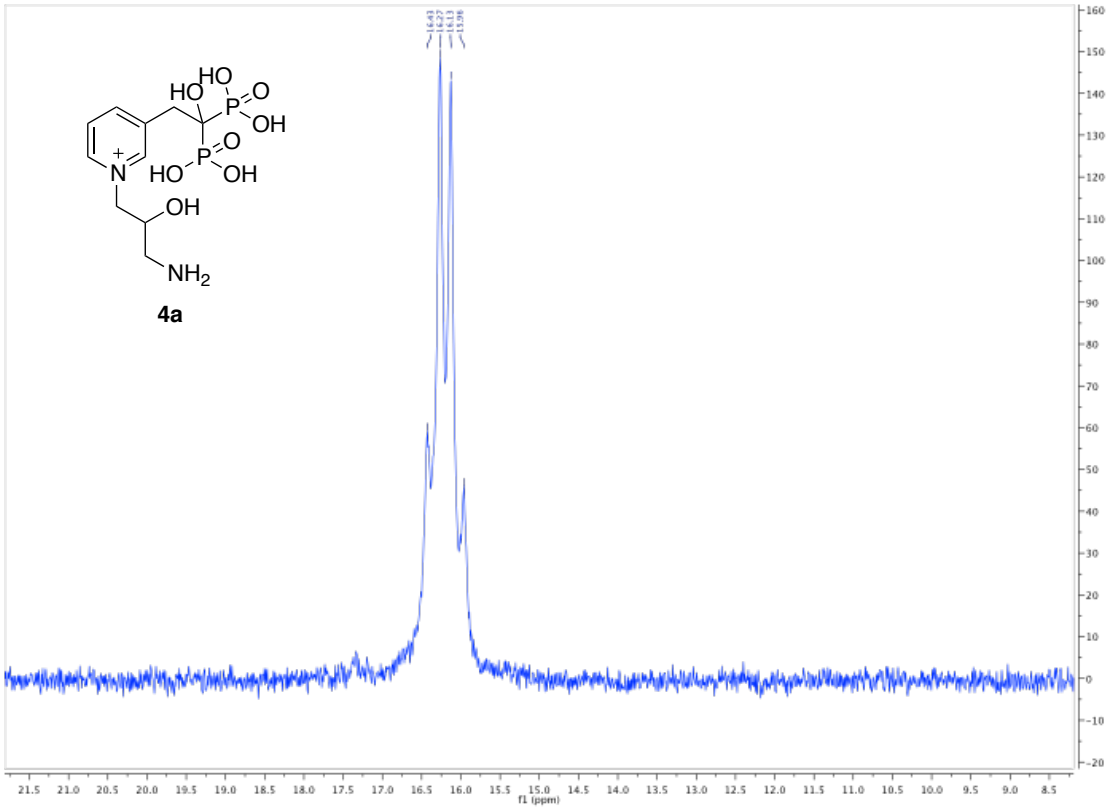


Figure A4. ^{31}P NMR (D_2O) spectrum of RIS-linker **4a**.

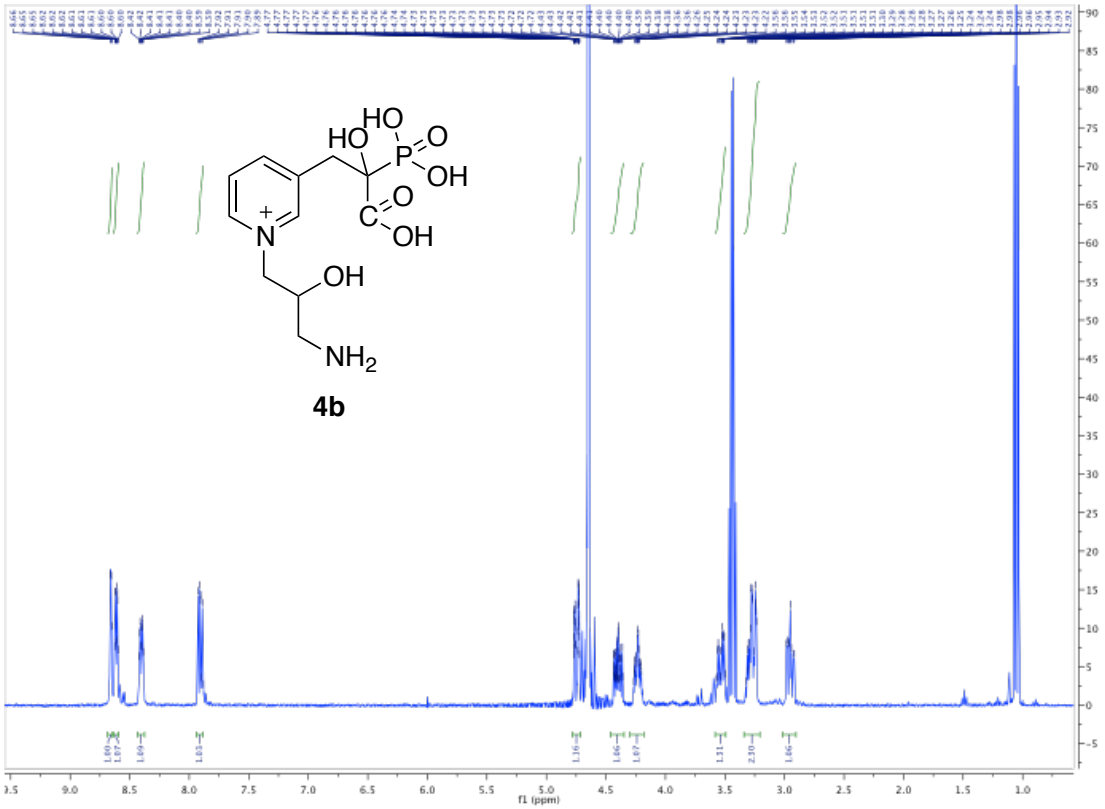


Figure A5. ^{1}H NMR (D_2O) spectrum of RISPC-linker **4b**.

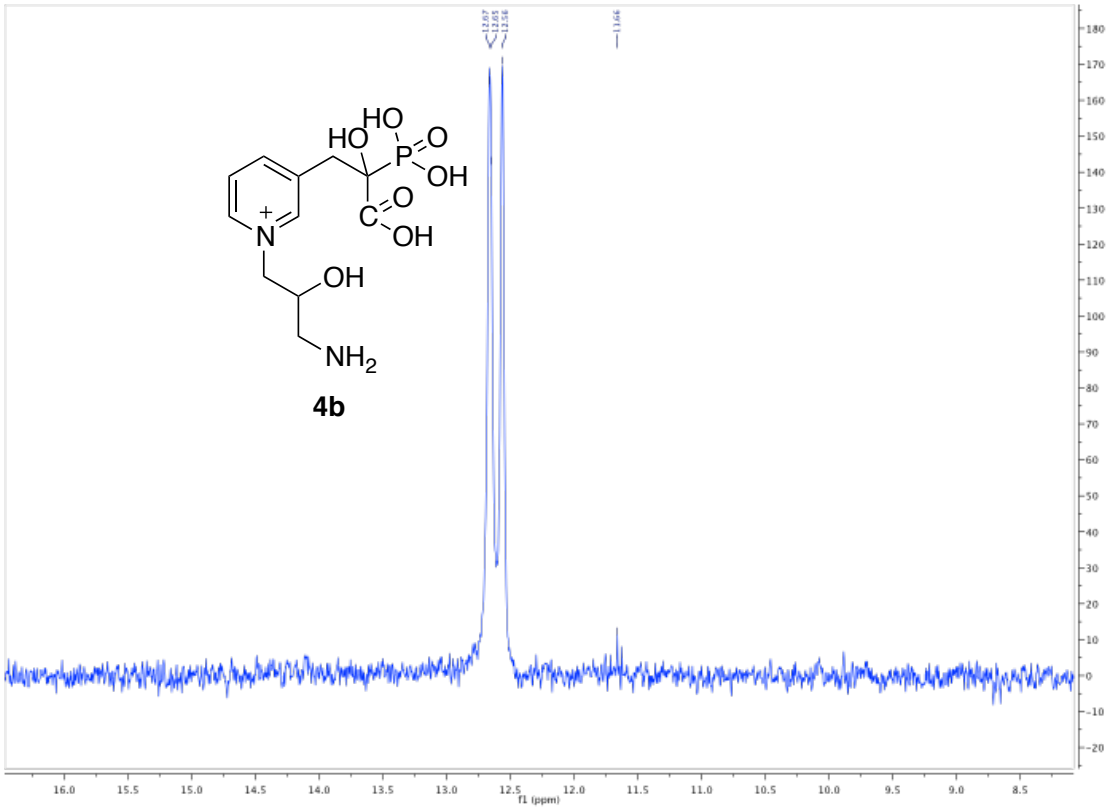


Figure A6. ^{31}P NMR (D_2O) spectrum of RISPC-linker **4b**.

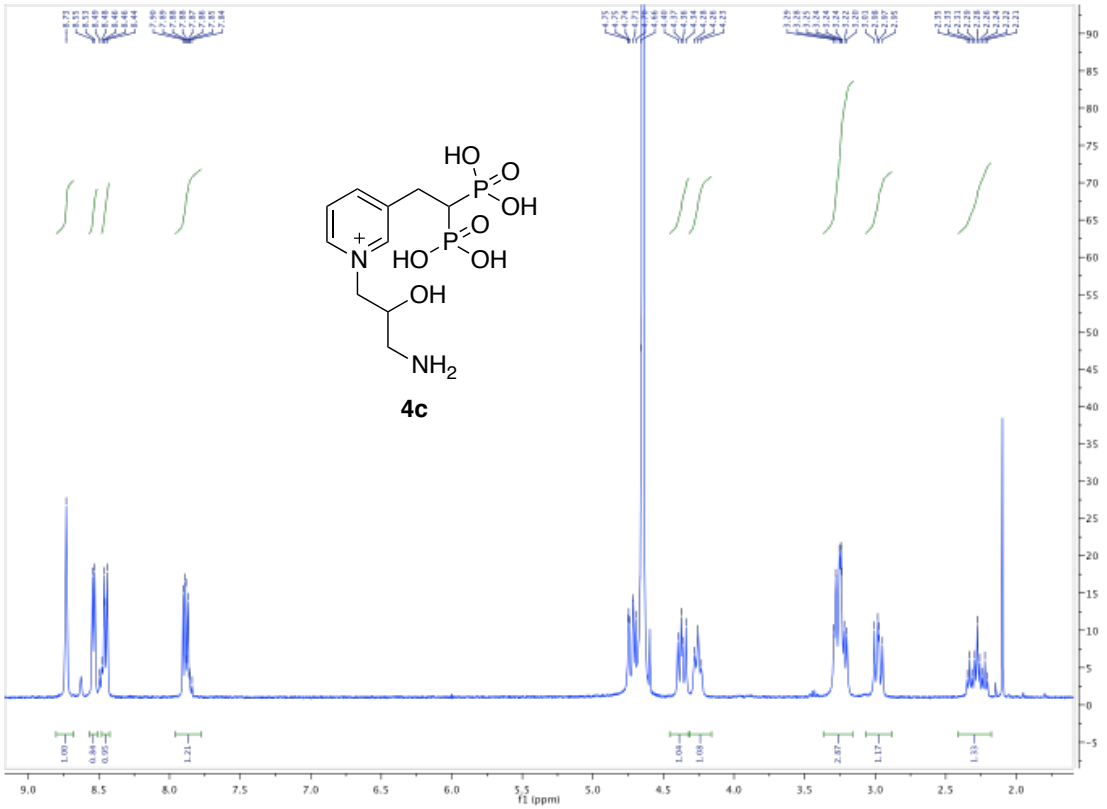


Figure A7. ^{1}H NMR (D_2O) spectrum of dRIS-linker **4c**.

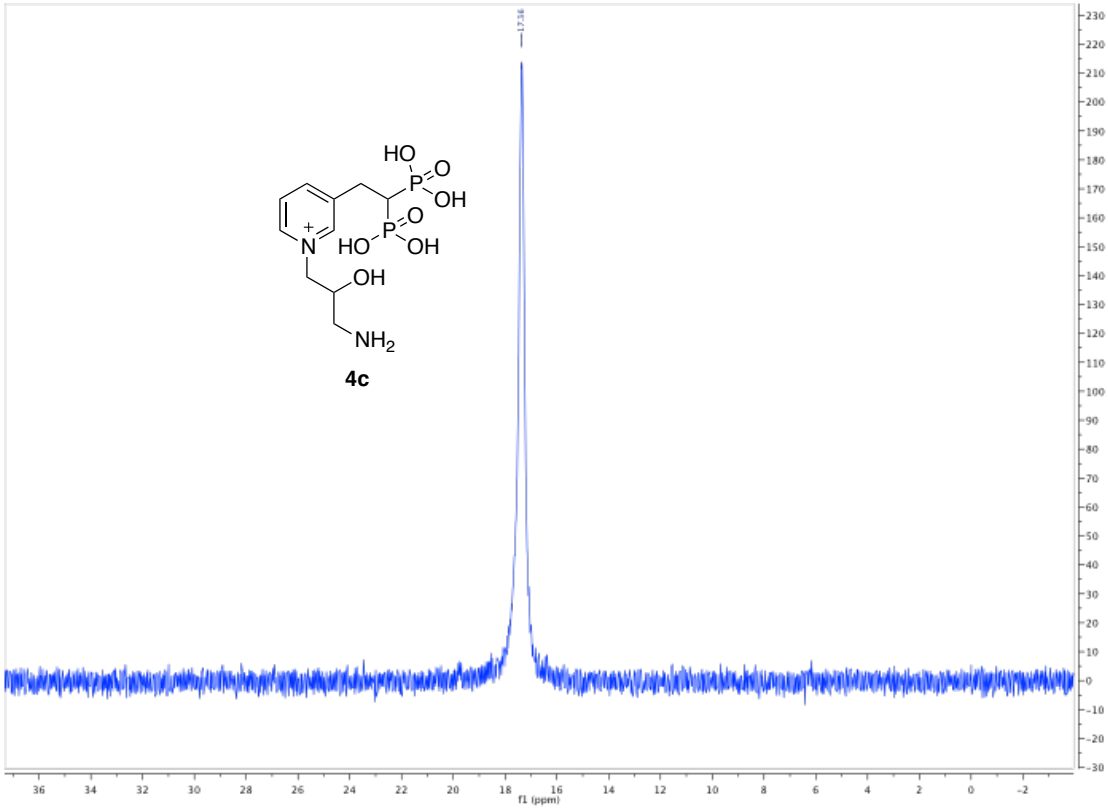


Figure A8. ^{31}P NMR (D_2O) spectrum of dRIS-linker **4c**.

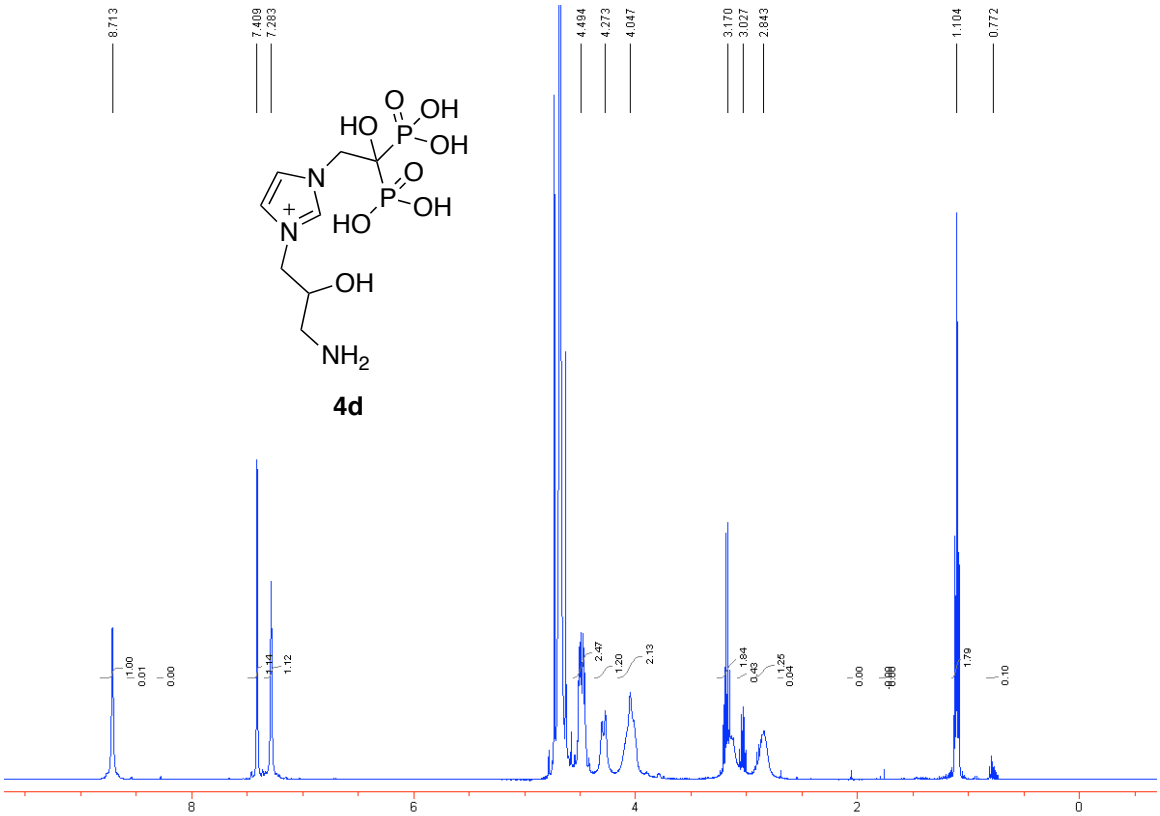


Figure A9. ^{1}H NMR (D_2O) spectrum of ZOL-linker **4d**.

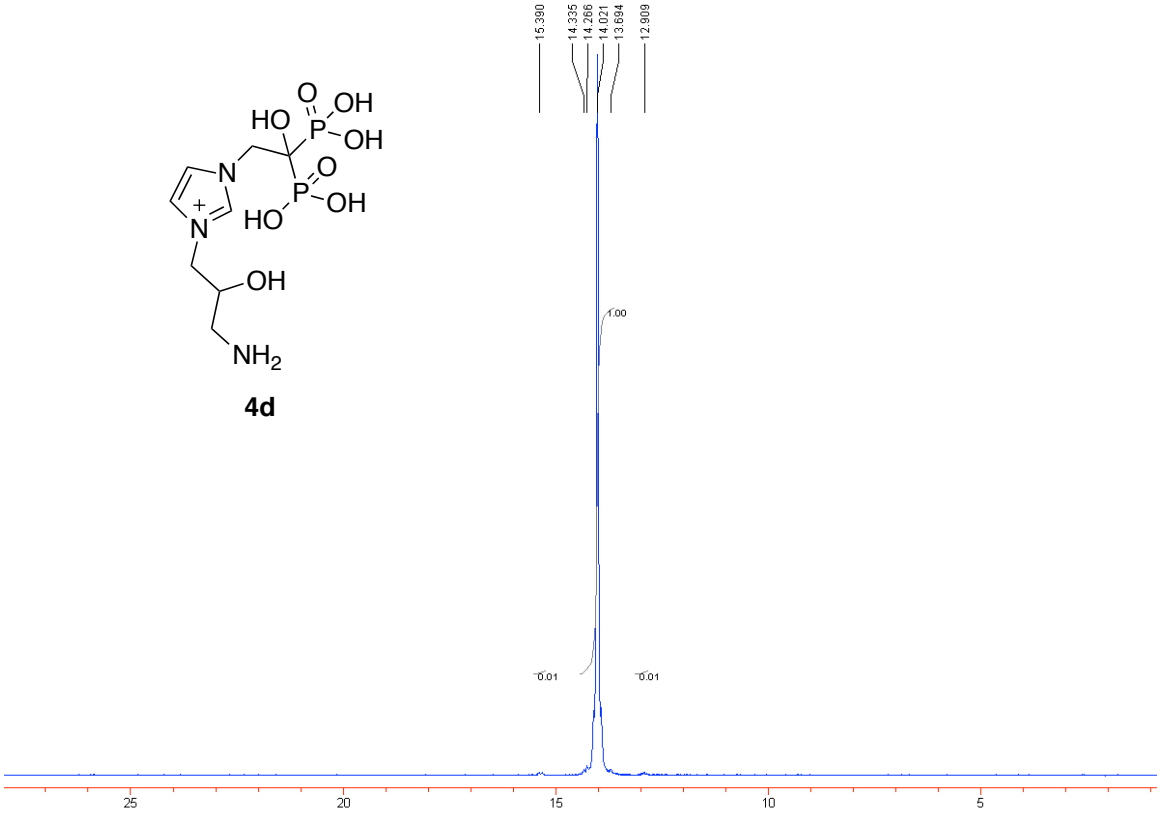


Figure A10. ^{31}P NMR (D_2O) spectrum of ZOL-linker **4d**.

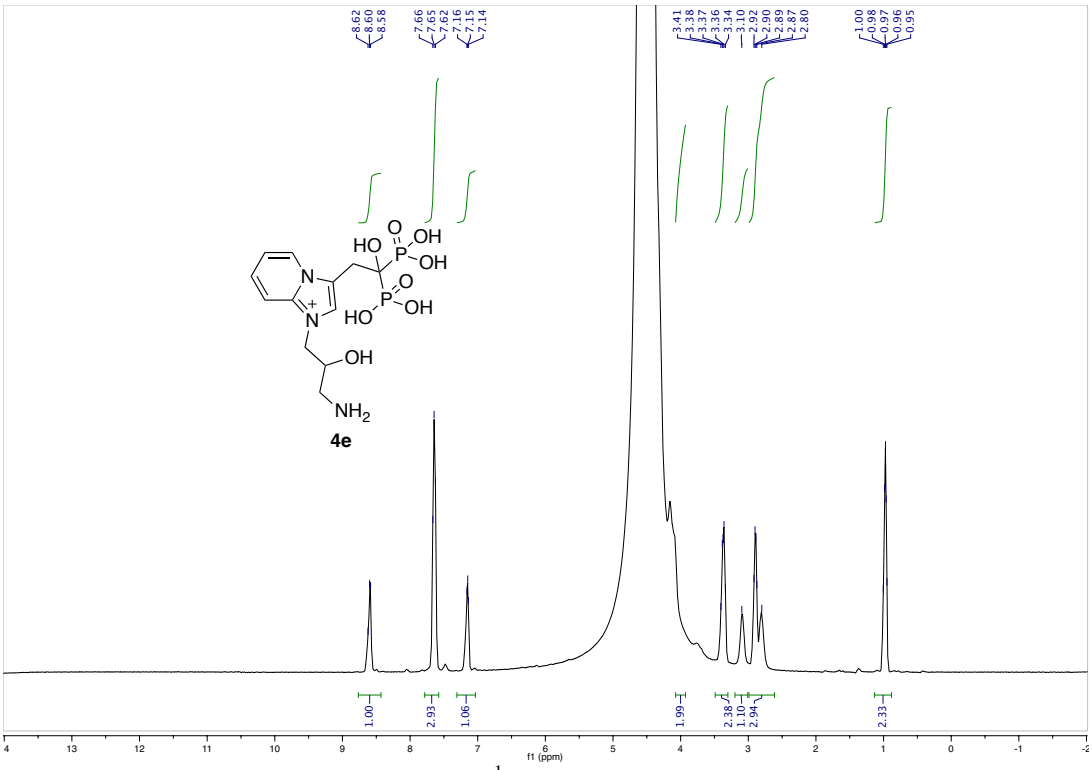


Figure A11. ^{1}H NMR (D_2O) spectrum of MIN-linker **4e**.

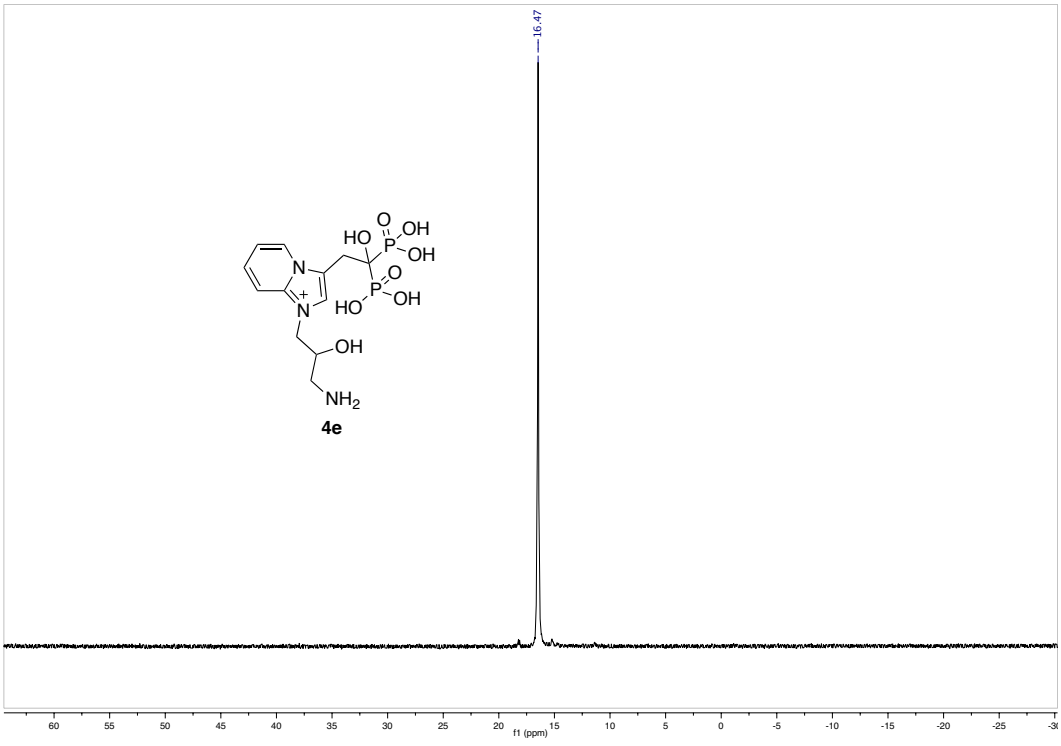


Figure A12. ^{31}P NMR (D_2O) spectrum of MIN-linker **4e**.

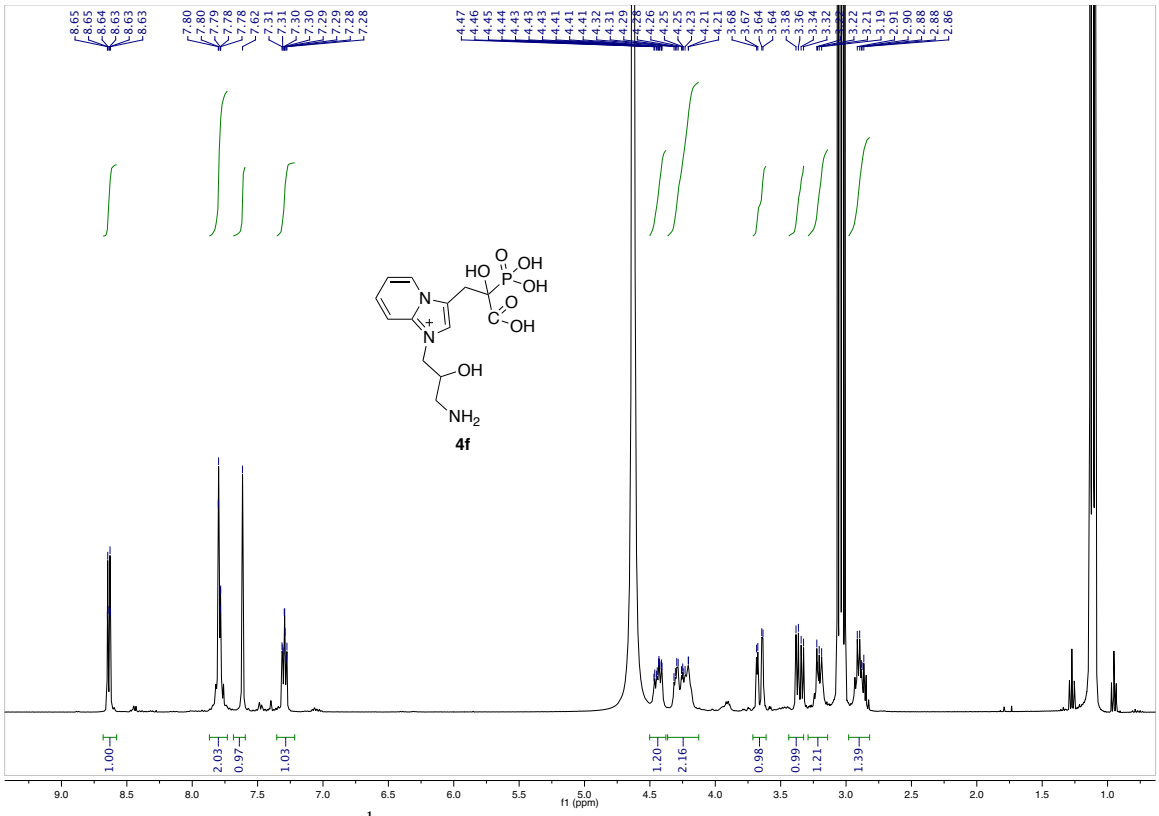


Figure A13. ^{1}H NMR (D_2O) spectrum of MINPC-linker **4f**.

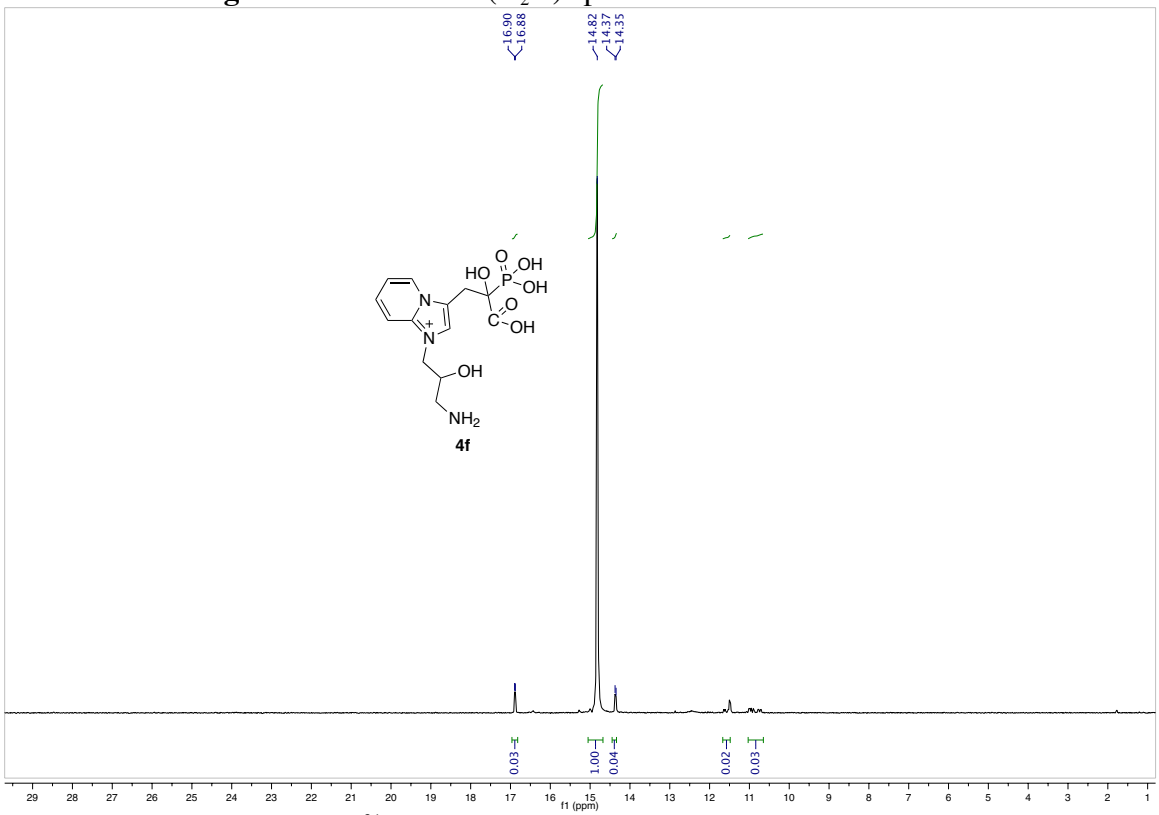


Figure A14. ^{31}P NMR (D_2O) spectrum of MINPC-linker **4f**.

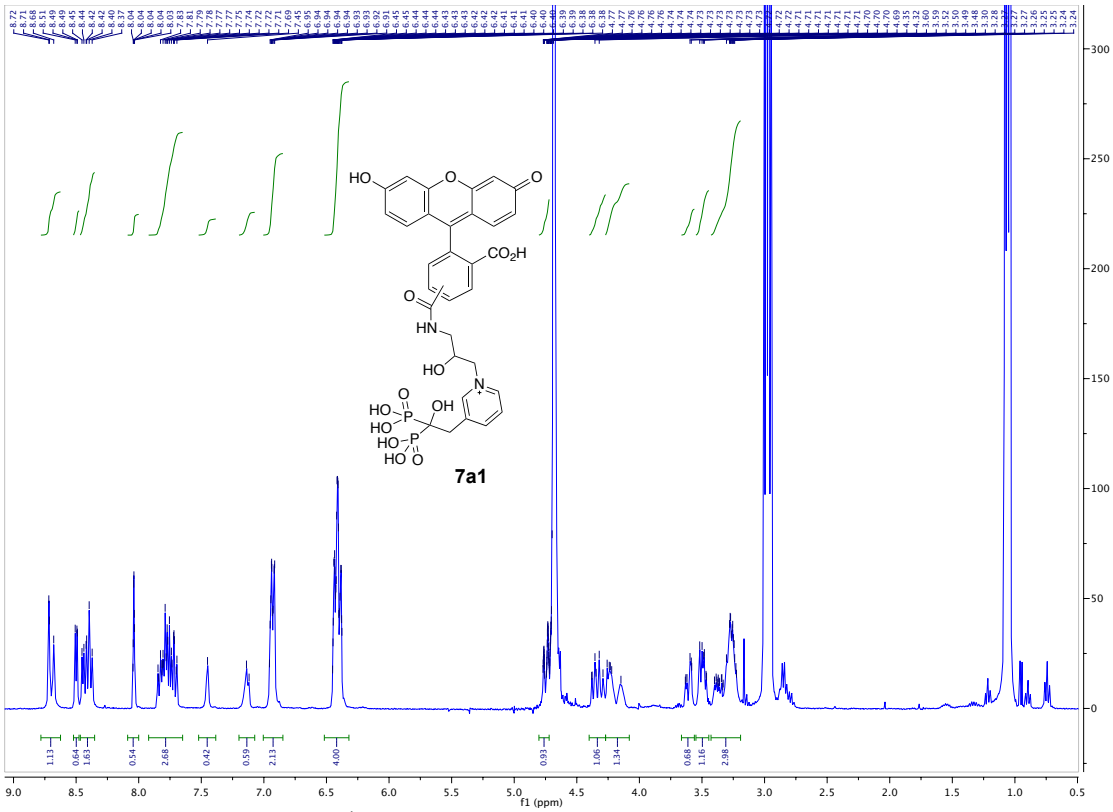


Figure A15. ^{1}H NMR (D_2O) spectrum of 5(6)-FAM-RIS, **7a1**.

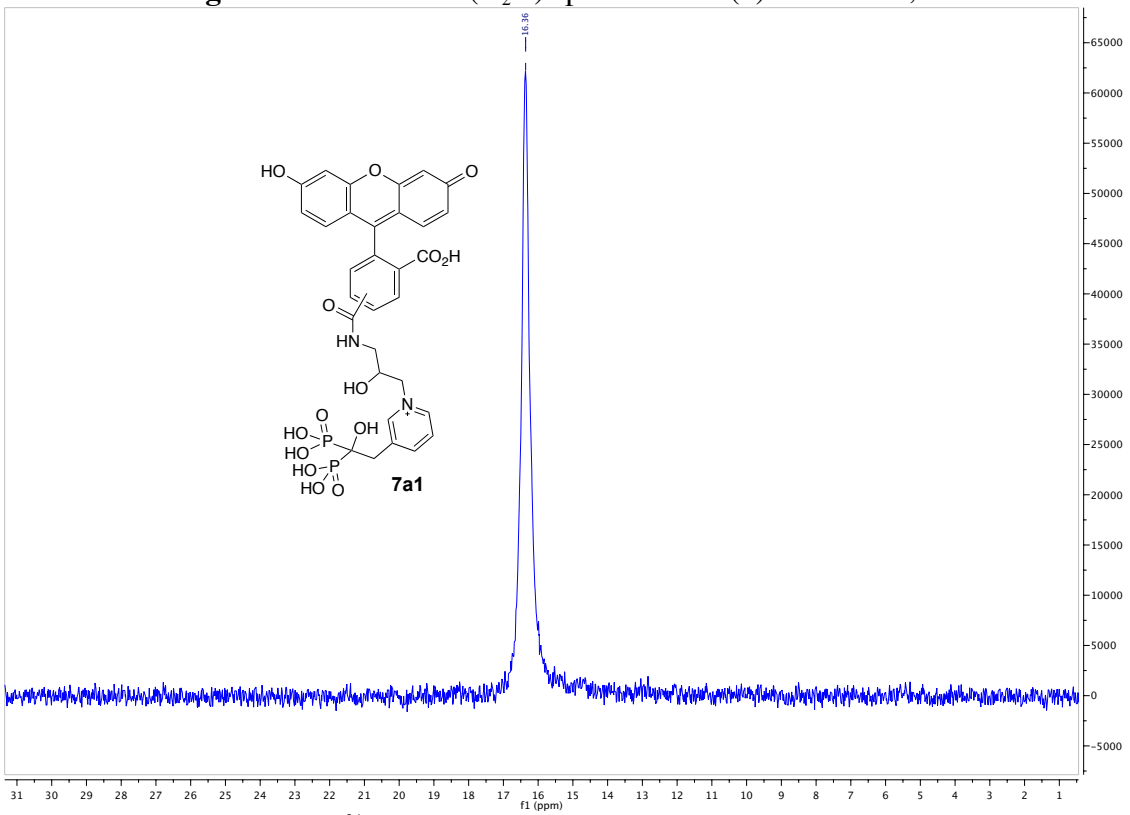


Figure A16. ^{31}P NMR (D_2O) spectrum of 5(6)-FAM-RIS, **7a1**.

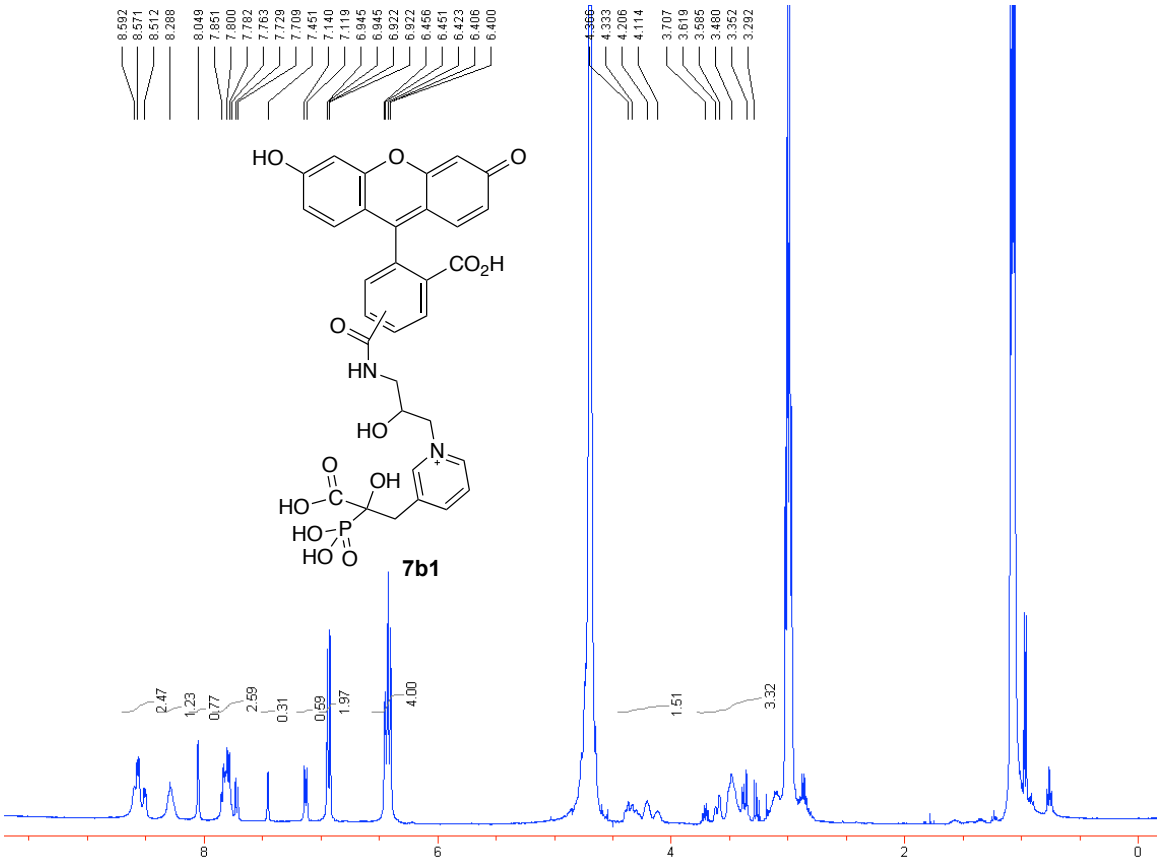


Figure A17. ^{1}H NMR (D_2O) spectrum of 5(6)-FAM-RISPC, **7b1**.

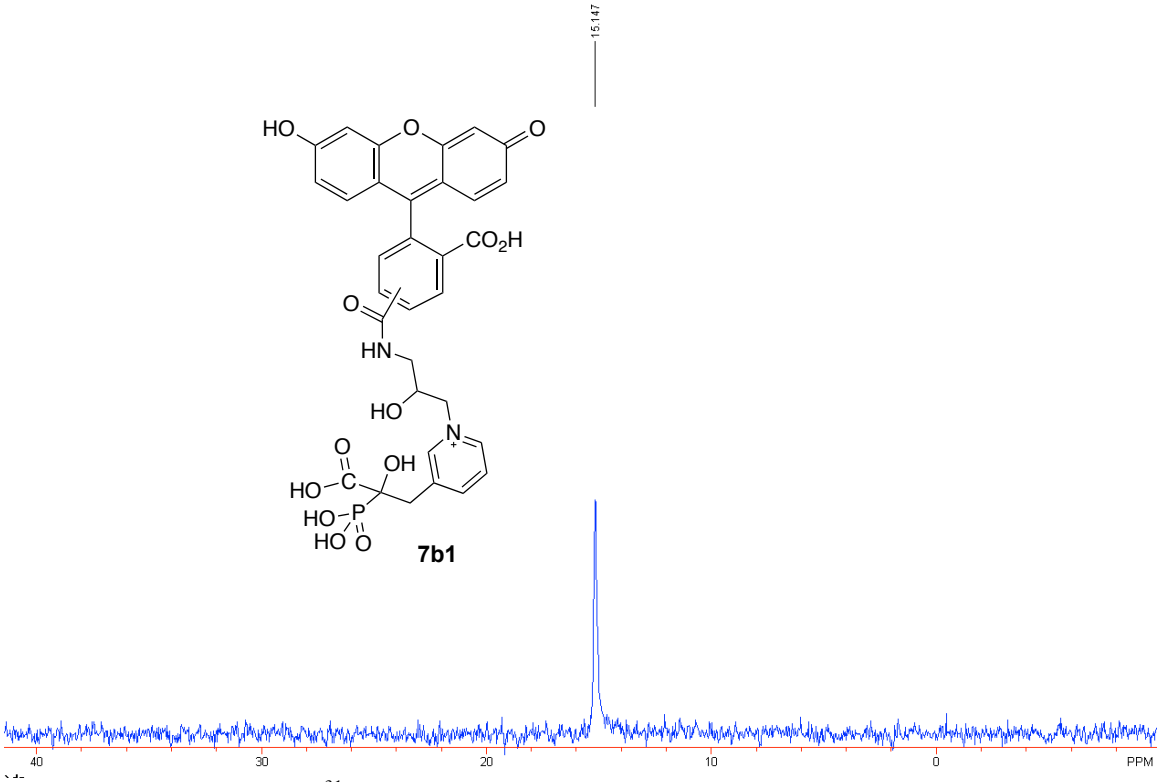


Figure A18. ^{31}P NMR (D_2O) spectrum of 5(6)-FAM-RISPC, **7b1**.

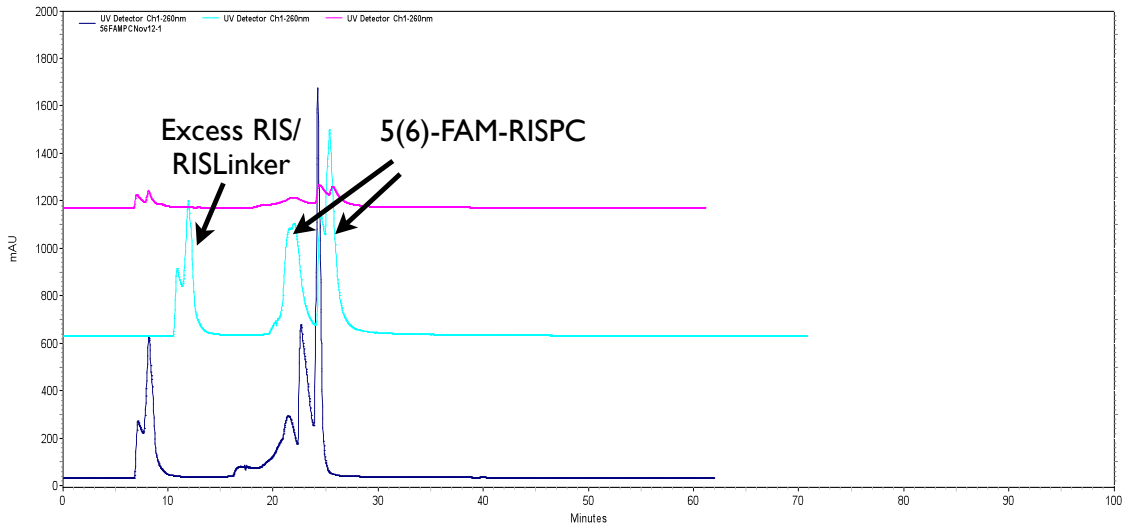


Figure A19. HPLC trace of 5(6)-FAM-RISPC, **7b1**.

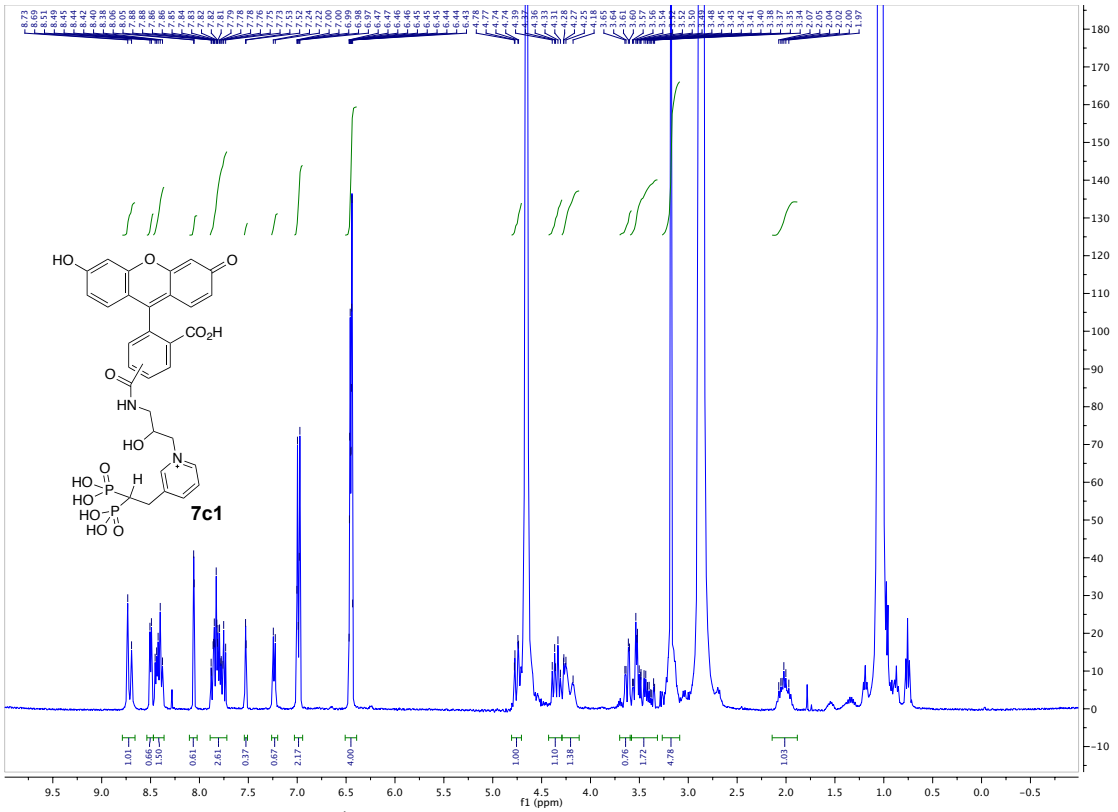


Figure A20. ^{1}H NMR (D_2O) spectrum of 5(6)-FAM-dRIS, **7c1**.

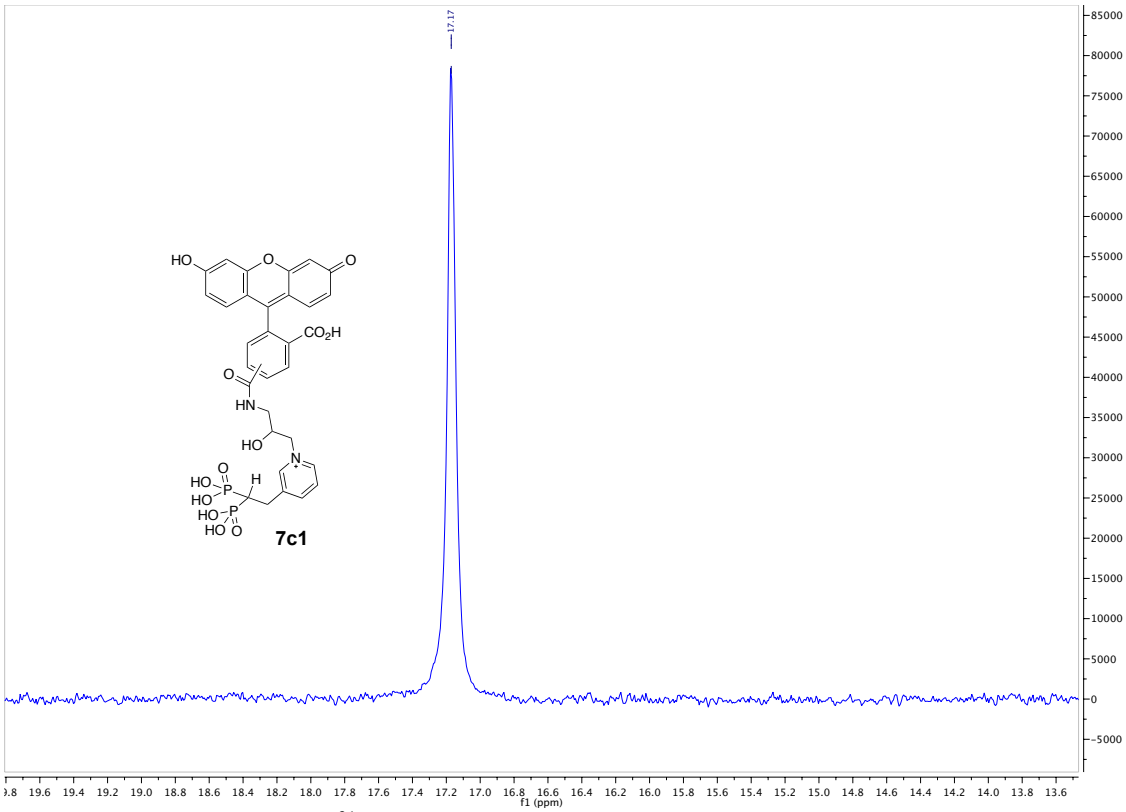


Figure A21. ^{31}P NMR (D_2O) spectrum of 5(6)-FAM-dRIS, **7c1**.

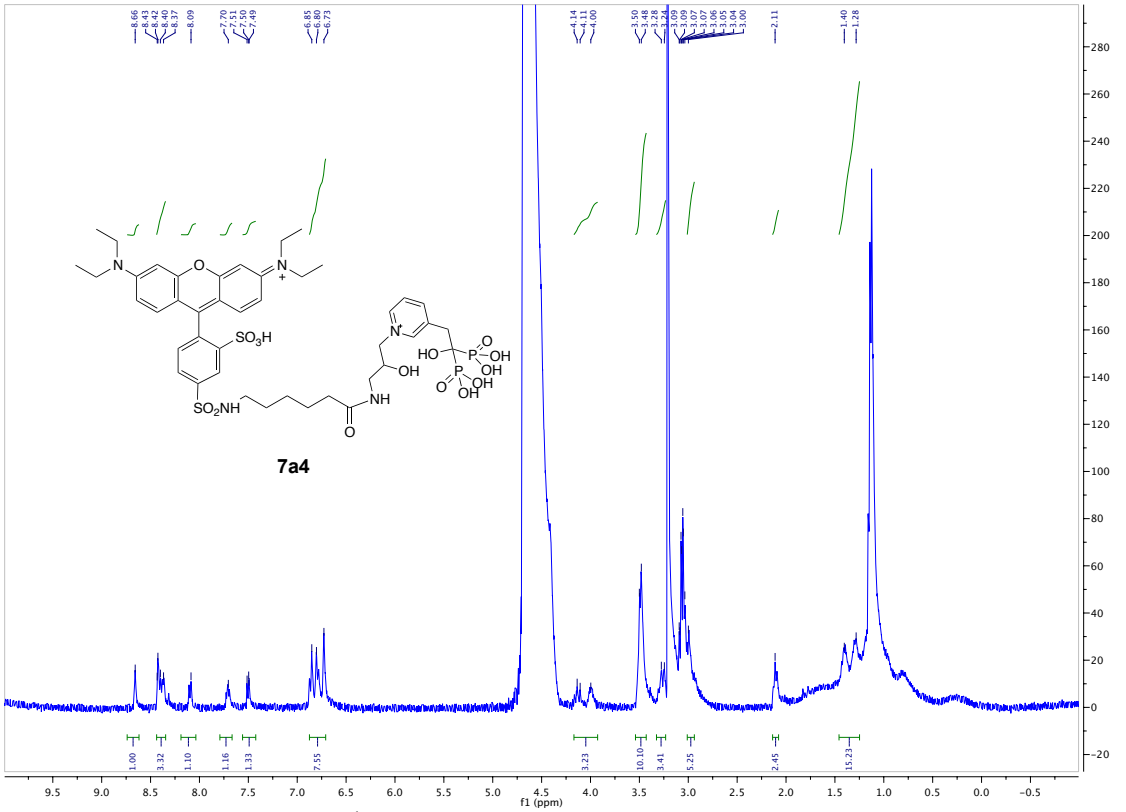


Figure A22. ^{1}H NMR (D_2O) spectrum of 5(6)-RhR-RIS, **7a4**.

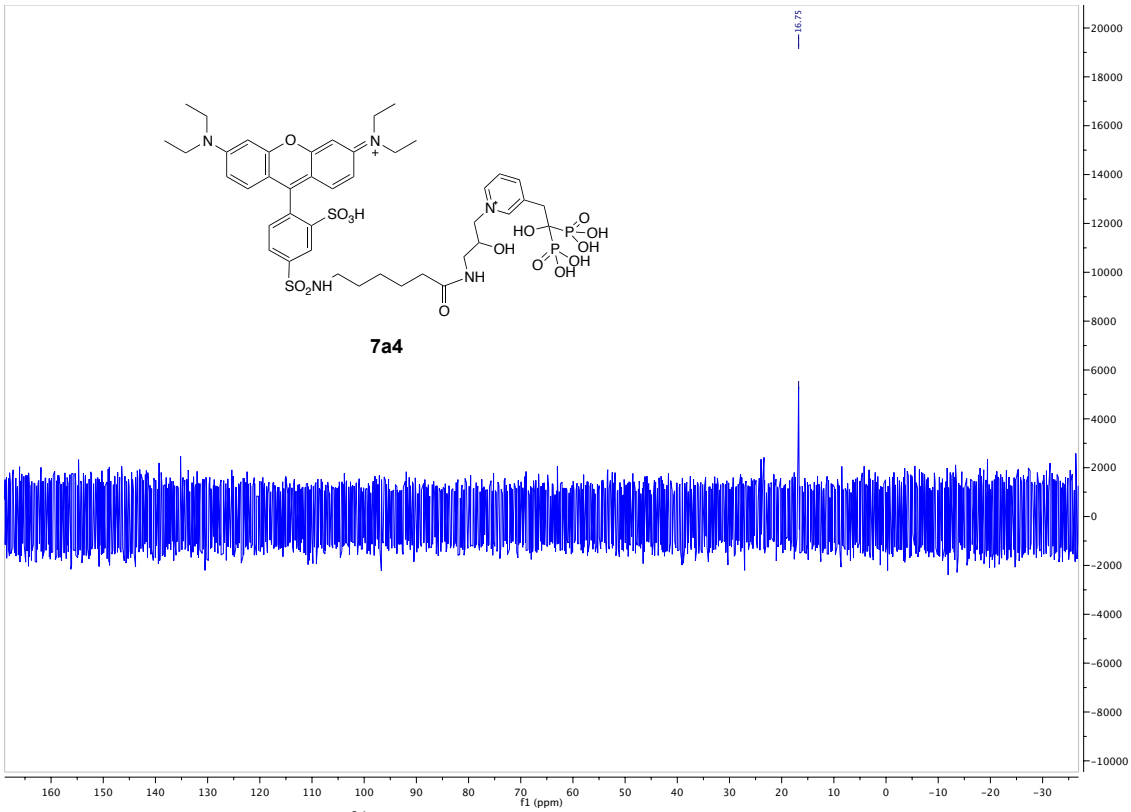
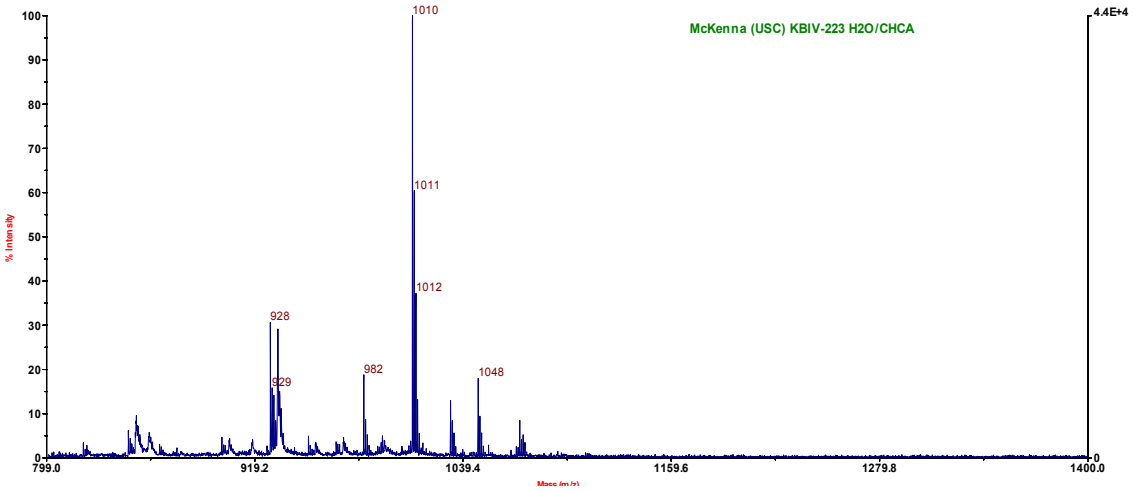
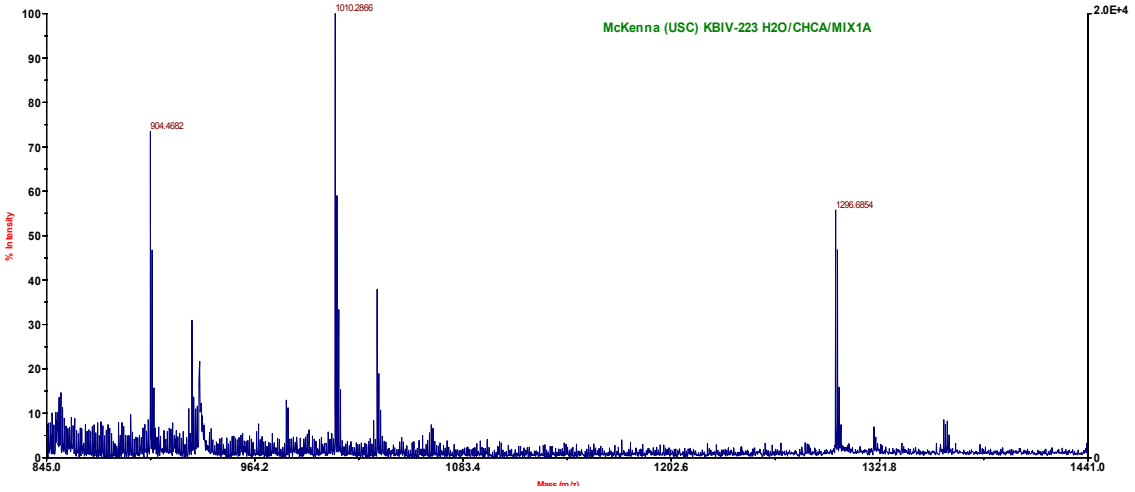


Figure A23. ^{31}P NMR (D_2O) spectrum of 5(6)-RhR-RIS, **7a4**.





Measured Mass 1010.2866

Element	Low Limit	High Limit
C	38	48
H	50	70
N	4	6
O	14	16
P	0	2
S	0	3

Formula	Calculated Mass	mDaError	ppmError	RDB
C44 H59 N4 O15 P S3	1010.2871	-0.5	-0.5	18
C40 H62 N5 O15 P2 S3	1010.2874	-0.8	-0.8	13.5
C46 H54 N6 O14 S3	1010.2855	1.1	1.1	23
C43 H58 N5 O15 P2 S2	1010.2841	2.5	2.5	18.5
C47 H55 N4 O15 P S2	1010.2838	2.8	2.8	23

Figure A24. Mass spectrum of 5(6)-RhR-RIS, **7a4**.

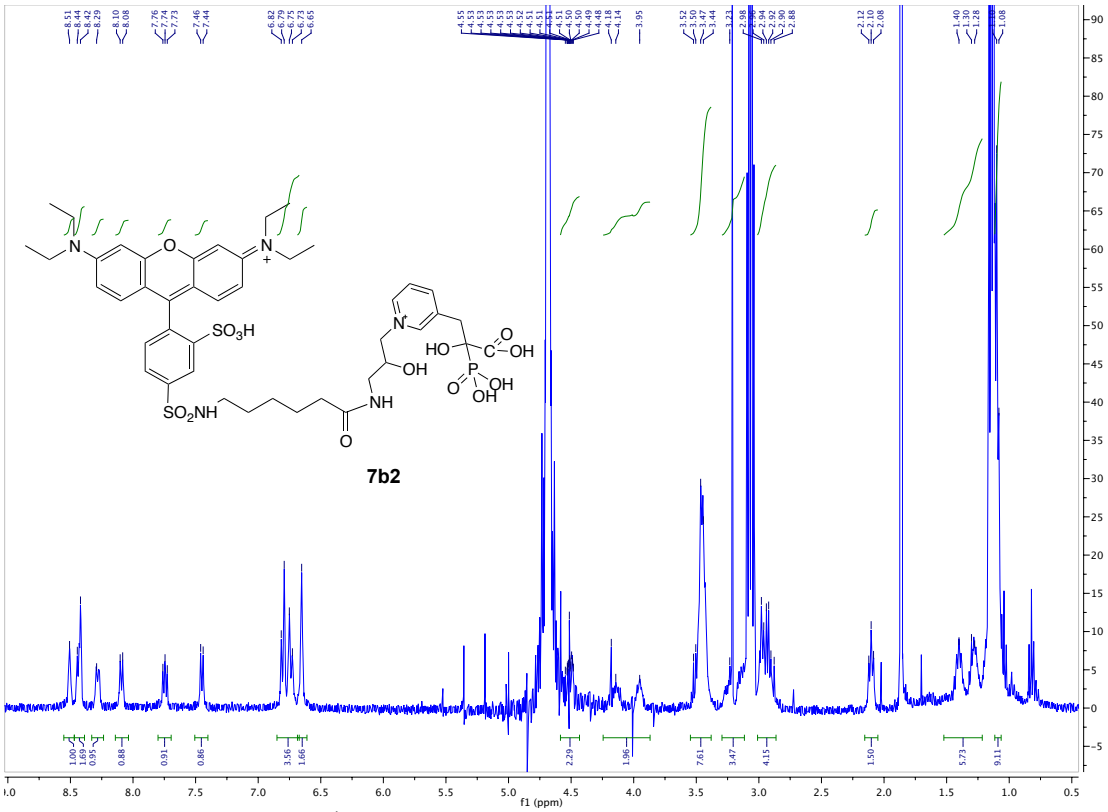


Figure A25. ^{1}H NMR (D_2O) spectrum of 5(6)-RhR-RISPC, **7b2**.

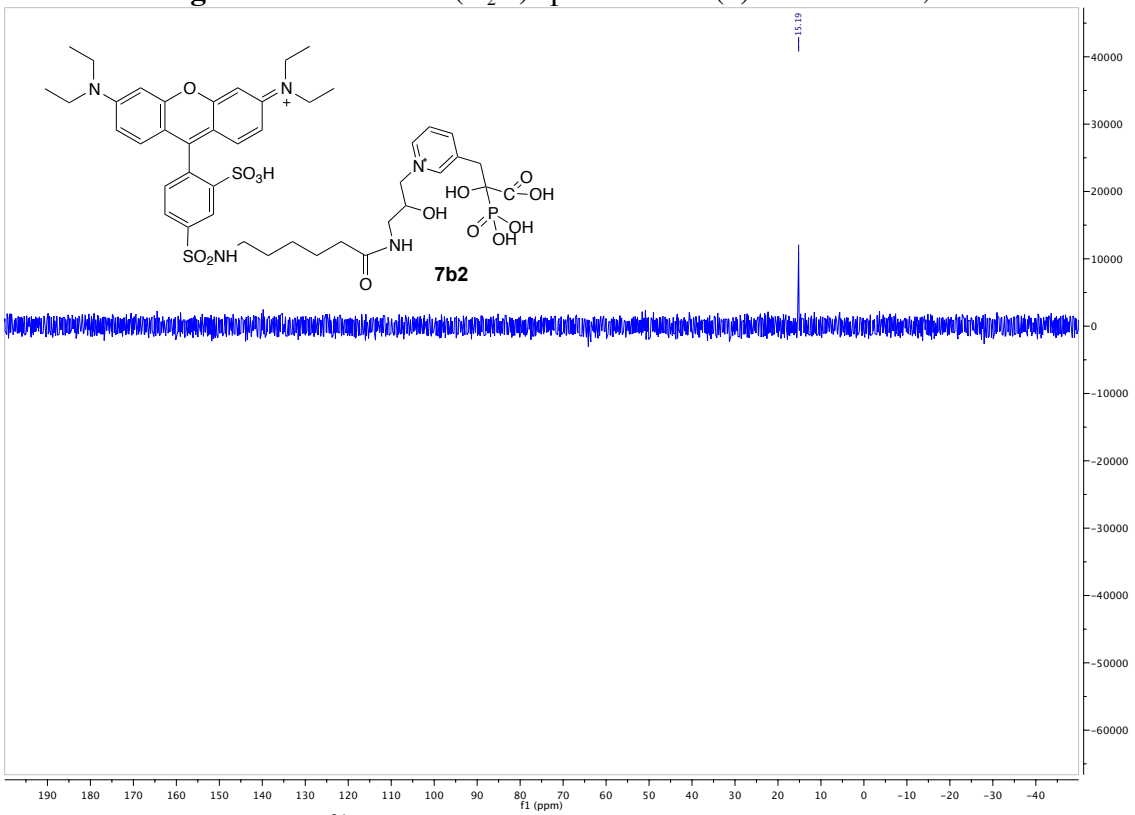


Figure A26. ^{31}P NMR (D_2O) spectrum of 5(6)-RhR-RISPC, **7b2**.

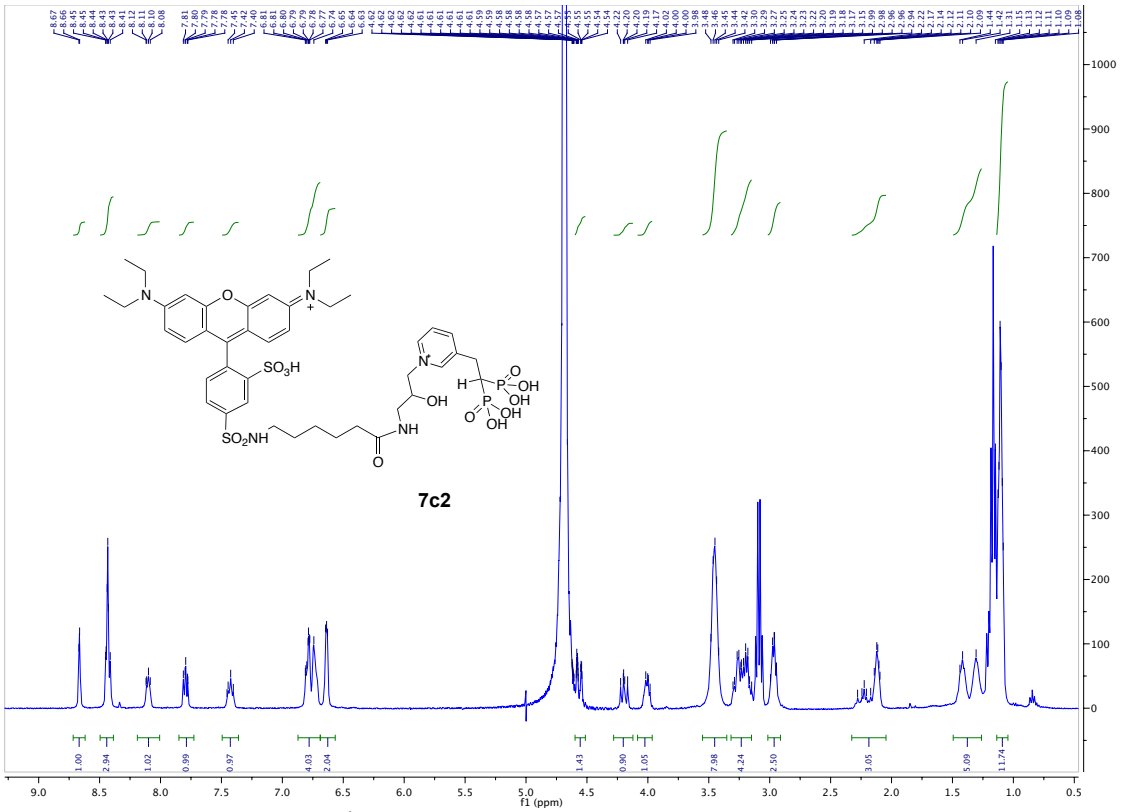


Figure A27. ^{1}H NMR (D_2O) spectrum of 5(6)-RhR-dRIS, **7c2**.

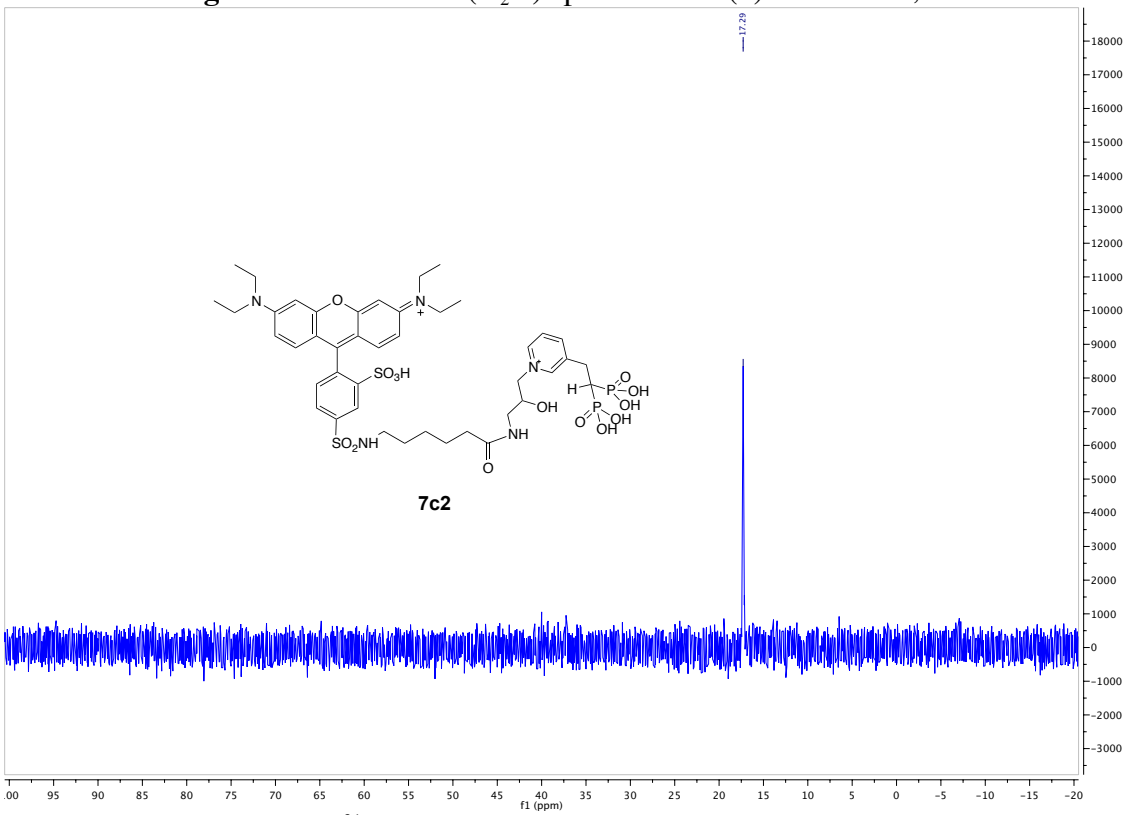
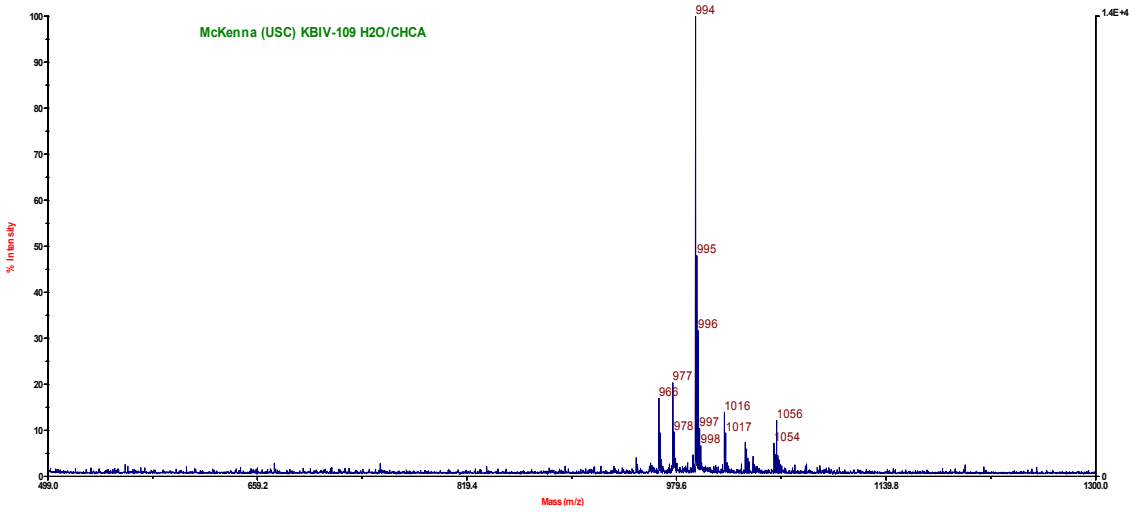
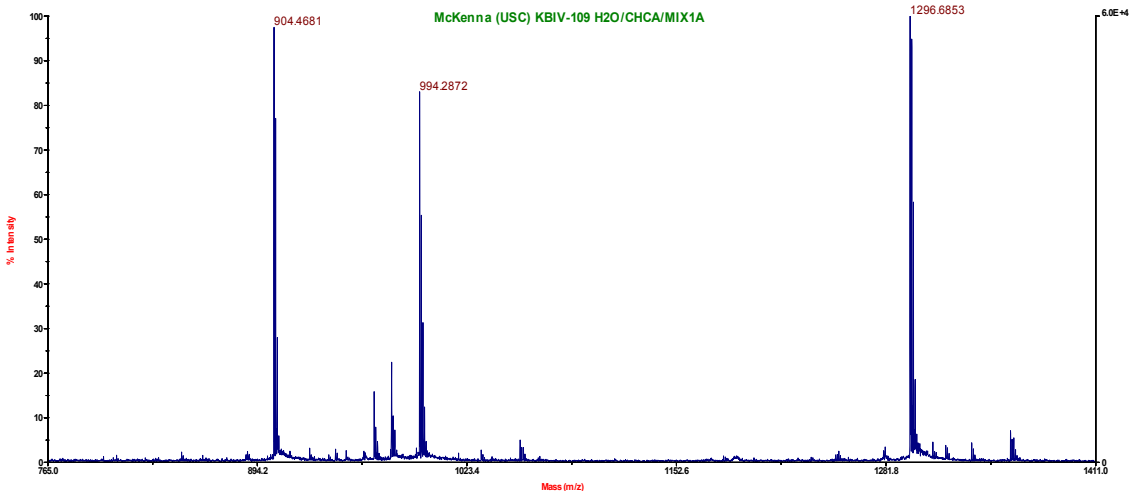


Figure A28. ^{31}P NMR (D_2O) spectrum of 5(6)-RhR-dRIS, **7c2**.





Measured Mass 994.2872

Element	Low Limit	High Limit
C	38	48
H	50	70
N	3	7
O	12	16
P	0	2
S	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C45 H53 N7 O13 P S2	994.2875	-0.3	-0.3	23.5
C46 H54 N5 O14 P2 S	994.2858	1.4	1.4	23.5
C47 H55 N4 O14 P S2	994.2888	-1.6	-1.6	23
C43 H58 N5 O14 P2 S2	994.2891	-1.9	-2.0	18.5
C38 H58 N7 O16 P2 S2	994.2851	2.1	2.1	14.5
C42 H55 N6 O16 P S2	994.2848	2.4	2.4	19
C46 H52 N5 O16 S2	994.2845	2.7	2.7	23.5
C45 H52 N6 O16 P2	994.2910	-3.8	-3.8	24

Figure A29. Mass spectrum of 5(6)-RhR-dRIS, **7c2**.

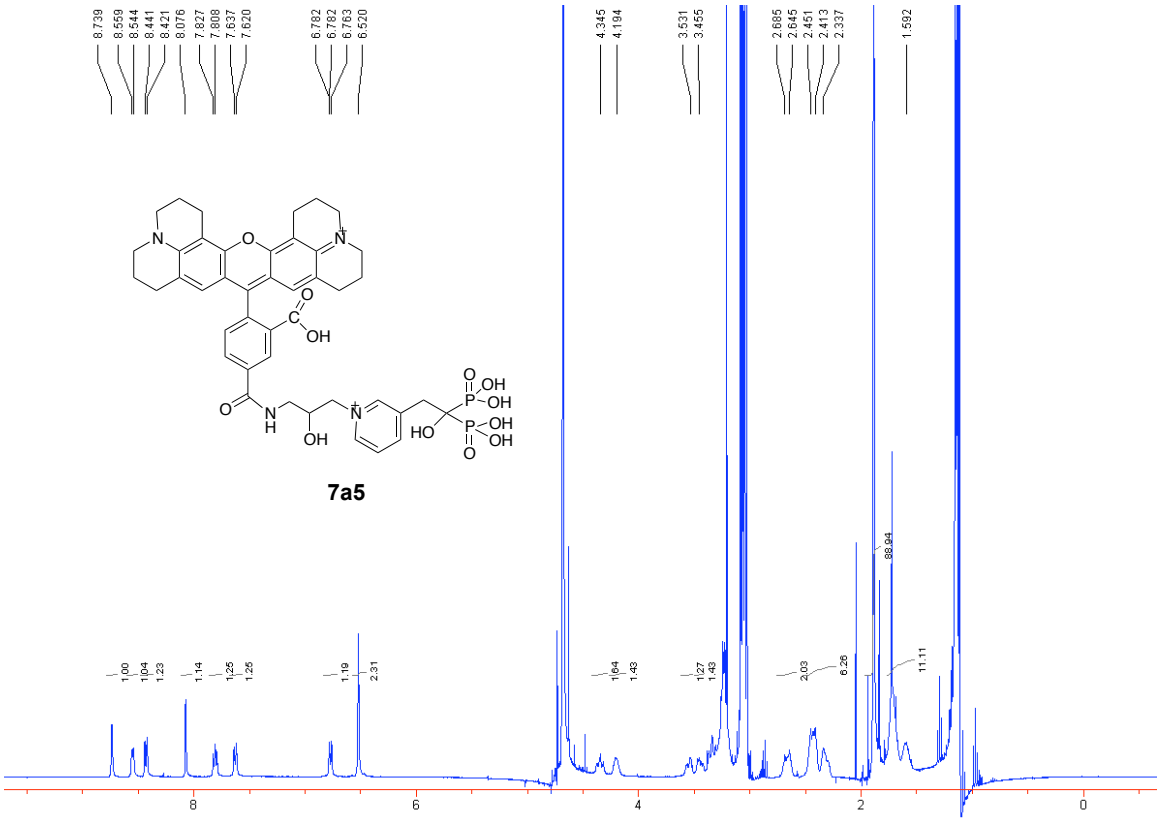


Figure A30. ^{1}H NMR (D_2O) spectrum of 5-ROX-RIS, **7a5**.

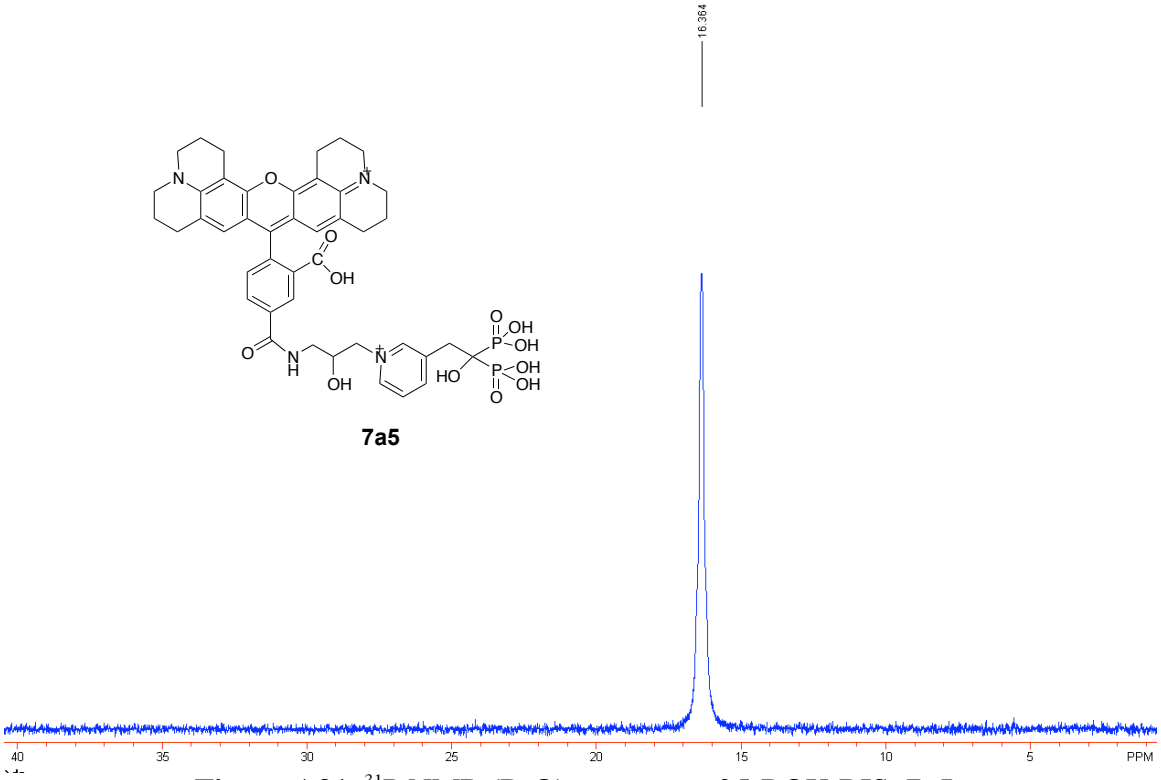
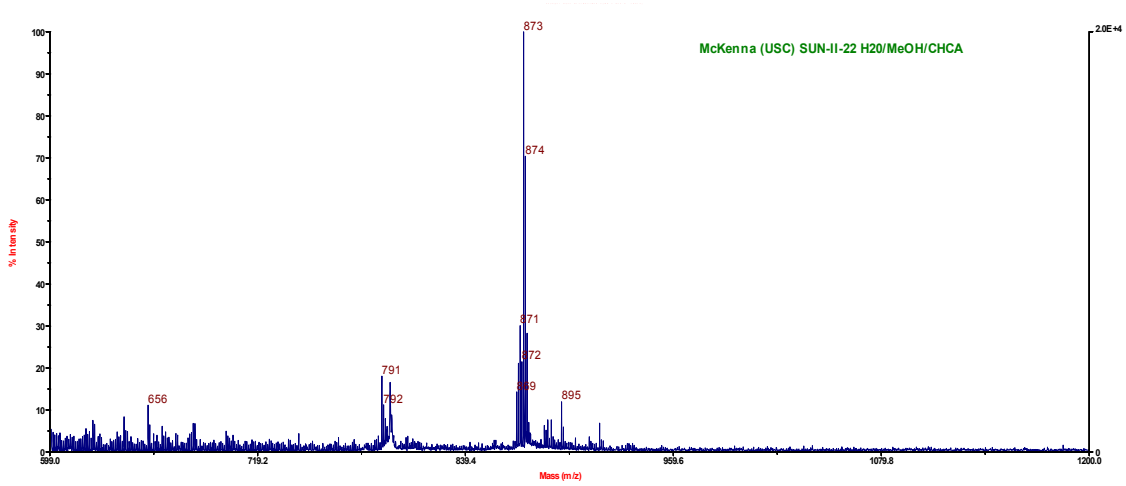
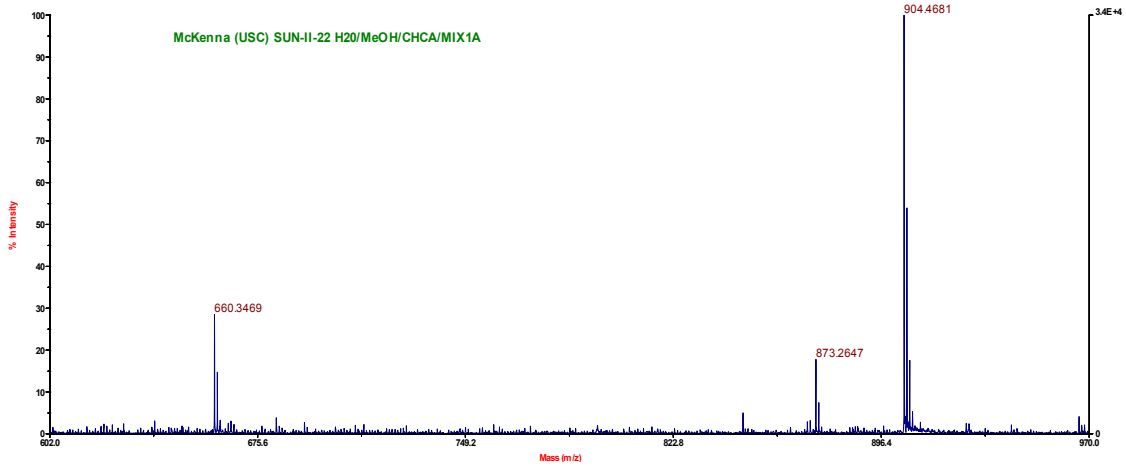


Figure A31. ^{31}P NMR (D_2O) spectrum of 5-ROX-RIS, **7a5**.





Measured Mass 873.2647

Element	Low Limit	High Limit
C	38	48
H	35	55
N	3	5
O	10	14
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C47 H44 N3 O12 P	873.2657	-1.0	-1.2	28
C43 H47 N4 O12 P2	873.2660	-1.3	-1.5	23.5
C42 H44 N5 O14 P	873.2617	3.0	3.4	24
C46 H41 N4 O14	873.2614	3.3	3.8	28.5
C47 H45 N3 O10 P2	873.2575	7.2	8.3	28

Figure A32. Mass spectrum of 5-ROX-RIS, **7a5**.

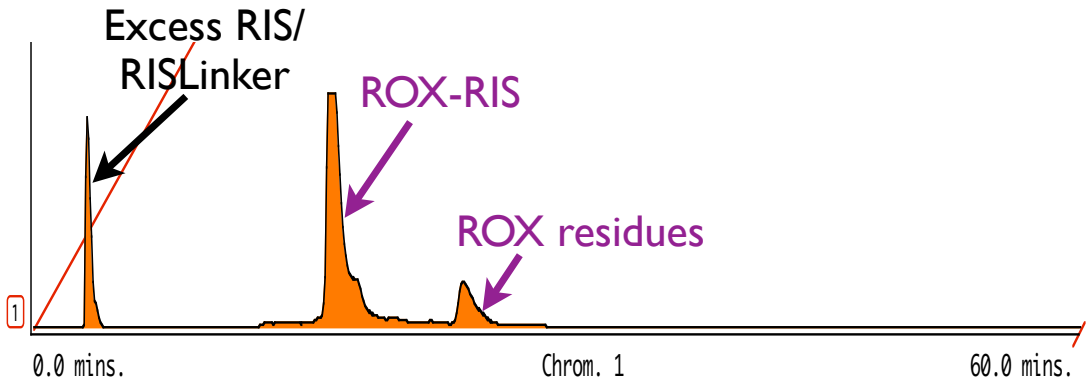


Figure A33. HPLC trace of 5-ROX-RIS, **7a5**.

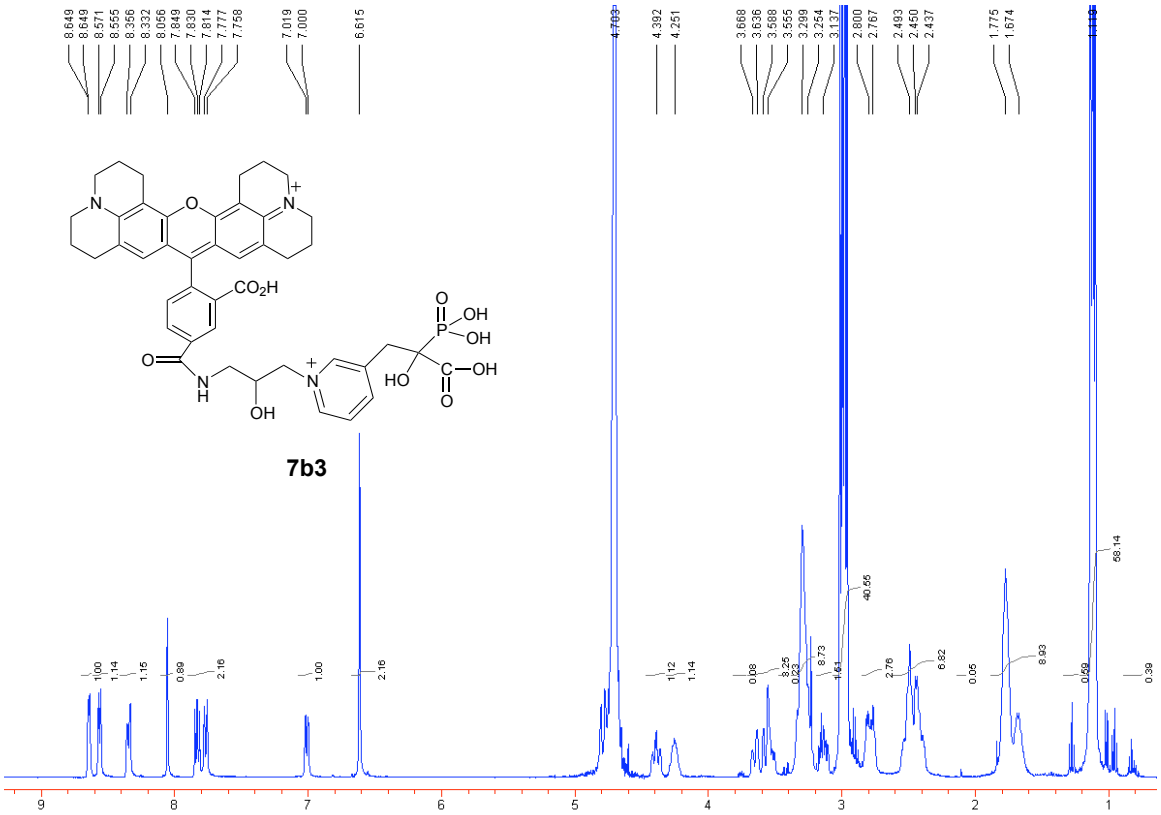
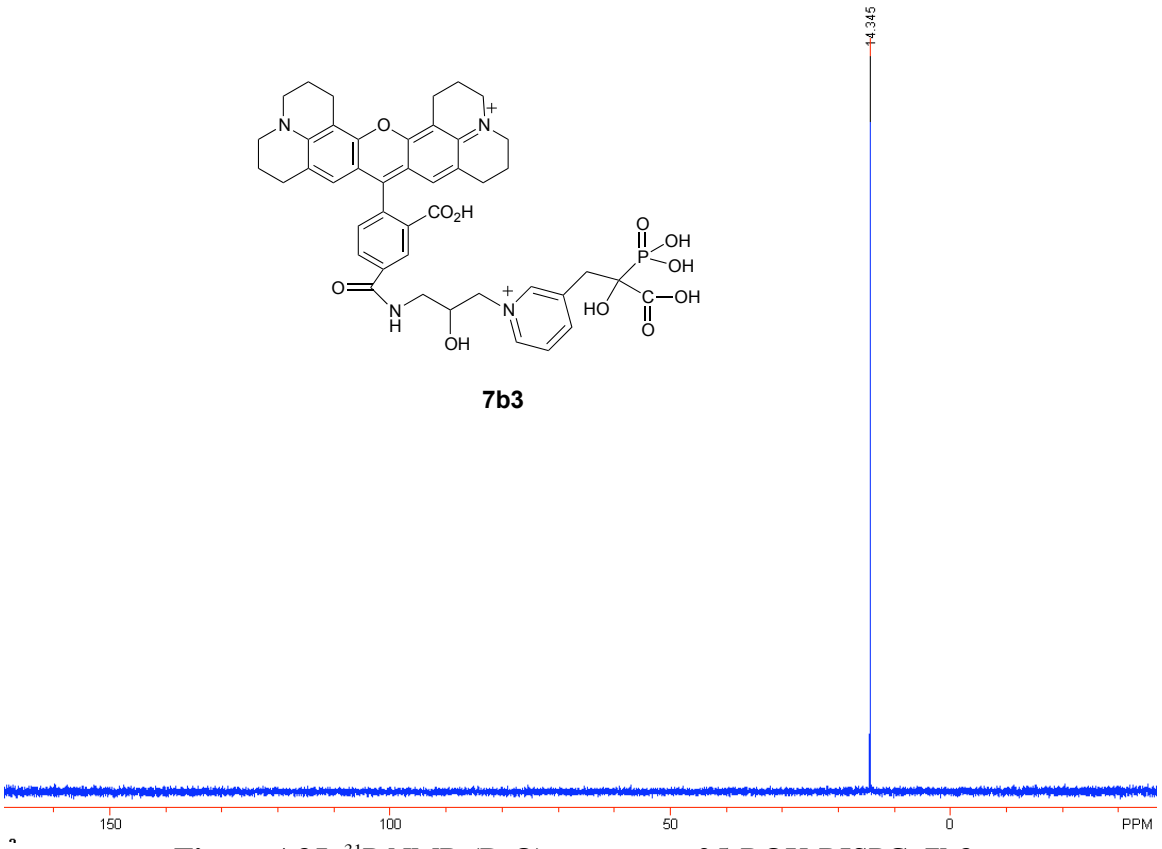
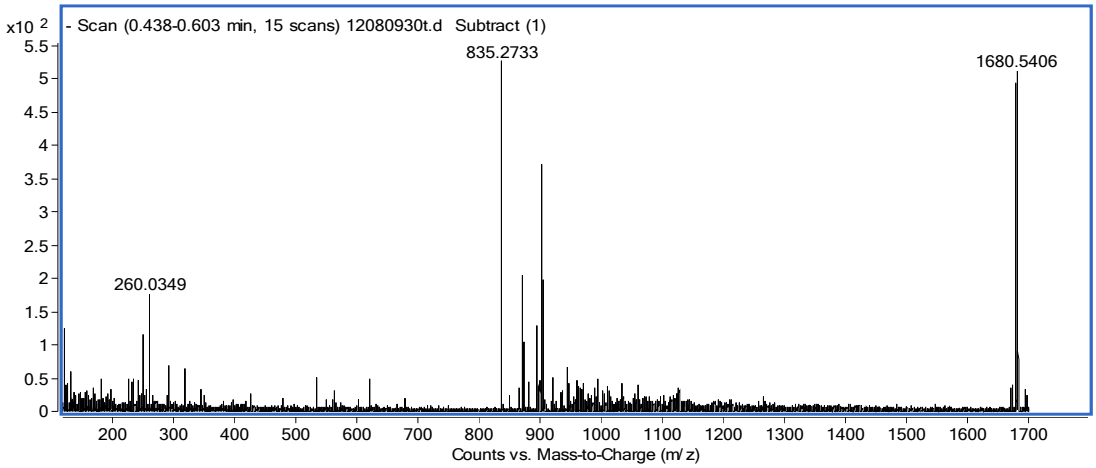


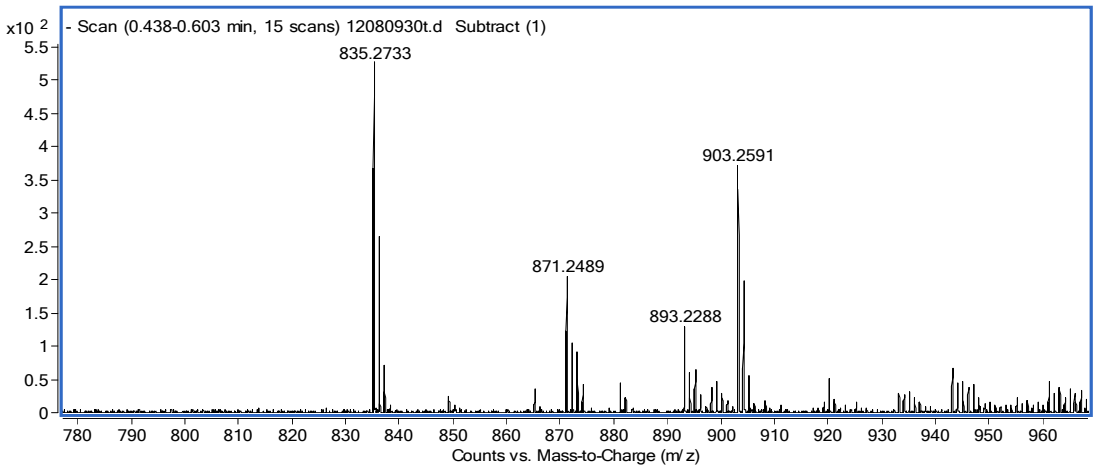
Figure A34. ^{1}H NMR (D_2O) spectrum of 5-ROX-RISPC, **7b3**.



7b3

Figure A35. ^{31}P NMR (D_2O) spectrum of 5-ROX-RISPC, **7b3**.





Measured Mass 835.2733

Element	Low Limit	High Limit
C	39	49
H	35	55
N	0	5
O	9	13
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C48 H41 N3 O11	835.2747	-1.4	-1.6	30
C44 H44 N4 O11 P	835.2750	-1.7	-2.0	25.5
C40 H47 N5 O11 P2	835.2753	-2.0	-2.4	21
C43 H41 N5 O13	835.2706	2.7	3.2	26
C46 H46 N O12 P	835.2763	-3.0	-3.6	25
C42 H49 N2 O12 P2	835.2766	-3.3	-4.0	20.5

Figure A36. Mass spectrum of 5-ROX-RISPC, **7b3**.

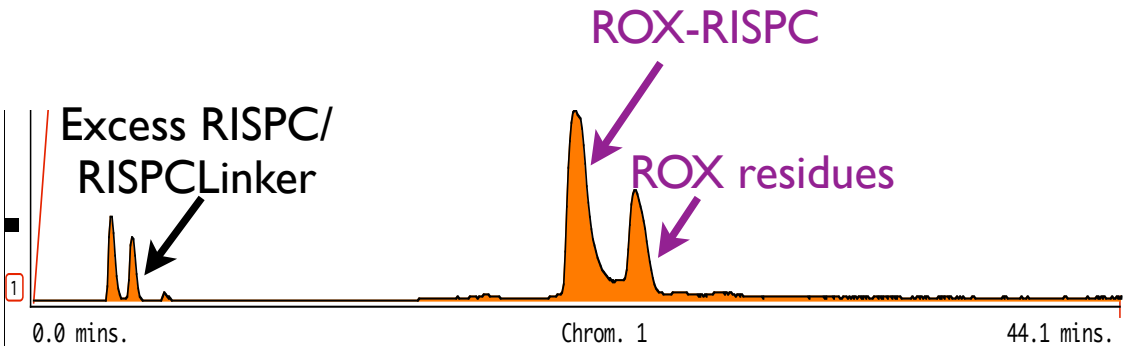


Figure A37. HPLC trace of 5-ROX-RISPC, **7b3**.

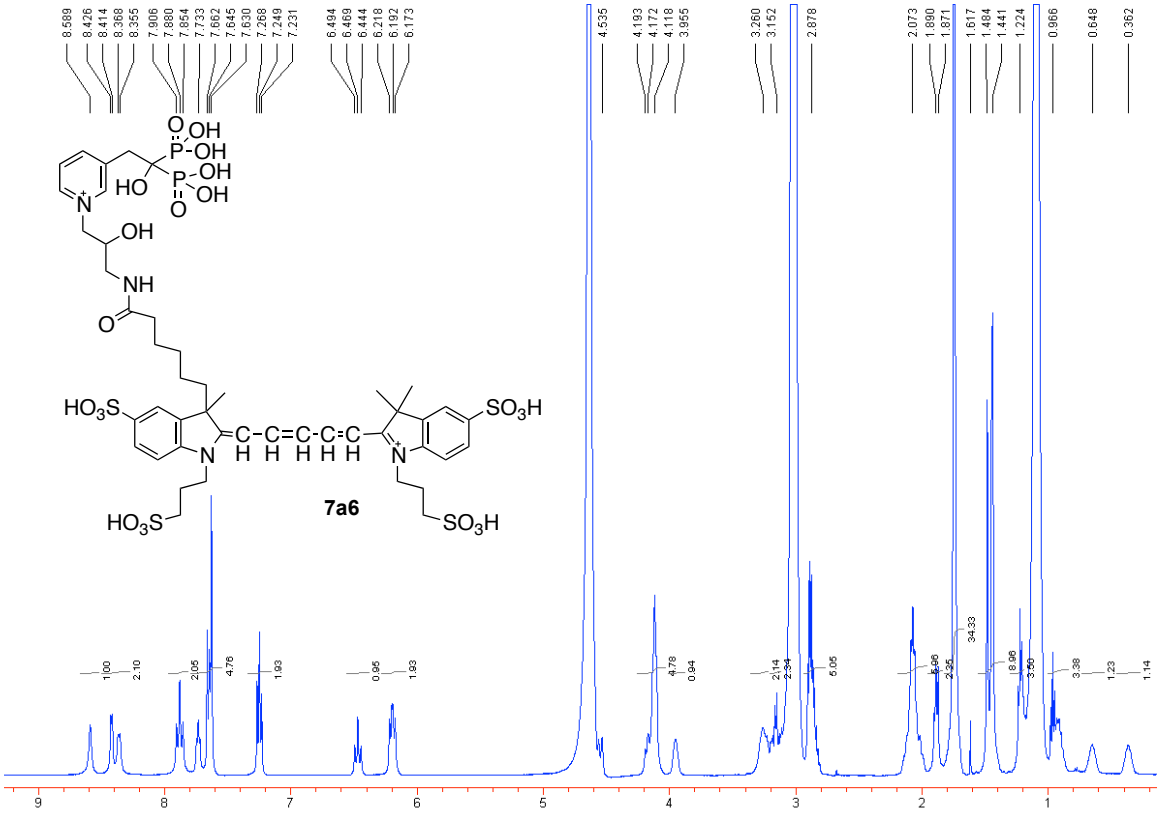


Figure A38. ^{1}H NMR (D_2O) spectrum of AF647-RIS, **7a6**.

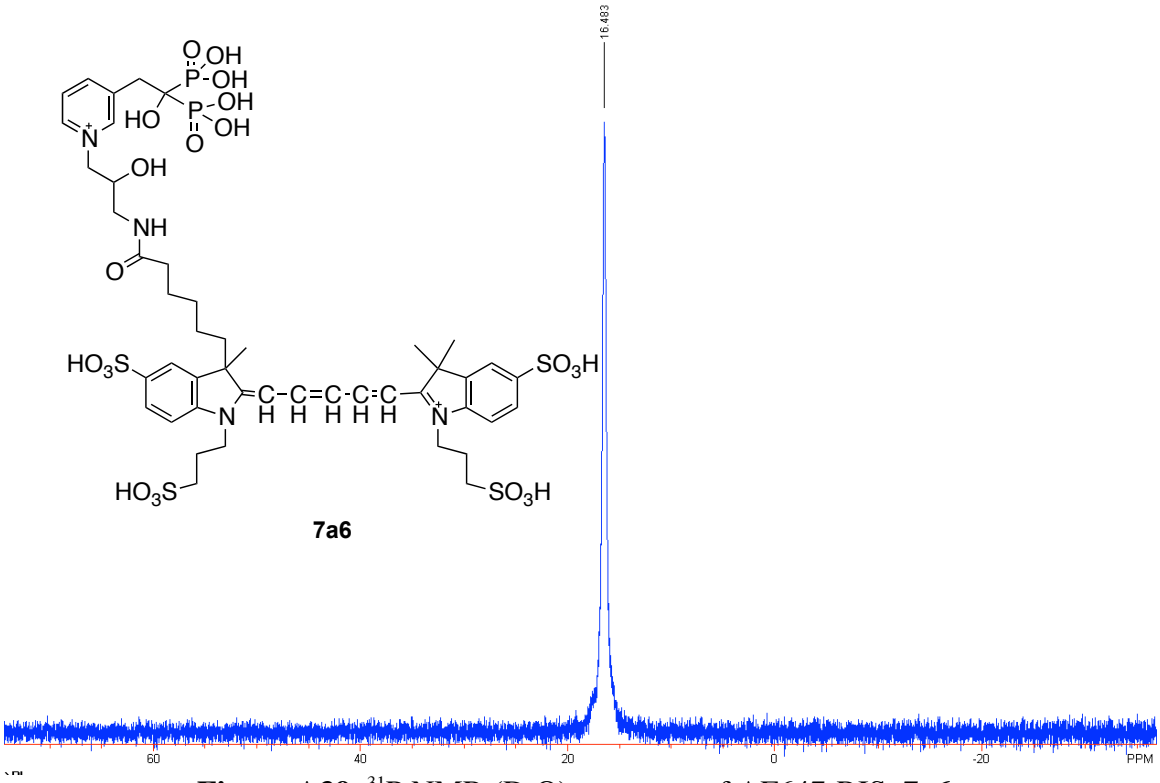
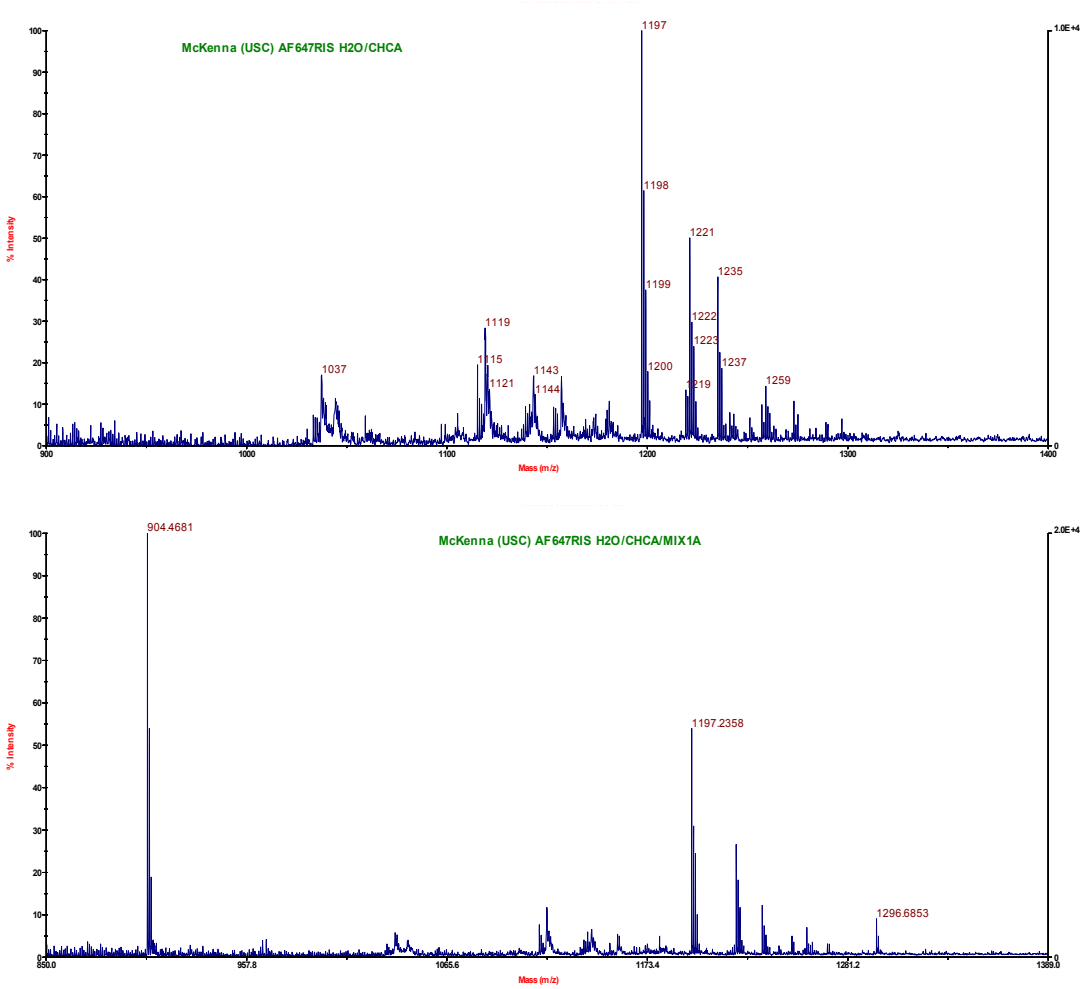


Figure A39. ^{31}P NMR (D_2O) spectrum of AF647-RIS, **7a6**.



Measured Mass 1197.2358

Element	Low Limit	High Limit
C	41	51
H	55	75
N	3	5
O	19	23
P	0	2
S	3	5

Formula	Calculated Mass	mDaError	ppmError	RDB
C49 H59 N5 O20 S5	1197.2352	0.6	0.5	23
C47 H64 N3 O21 P S5	1197.2368	-1.0	-0.8	18
C43 H67 N4 O21 P2 S5	1197.2371	-1.3	-1.1	13.5
C46 H63 N4 O21 P2 S4	1197.2337	2.1	1.7	18.5
C50 H60 N3 O21 P S4	1197.2334	2.4	2.0	23
C49 H58 N4 O23 P S3	1197.2386	-2.8	-2.4	23.5
C42 H64 N5 O23 P S5	1197.2328	3.0	2.5	14
C45 H61 N5 O23 P2 S3	1197.2389	-3.1	-2.6	19
C46 H61 N4 O23 S5	1197.2325	3.3	2.8	18.5
C49 H59 N4 O21 P2 S3	1197.2304	5.4	4.5	23.5
C50 H59 N3 O23 S4	1197.2417	-5.9	-4.9	23

Figure A40. Mass spectrum of AF647-RIS, **7a6**.

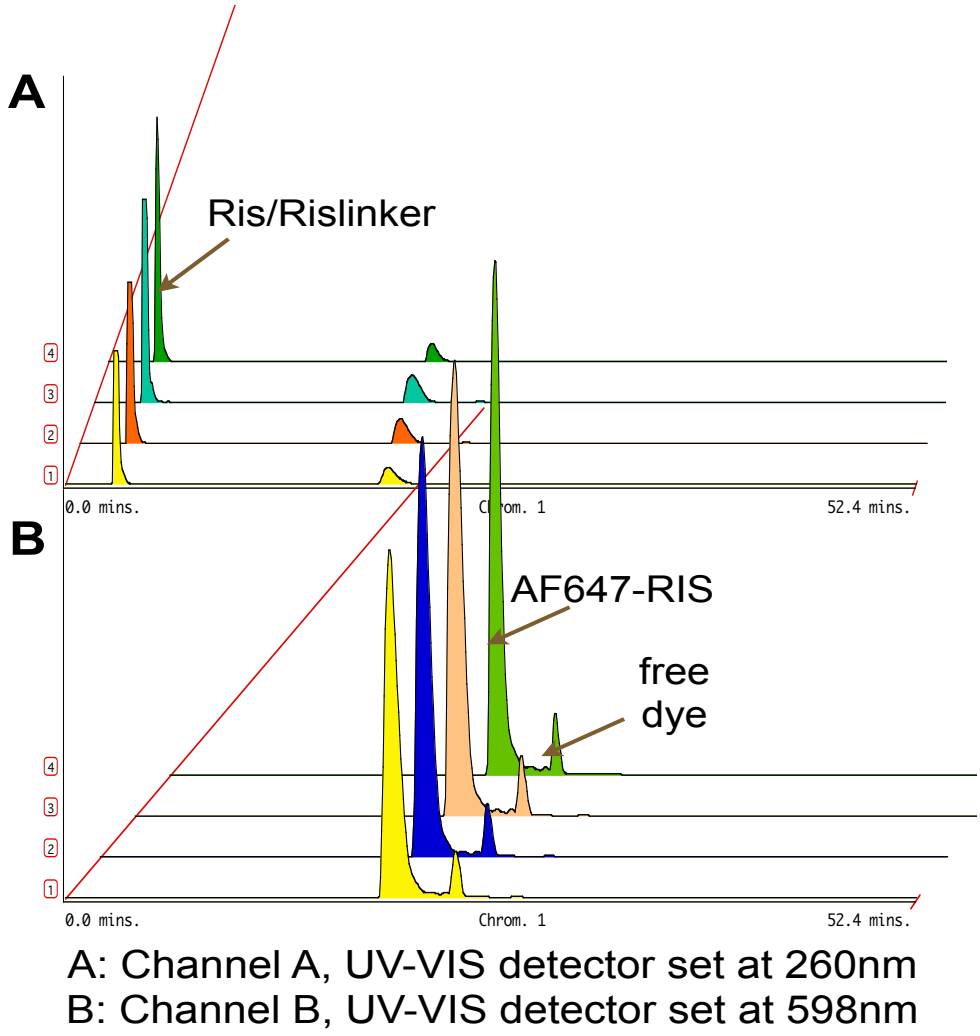


Figure A41. HPLC trace of AF647-RIS, **7a6**.

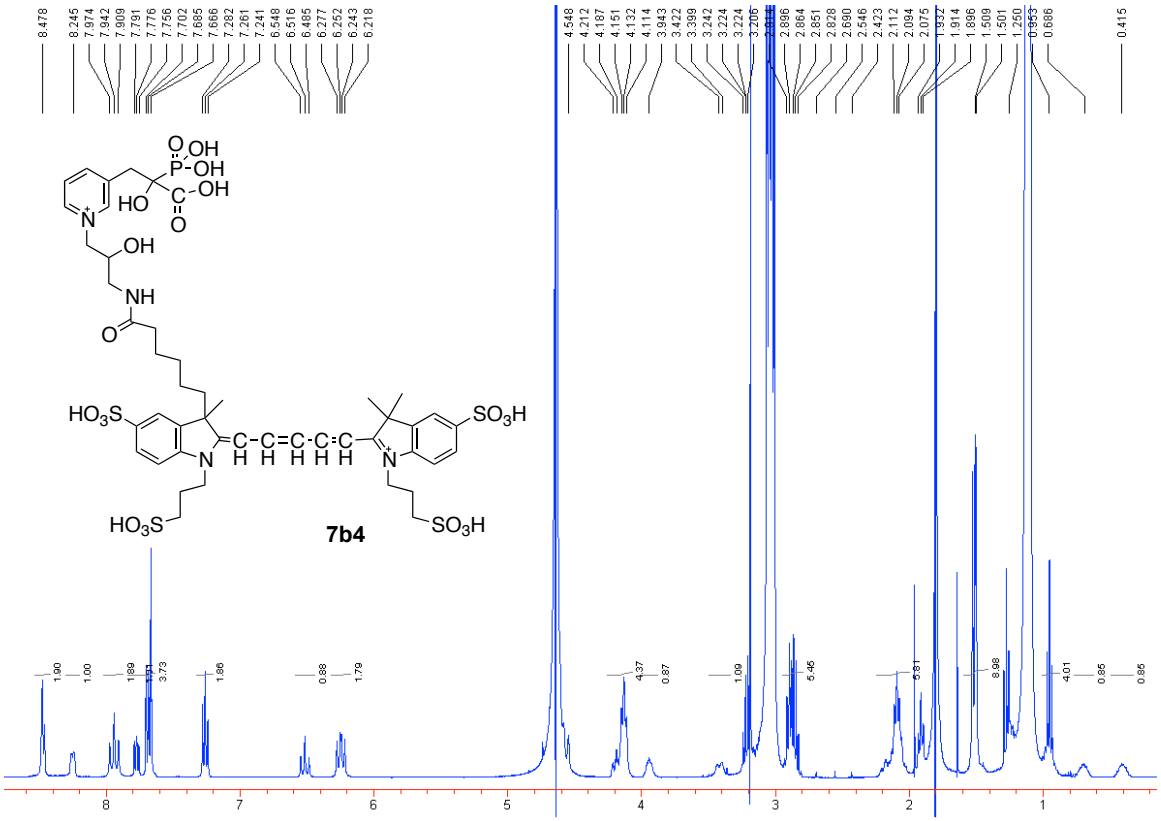


Figure A42. ^{1}H NMR (D_2O) spectrum of AF647-RISPC, **7b4**.

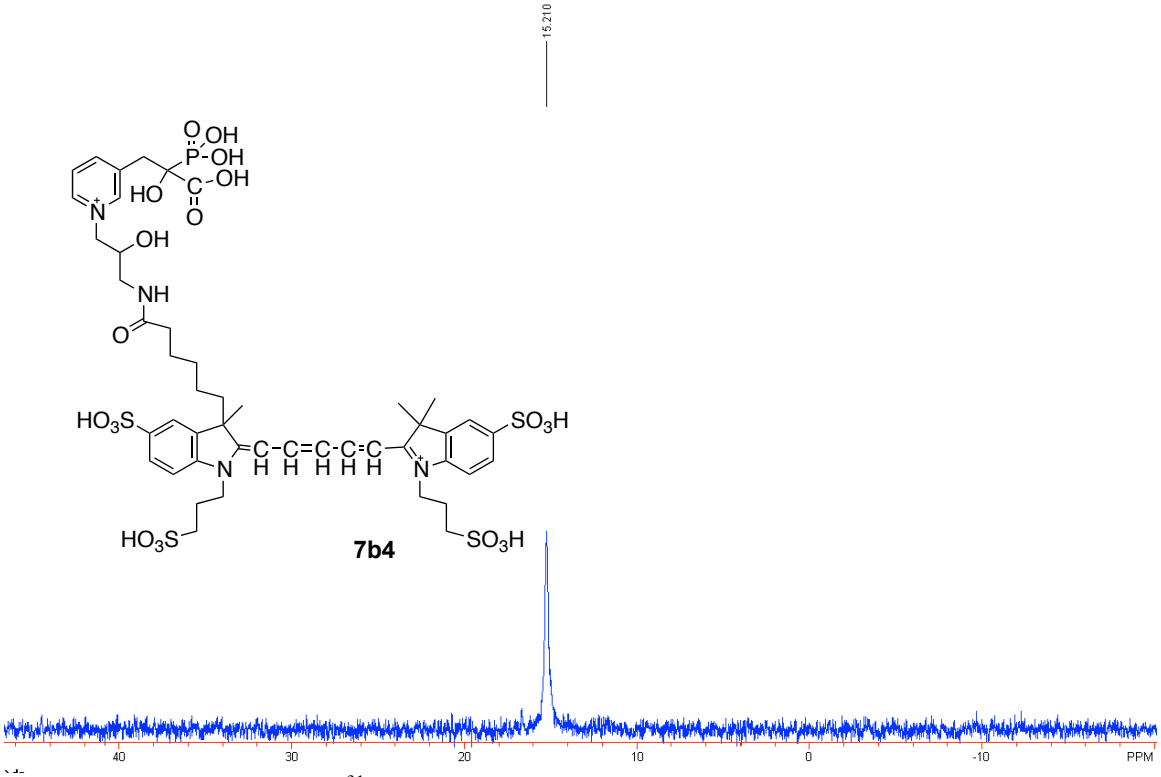
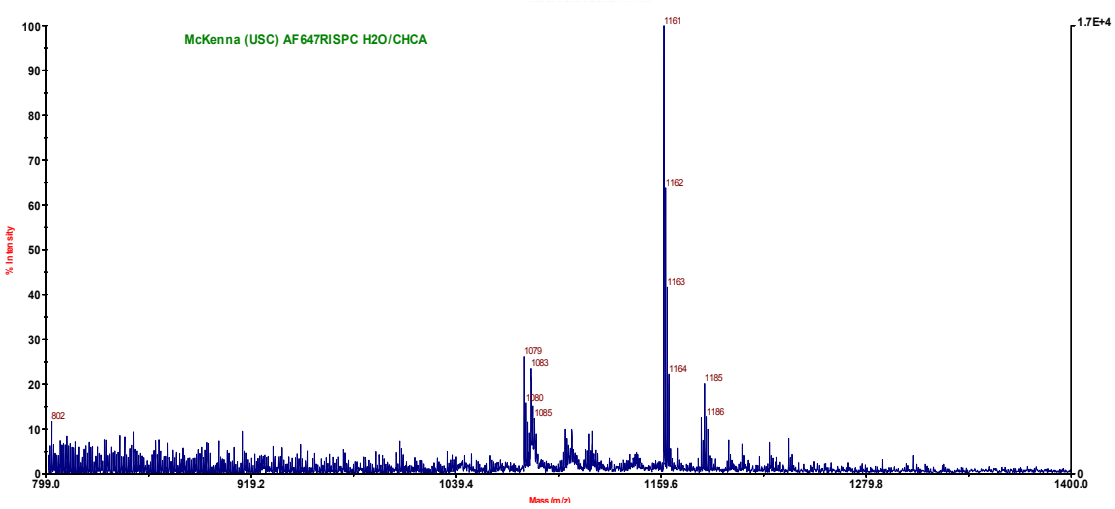
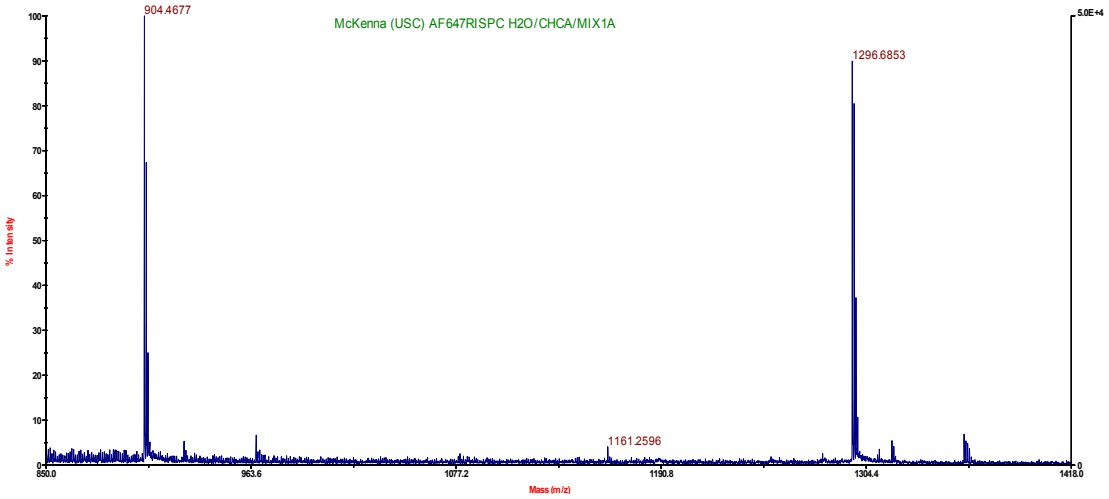


Figure A43. ^{31}P NMR (D_2O) spectrum of AF647-RISPC, **7b4**.



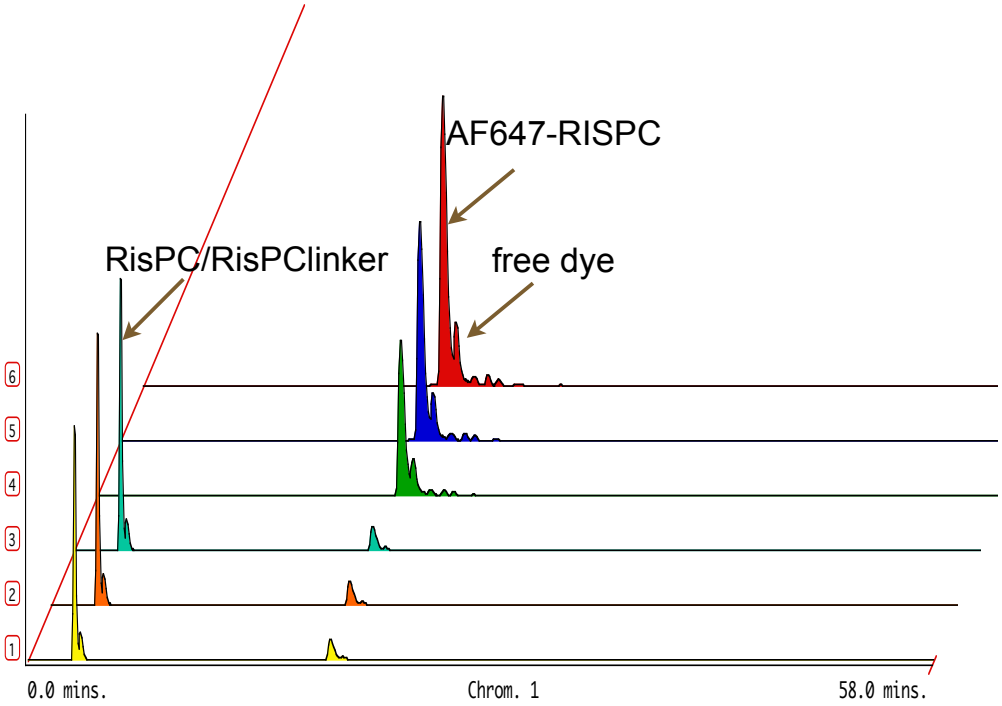


Measured Mass 1161.2596

Element	Low Limit	High Limit
C	42	52
H	55	75
N	3	5
O	19	21
P	0	1
S	3	5

Formula	Calculated Mass	mDaError	ppmError	RDB
C48 H63 N3 O20 S5	1161.2603	-0.7	-0.6	19
C44 H66 N4 O20 P S5	1161.2606	-1.0	-0.9	14.5
C47 H62 N4 O20 P S4	1161.2572	2.4	2.0	19.5
C51 H59 N3 O20 S4	1161.2569	2.7	2.3	24
C50 H58 N4 O20 P S3	1161.2539	5.7	4.9	24.5

Figure A44. Mass spectrum of AF647-RISPC, **7b4**.



HPLC trace of AF647-RISPC
1-3: channel A 260nm
4-6: channel B 598nm

Figure A45. HPLC trace of AF647-RISPC, **7b4**.

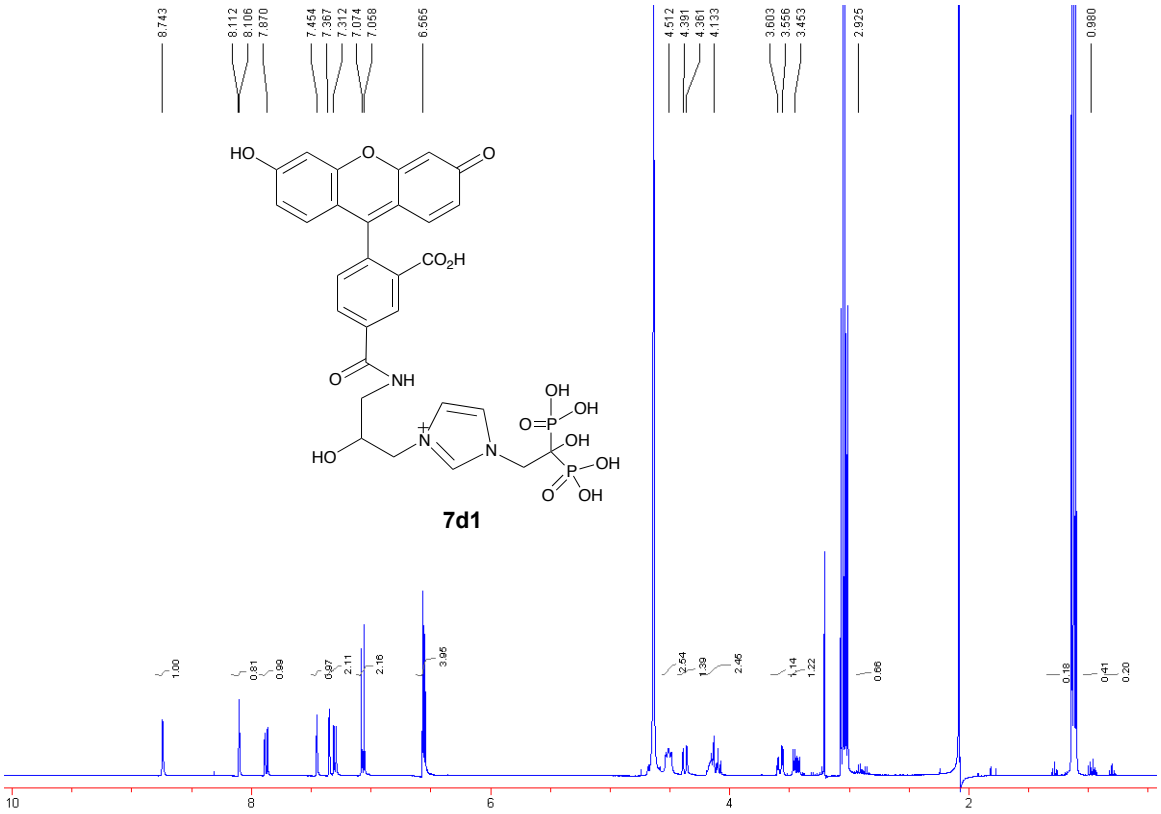


Figure A46. ^{1}H NMR (D_2O) spectrum of 5-FAM-ZOL, **7d1**.

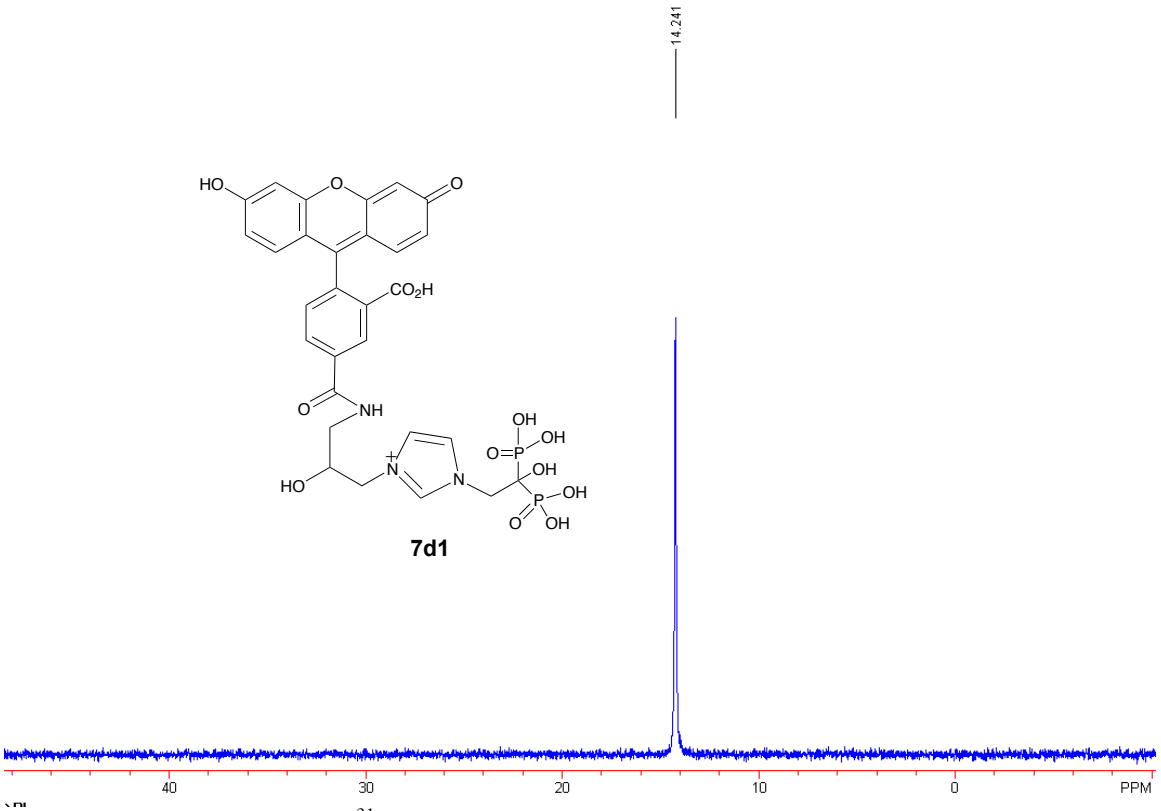
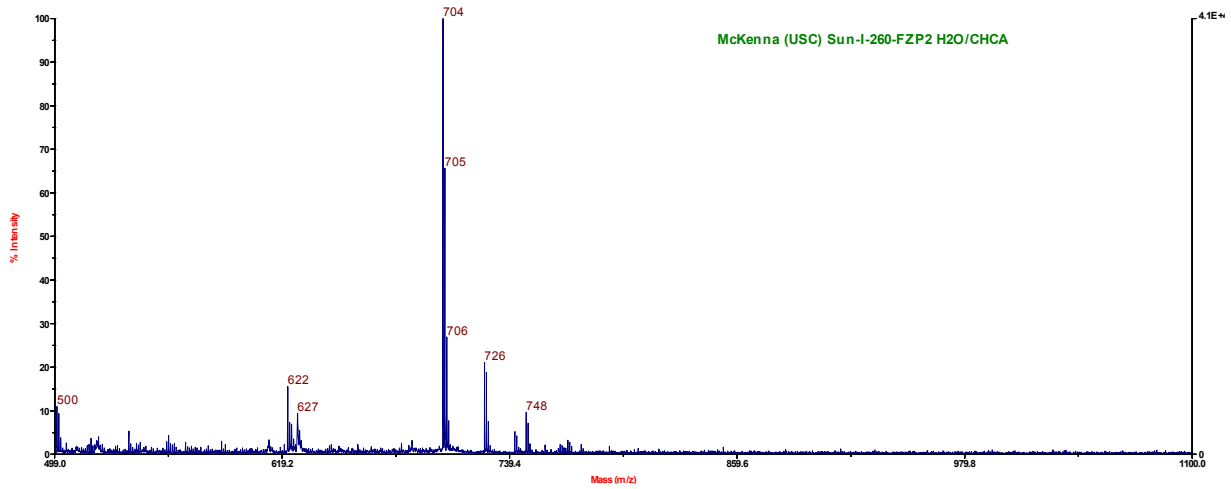
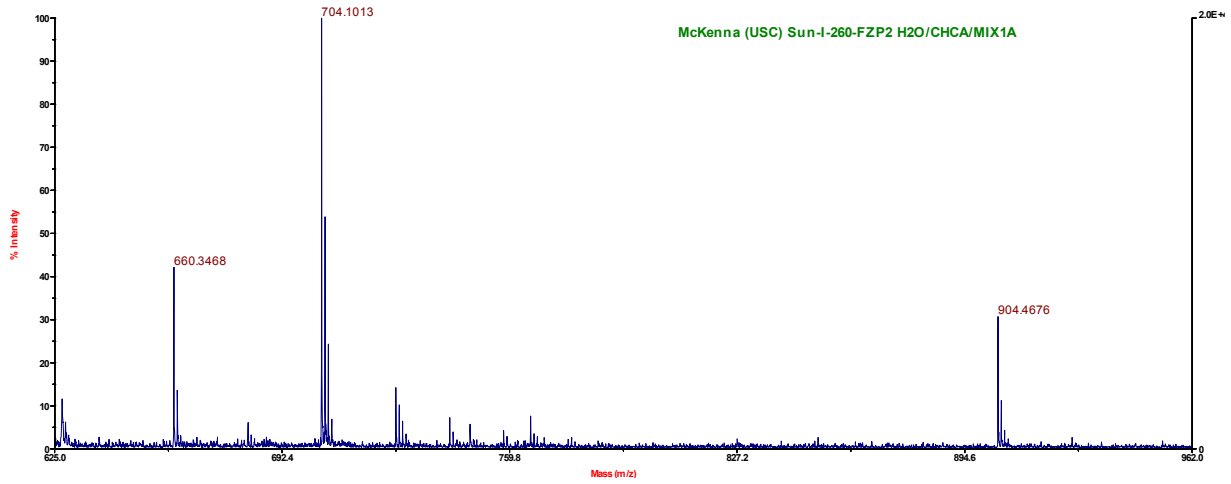


Figure A47. ^{31}P NMR (D_2O) spectrum of 5-FAM-ZOL, **7d1**.





Measured Mass 704.1013

Element	Low Limit	High Limit
C	24	34
H	20	40
N	0	5
O	12	16
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C31 H23 N5 O13 P	704.1025	-1.2	-1.6	23.5
C24 H28 N5 O16 P2	704.1001	1.2	1.7	14.5
C28 H25 N4 O16 P	704.0998	1.5	2.2	19
C32 H22 N3 O16	704.0995	1.8	2.6	23.5
C33 H25 N2 O14 P	704.1038	-2.5	-3.5	23
C29 H28 N3 O14 P2	704.1041	-2.8	-4.0	18.5

Figure A48. Mass spectrum of 5-FAM-ZOL, **7d1**.

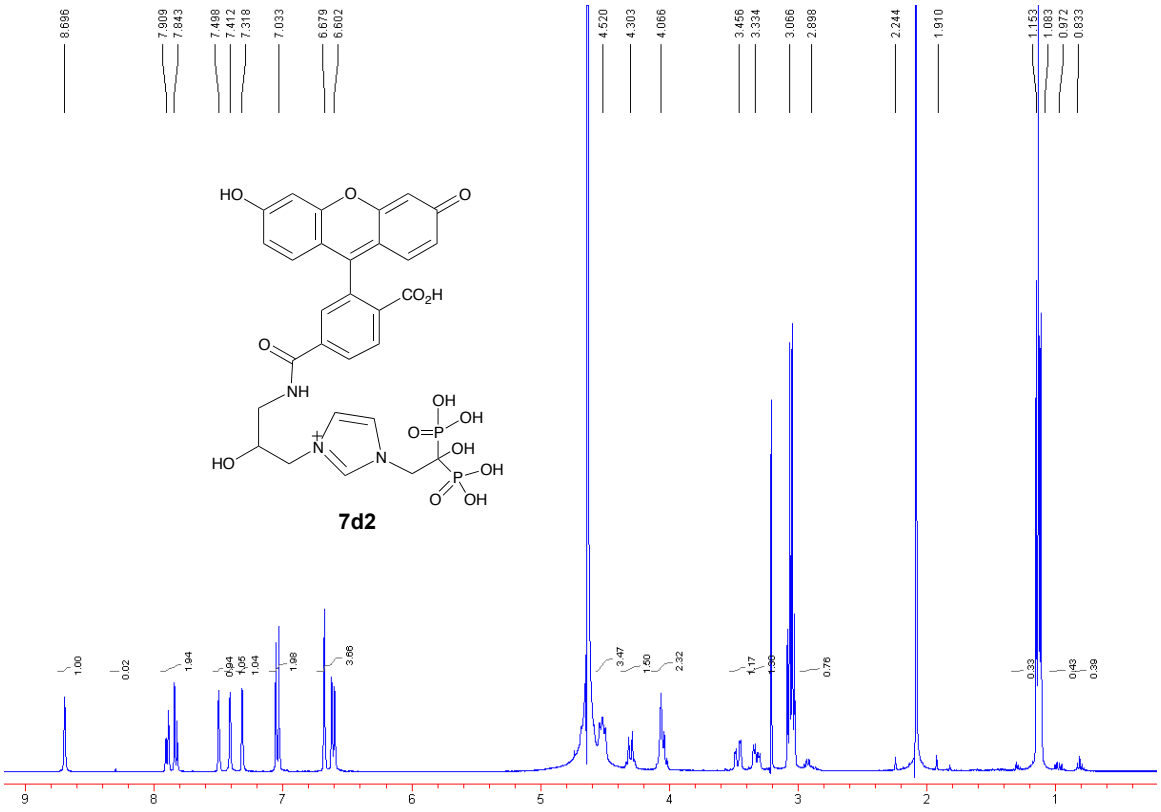


Figure A49. ^{1}H NMR (D_2O) spectrum of 6-FAM-ZOL, **7d2**.

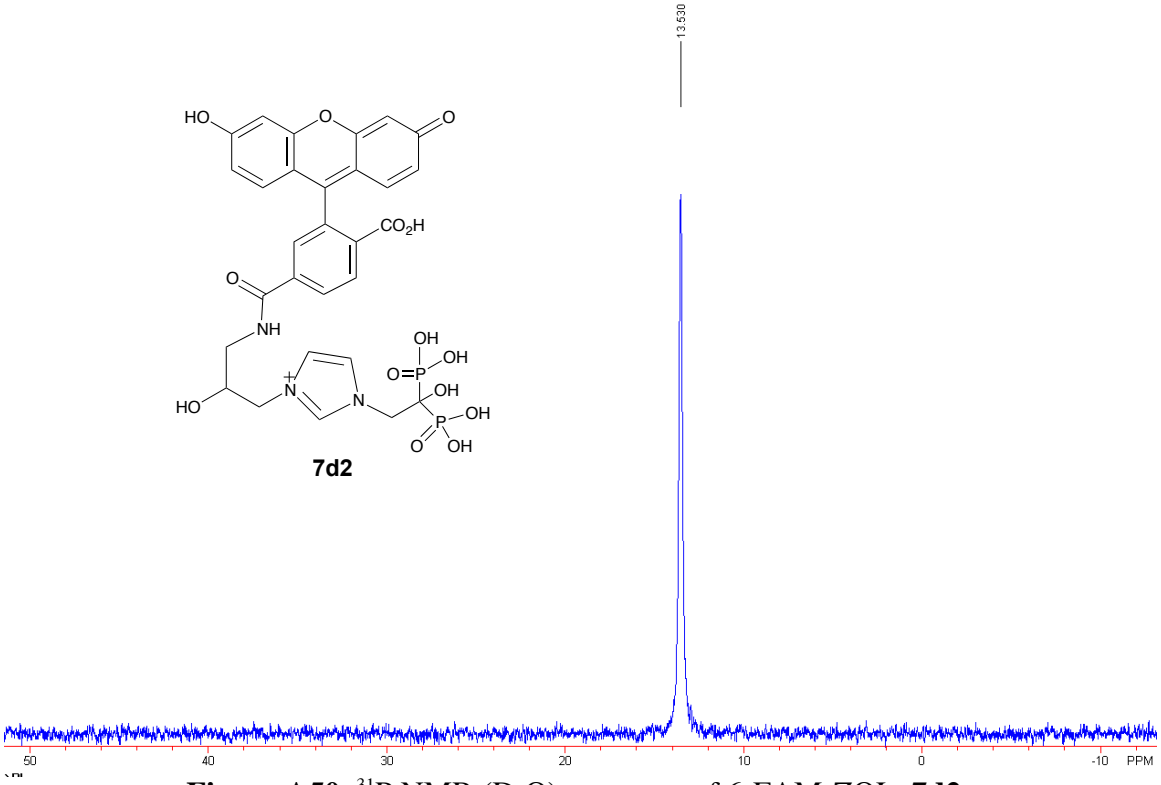
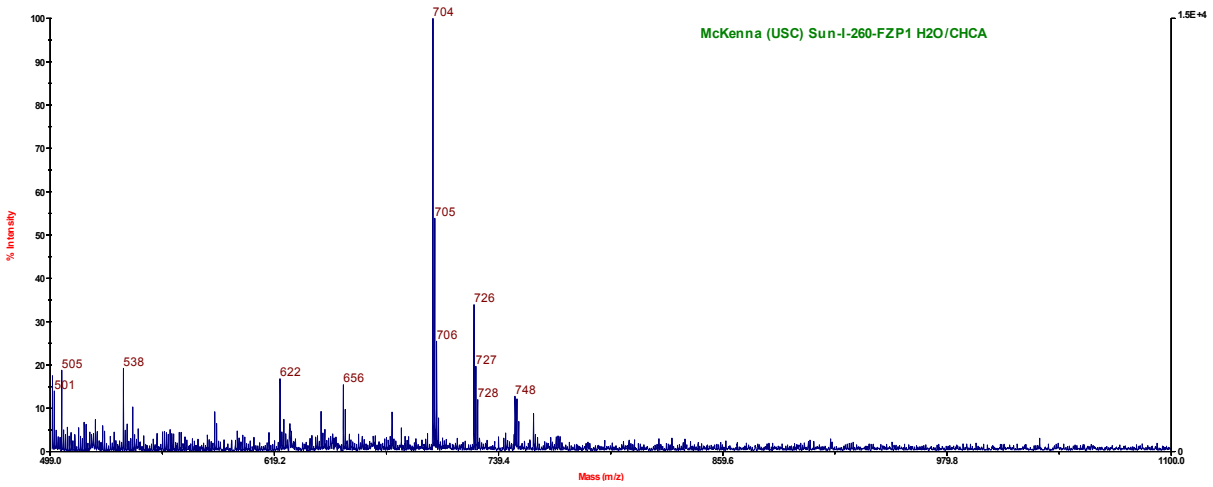
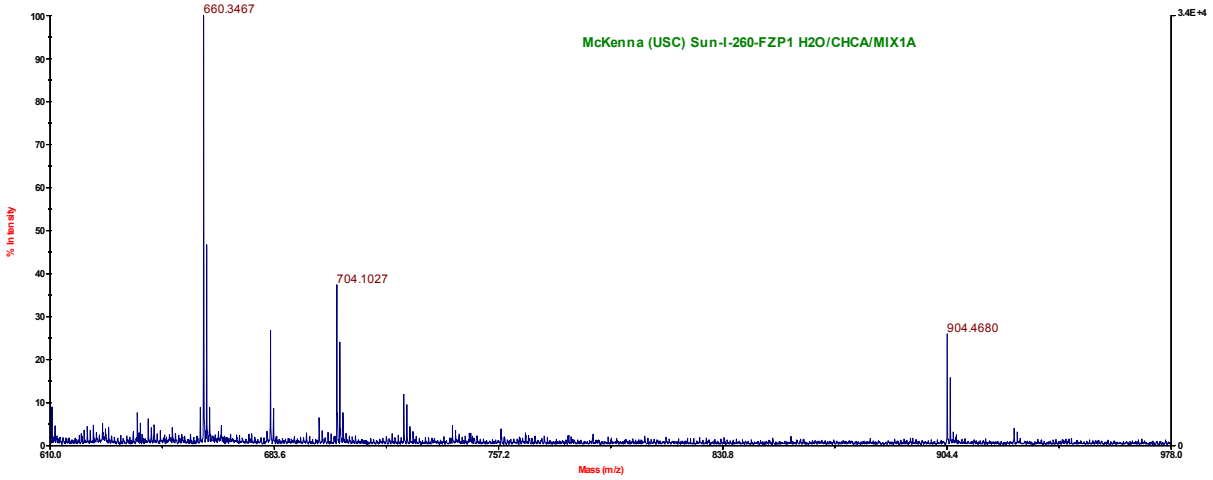


Figure A50. ^{31}P NMR (D_2O) spectrum of 6-FAM-ZOL, **7d2**.





Measured Mass 704.1027

Element	Low Limit	High Limit
C	24	34
H	20	40
N	0	5
O	12	16
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C31 H23 N5 O13 P	704.1025	0.2	0.4	23.5
C33 H25 N2 O14 P	704.1038	-1.1	-1.6	23
C29 H28 N3 O14 P2	704.1041	-1.4	-2.0	18.5
C24 H28 N5 O16 P2	704.1001	2.6	3.7	14.5
C31 H30 O15 P2	704.1054	-2.7	-3.9	18
C28 H25 N4 O16 P	704.0998	2.9	4.2	19
C32 H22 N3 O16	704.0995	3.2	4.6	23.5

Figure A51. Mass spectrum of 6-FAM-ZOL, **7d2**.

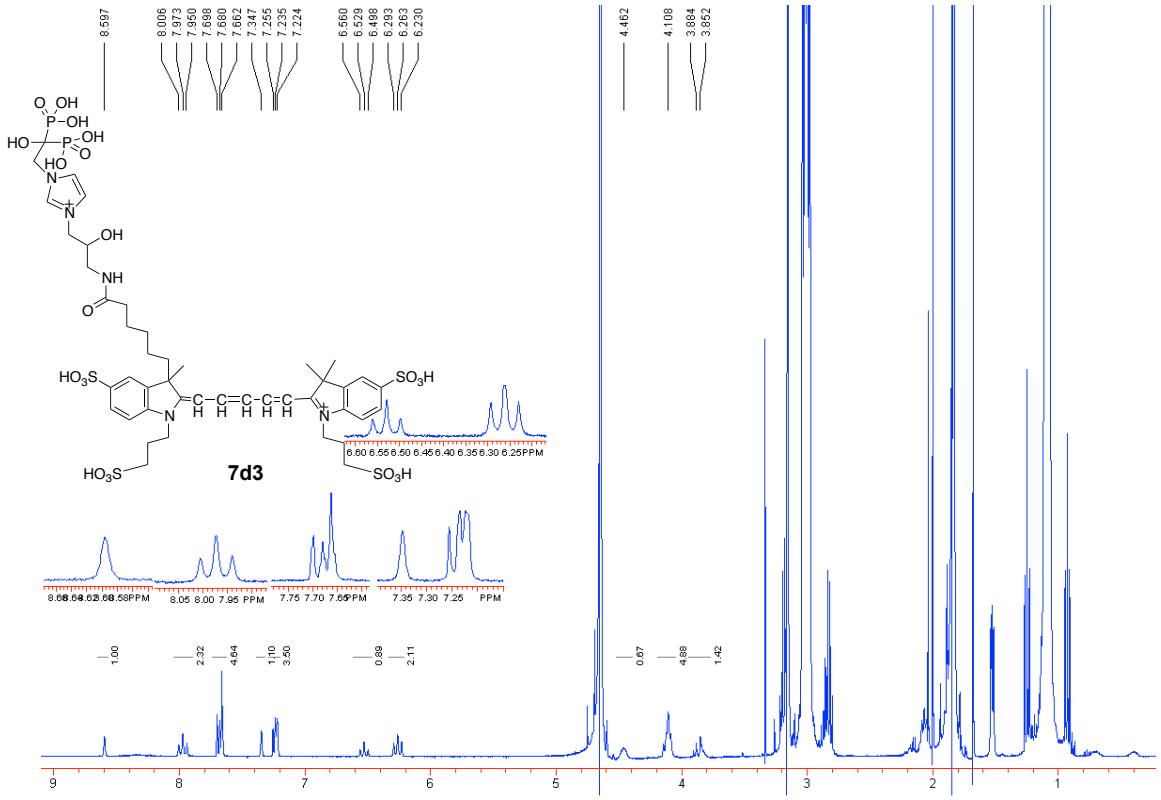


Figure A52. ^{1}H NMR (D_2O) spectrum of AF647-ZOL, **7d3**.

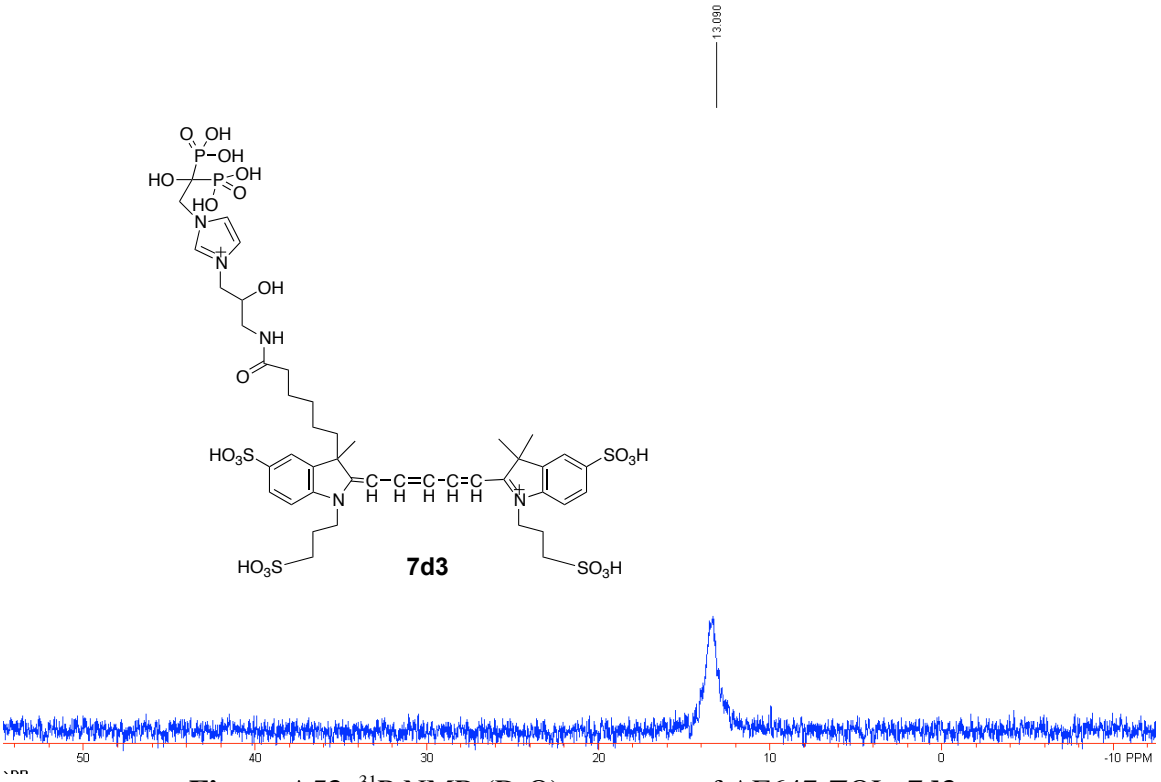
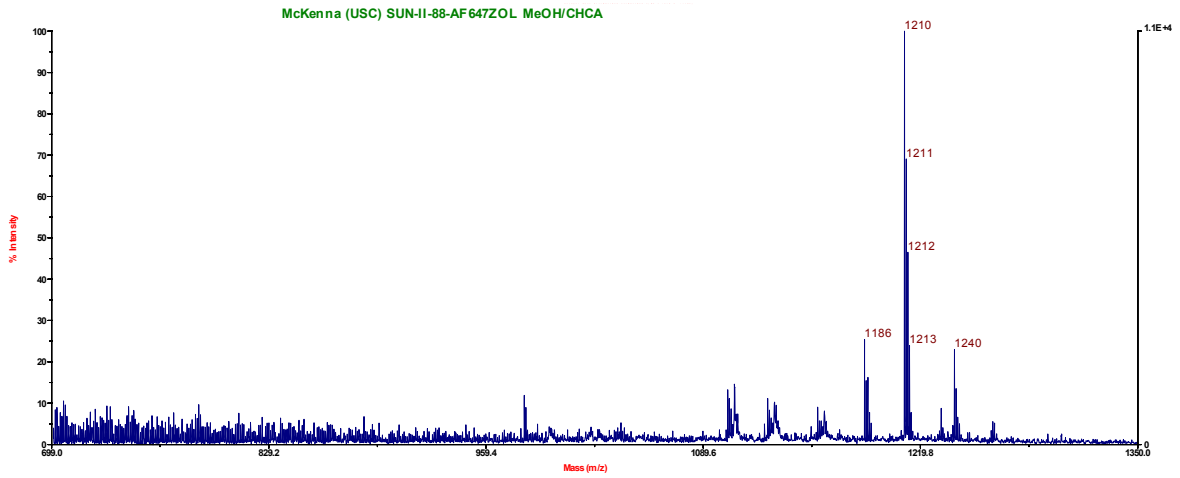
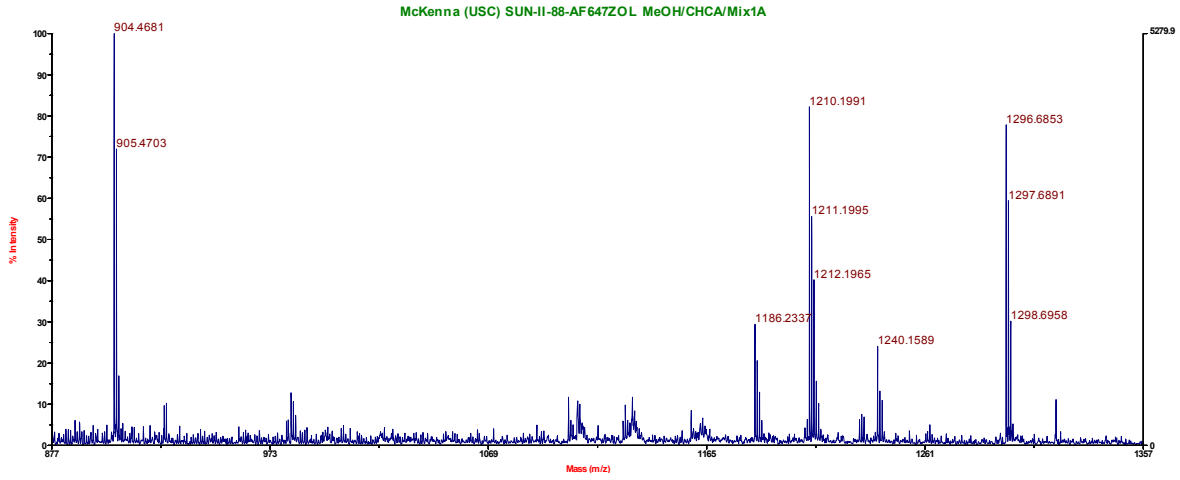


Figure A53. ^{31}P NMR (D_2O) spectrum of AF647-ZOL, **7d3**.





Measured Mass	1186.2337			
Element	Low Limit	High Limit		
C	39	49		
H	50	70		
N	4	6		
O	20	22		
P	1	2		
S	3	5		
Formula	Calculated Mass	mDaError	ppmError	RDB
C41 H66 N5 O21 P2 S5	1186.2324	1.3	1.1	12.5
C45 H63 N4 O21 P S5	1186.2321	1.6	1.4	17
C48 H60 N4 O21 P2 S3	1186.2382	-4.5	-3.8	22
C44 H62 N5 O21 P2 S4	1186.2290	4.7	4.0	17.5
C48 H59 N4 O21 P S4	1186.2287	5.0	4.2	22

Figure A54. Mass spectrum of AF647-ZOL, **7d3**.

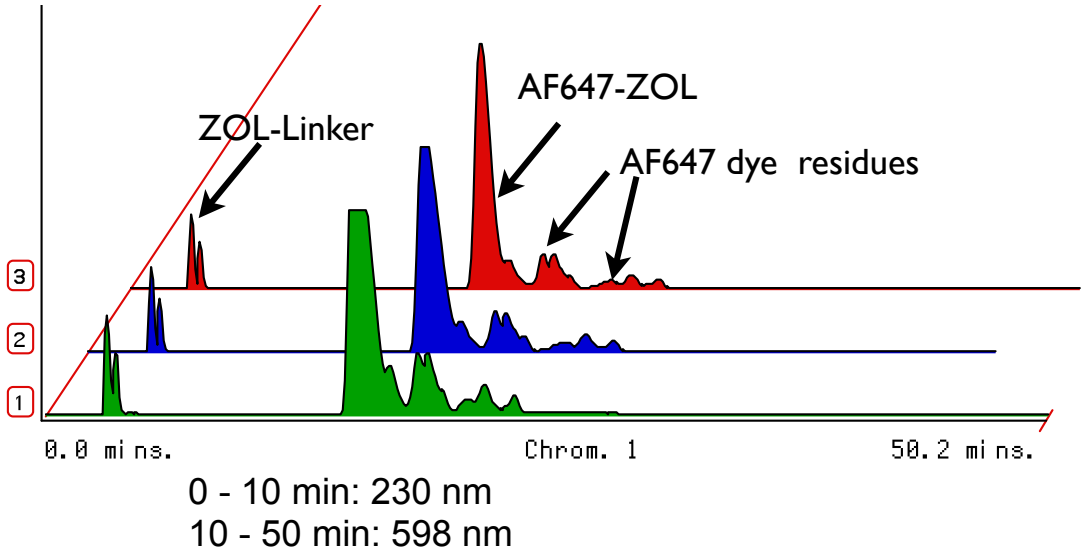


Figure A55. HPLC trace of AF647-ZOL, **7d3**.

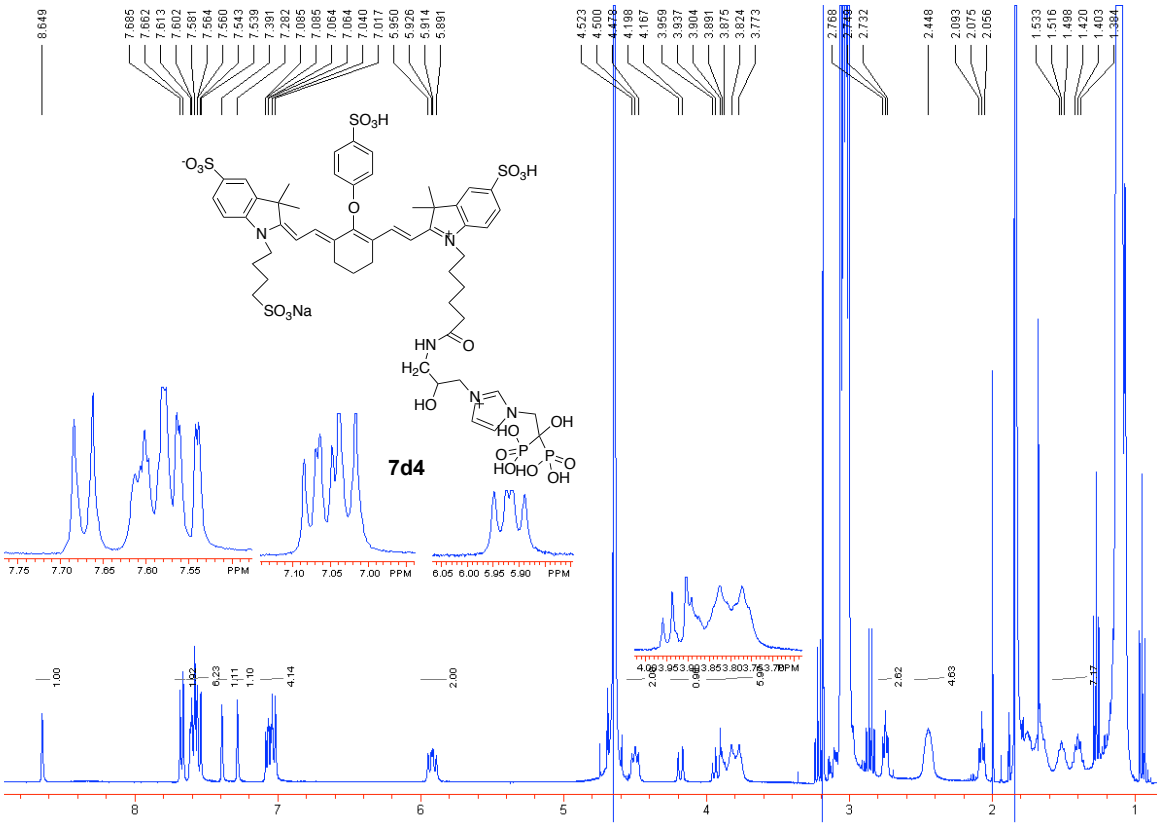


Figure A56. ^{1}H NMR (D_2O) spectrum of 800CW-ZOL, **7d4**.

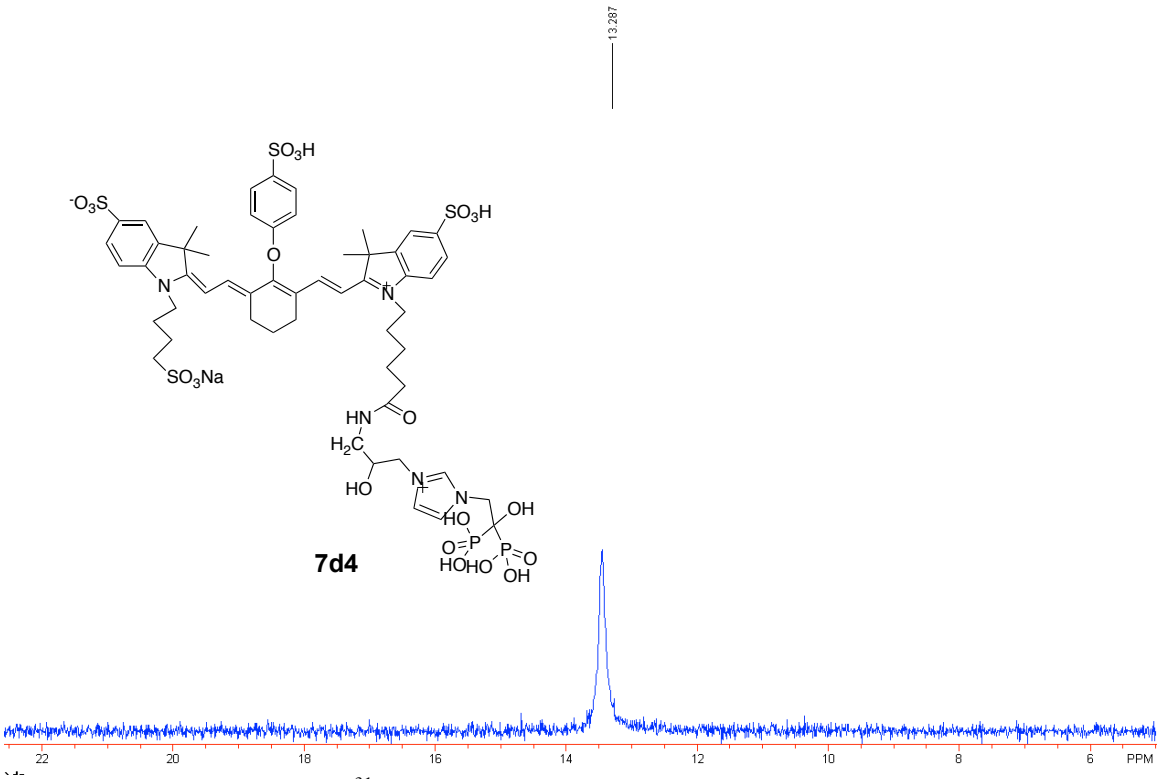
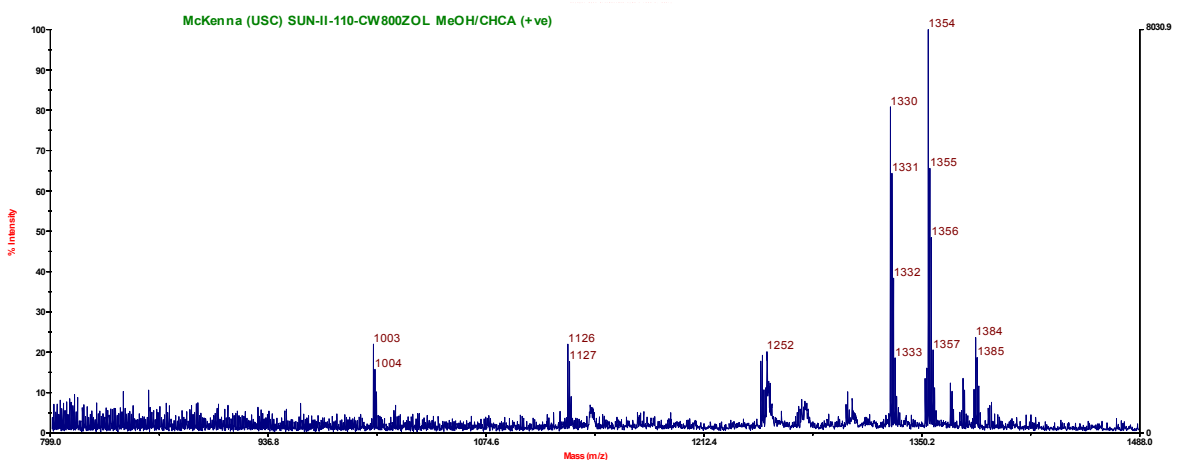
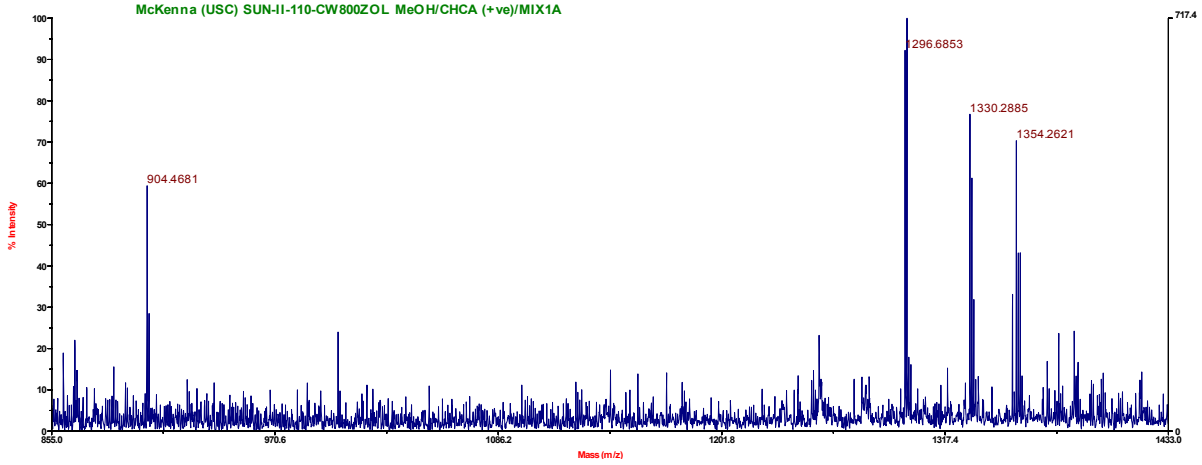


Figure A57. ^{31}P NMR (D_2O) spectrum of 800CW-ZOL, **7d4**.





Measured Mass	1330.2885	
Element	Low Limit	High Limit
C	49	59
H	60	80
N	4	6
O	21	23
S	3	5
P	1	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C55 H71 N4 O22 P S5	1330.2896	-1.1	-0.8	23
C51 H74 N5 O22 P2 S5	1330.2899	-1.4	-1.0	18.5
C54 H70 N5 O22 P2 S4	1330.2865	2.0	1.5	23.5
C58 H67 N4 O22 P S4	1330.2862	2.3	1.7	28
C57 H66 N5 O22 P2 S3	1330.2831	5.4	4.0	28.5

Figure A58. Mass spectrum of 800CW-ZOL, **7d4**.

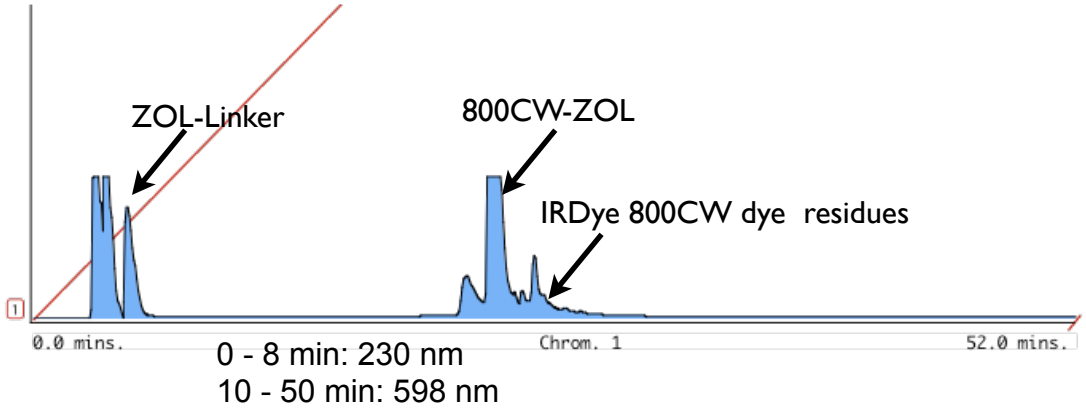


Figure A59. HPLC trace of 800CW-ZOL, **7d4**.

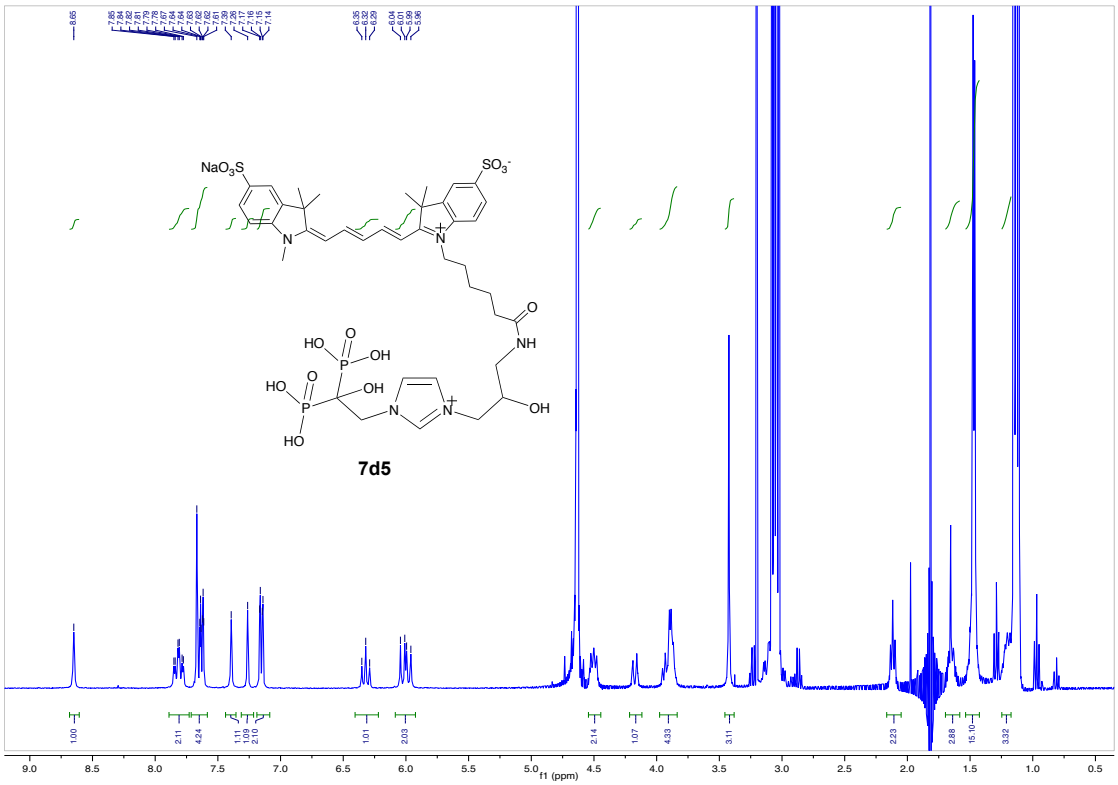


Figure A60. ^{1}H NMR (D_2O) spectrum of Sulfo-Cy5-ZOL, **7d5**.

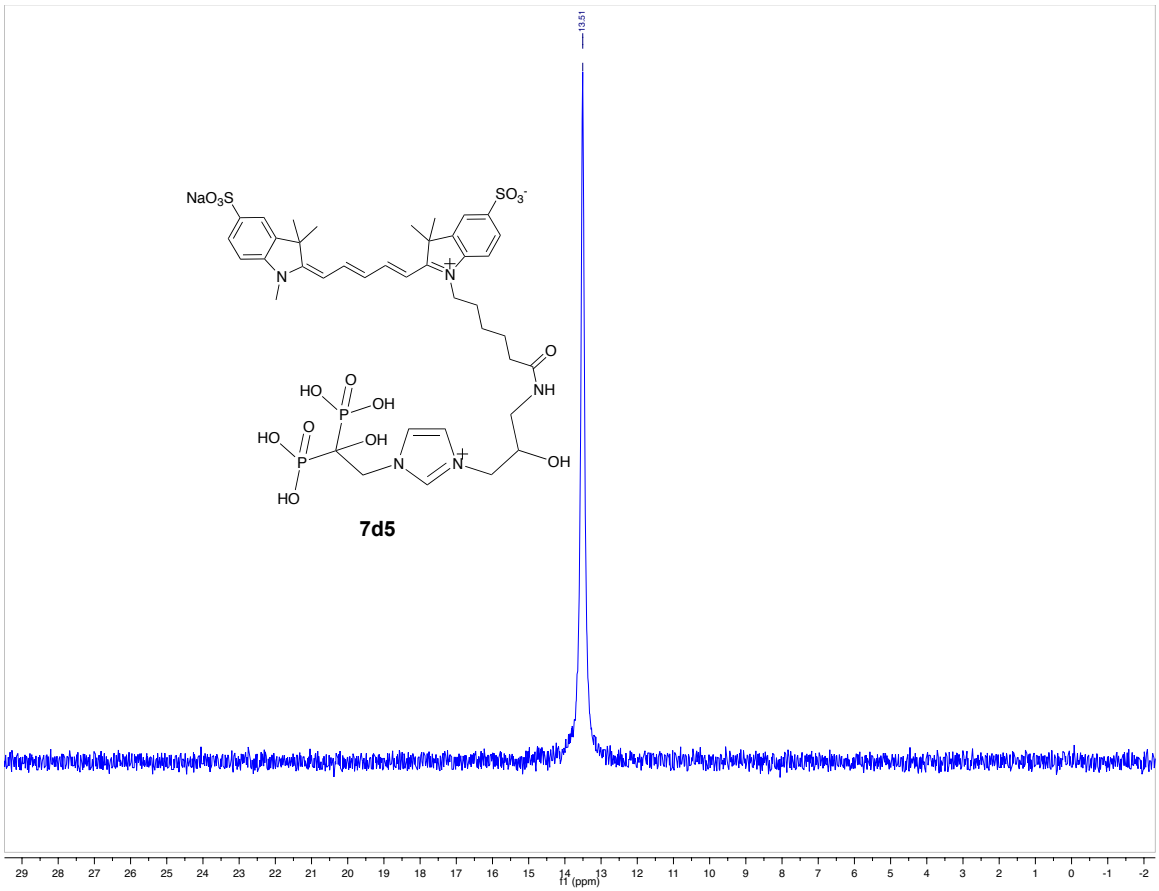


Figure A61. ^{31}P NMR (D_2O) spectrum of Sulfo-Cy5-ZOL, **7d5**.

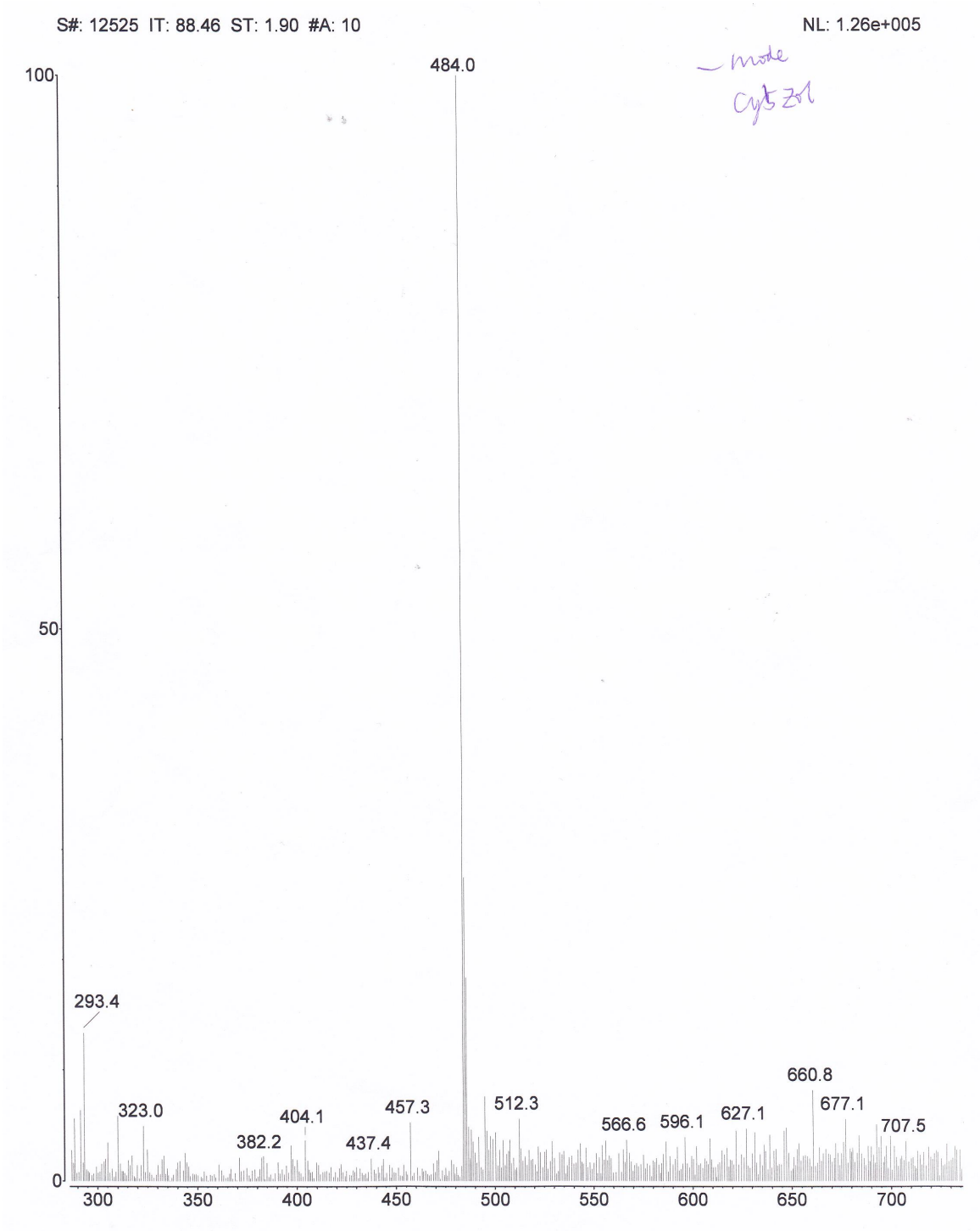


Figure A62. Mass spectrum (+) of Sulfo-Cy5-ZOL, **7d5**.

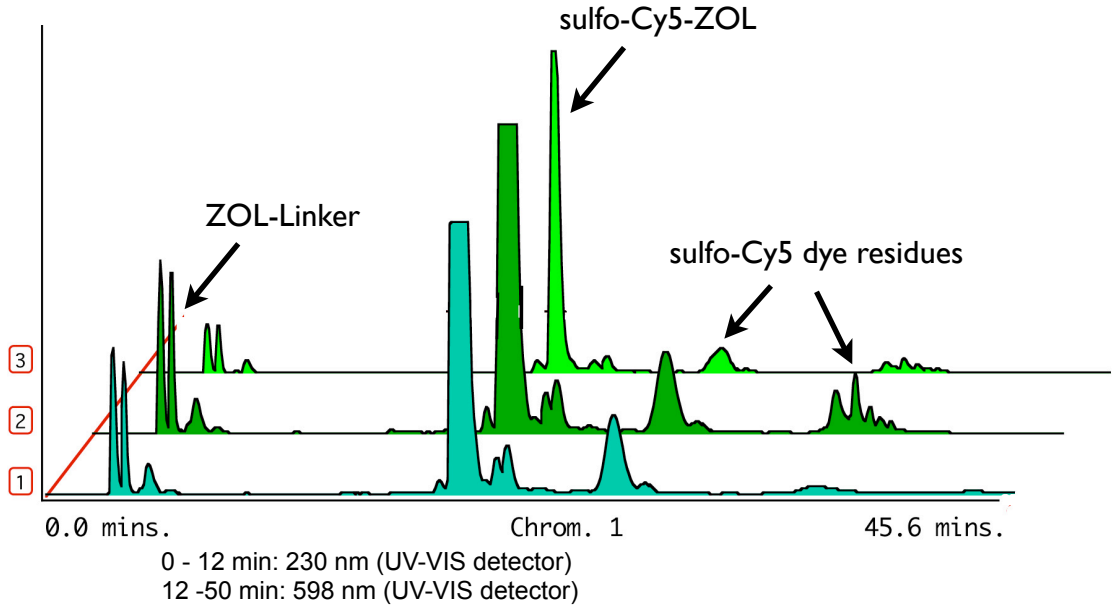


Figure A63. HPLC trace of Sulfo-Cy5-ZOL, **7d5**.

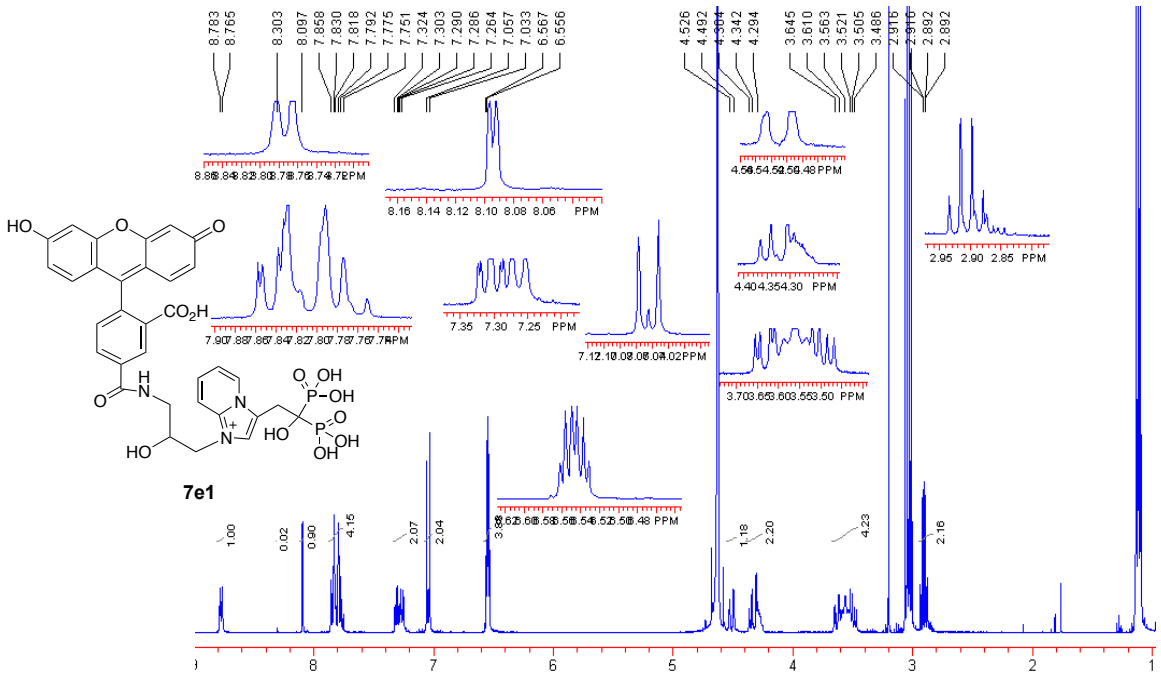


Figure A64. ^{1}H NMR (D_2O) spectrum of 5-FAM-MIN, **7e1**.

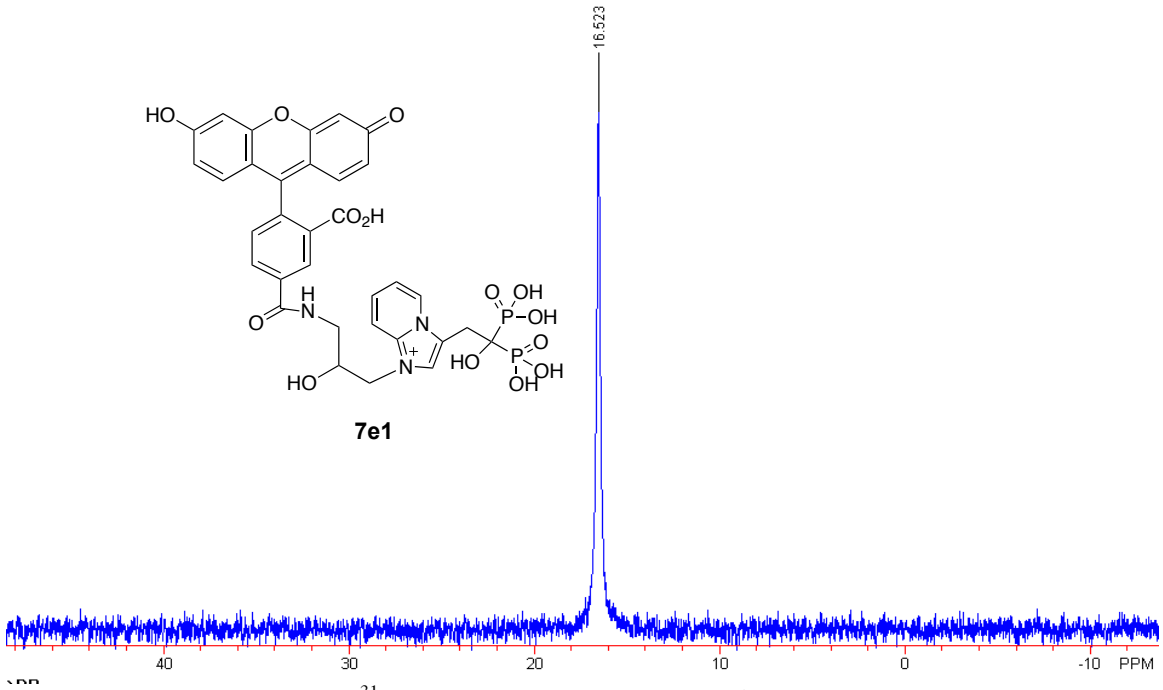
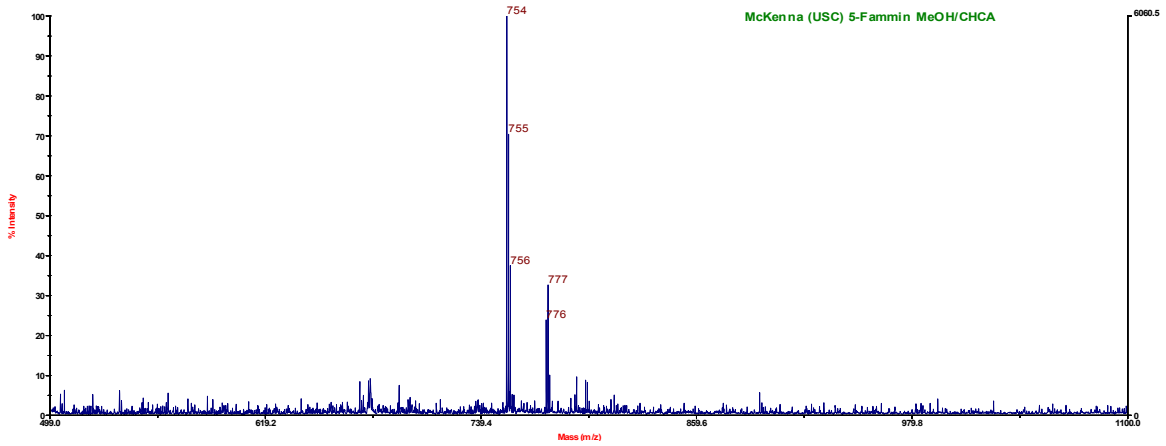
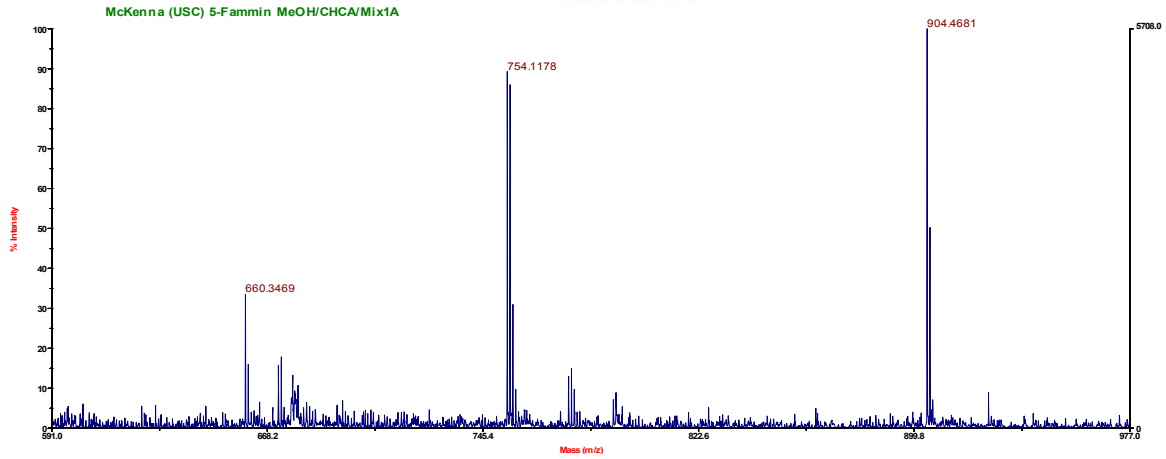


Figure A65. ^{31}P NMR (D_2O) spectrum of 5-FAM-MIN, **7e1**.





Measured Mass 754.1178

Element	Low Limit	High Limit
C	28	38
H	20	40
N	0	5
O	11	16
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C35 H25 N5 O13 P	754.1181	-0.3	-0.4	26.5
C37 H27 N2 O14 P	754.1194	-1.6	-2.2	26
C33 H30 N3 O14 P2	754.1198	-2.0	-2.6	21.5
C28 H30 N5 O16 P2	754.1157	2.1	2.7	17.5
C32 H27 N4 O16 P	754.1154	2.4	3.2	22
C36 H24 N3 O16	754.1151	2.7	3.6	26.5
C35 H32 O15 P2	754.1211	-3.3	-4.4	21

Figure A66. Mass spectrum of 5-FAM-MIN, **7e1**.

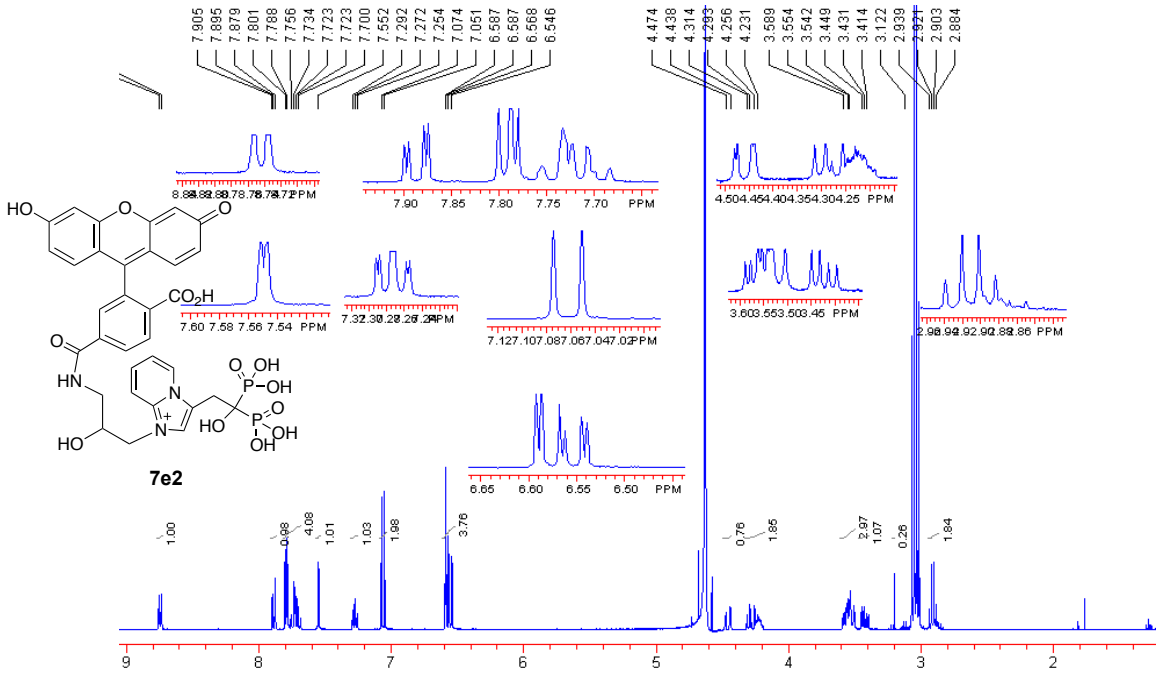


Figure A67. ^{1}H NMR (D_2O) spectrum of 6-FAM-MIN, **7e2**.

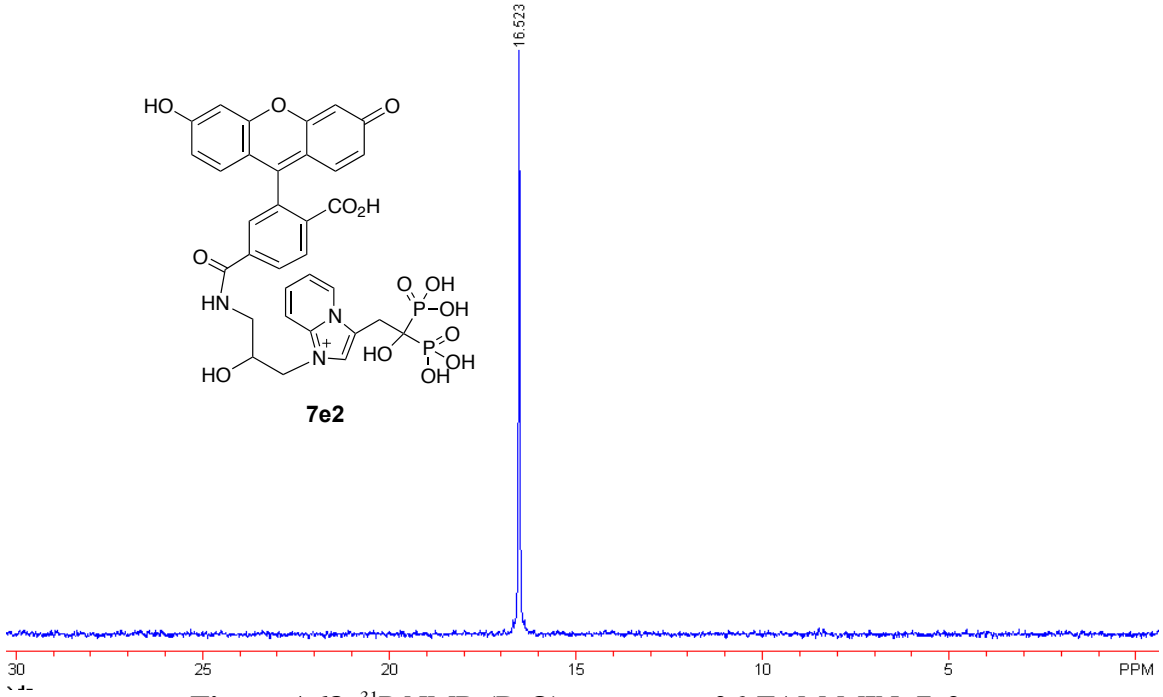
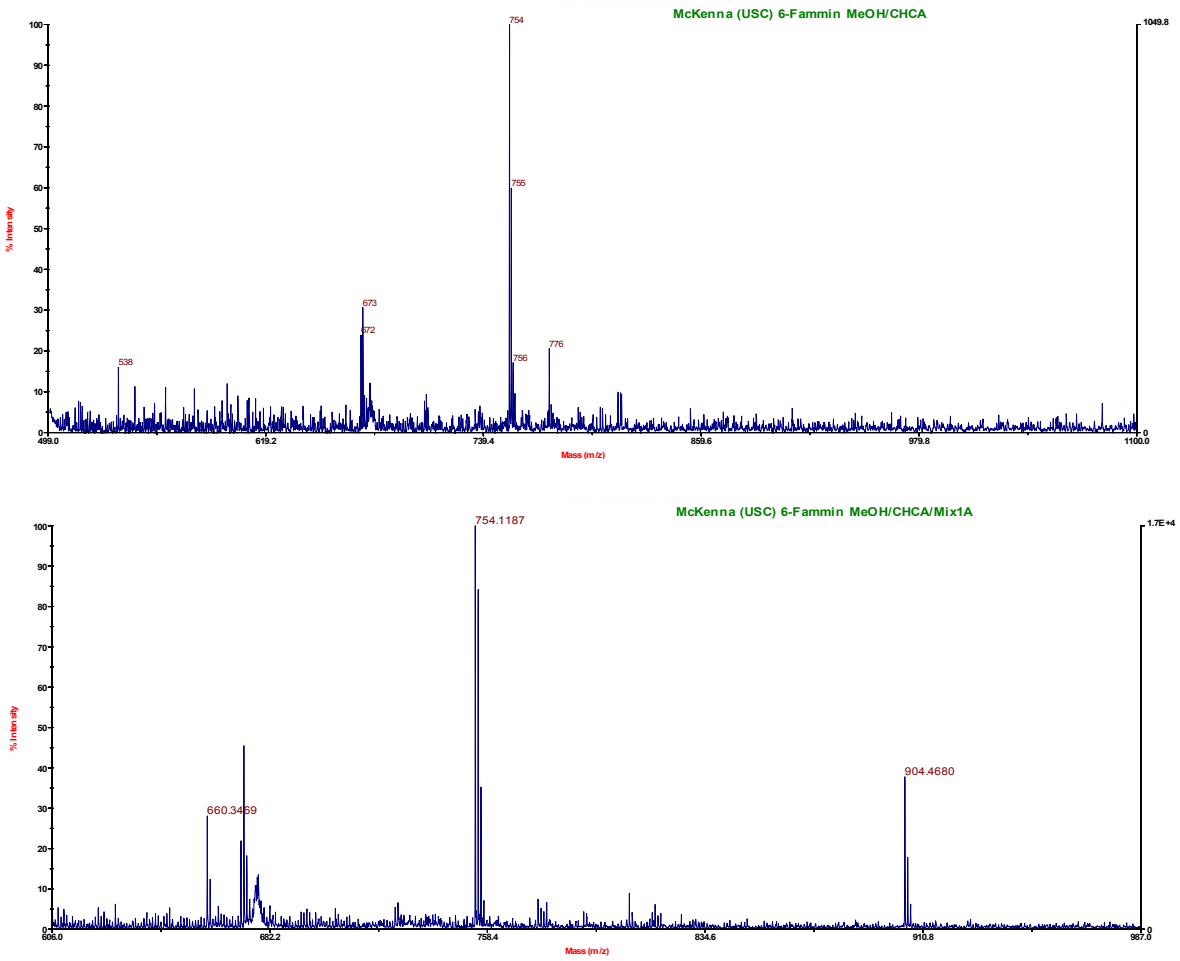


Figure A68. ^{31}P NMR (D_2O) spectrum of 6-FAM-MIN, **7e2**.



Measured Mass	754.1187	
Element	**Low Limit**	**High Limit**
C	28	38
H	20	40
N	0	5
O	11	16
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C35 H25 N5 O13 P	754.1181	0.6	0.8	26.5
C37 H27 N2 O14 P	754.1194	-0.7	-1.0	26
C33 H30 N3 O14 P2	754.1198	-1.1	-1.4	21.5
C35 H32 O15 P2	754.1211	-2.4	-3.2	21
C28 H30 N5 O16 P2	754.1157	3.0	3.9	17.5
C32 H27 N4 O16 P	754.1154	3.3	4.3	22
C36 H24 N3 O16	754.1151	3.6	4.8	26.5
C36 H28 N4 O11 P2	754.1224	-3.7	-5.0	26

Figure A69. Mass spectrum of 6-FAM-MIN, **7e2**.

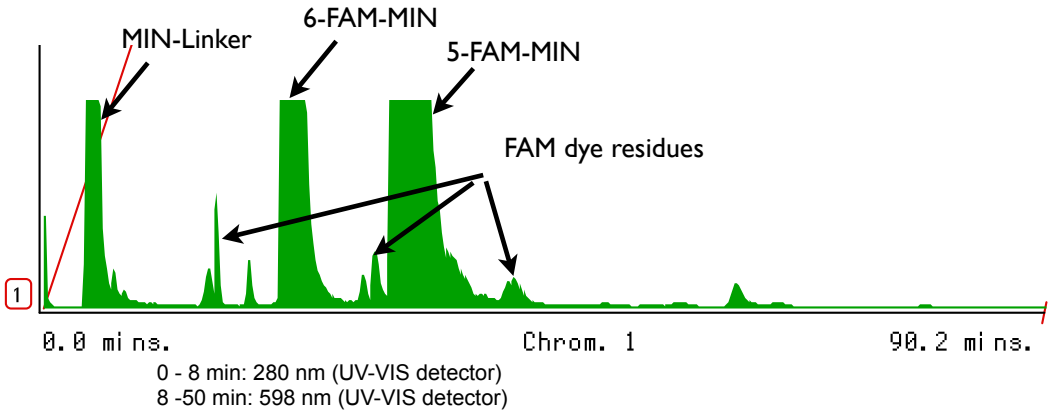


Figure A70. HPLC separation of 5-FAM-MIN (**7e1**) and 6-FAM-MIN (**7e2**).

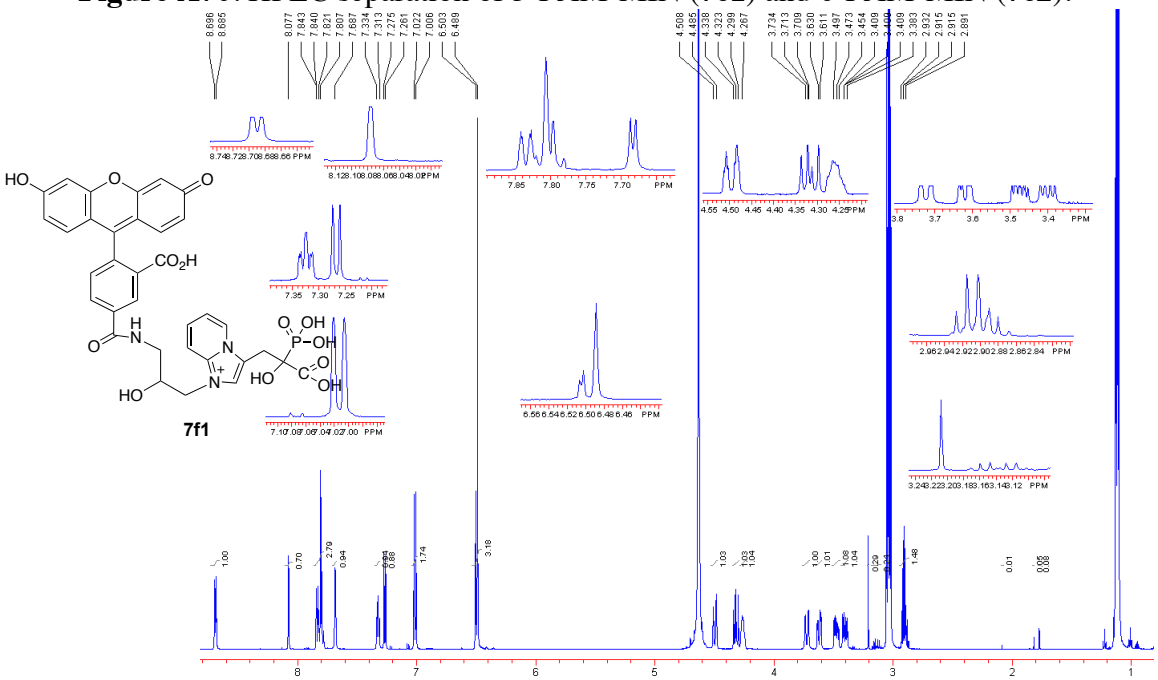


Figure A71. ^{1}H NMR (D_2O) spectrum of 5-FAM-MINPC, **7f1**.

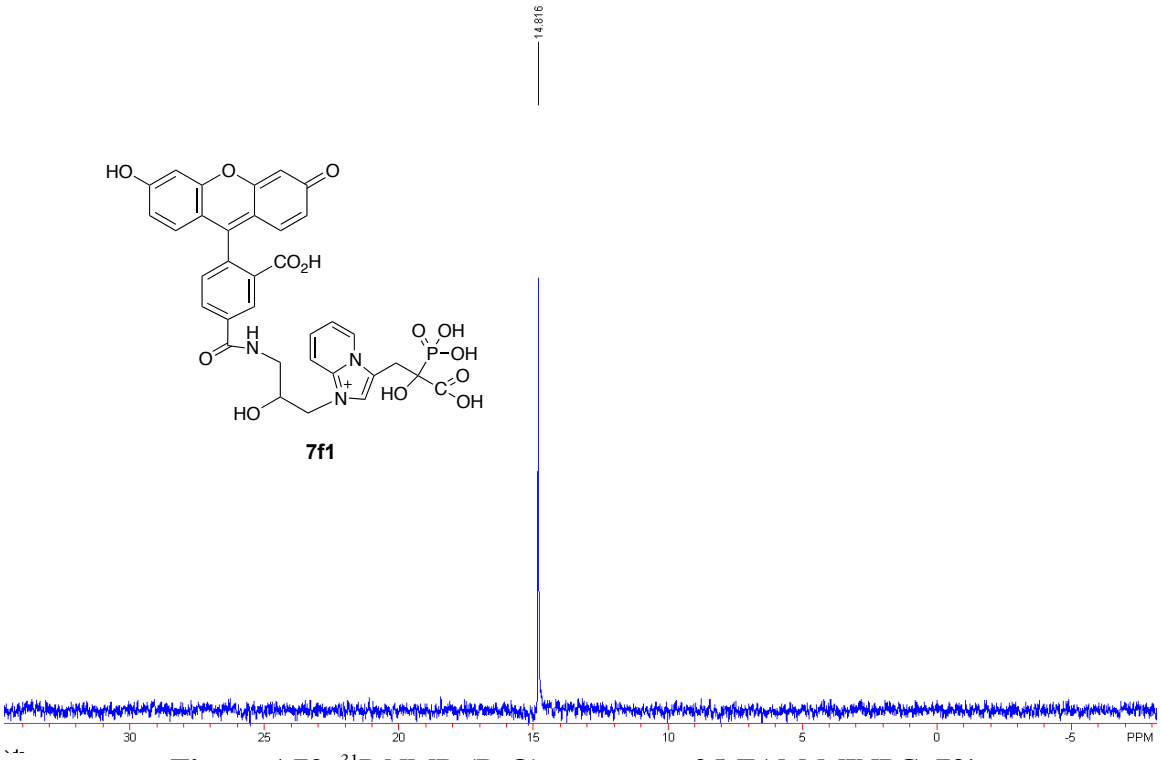
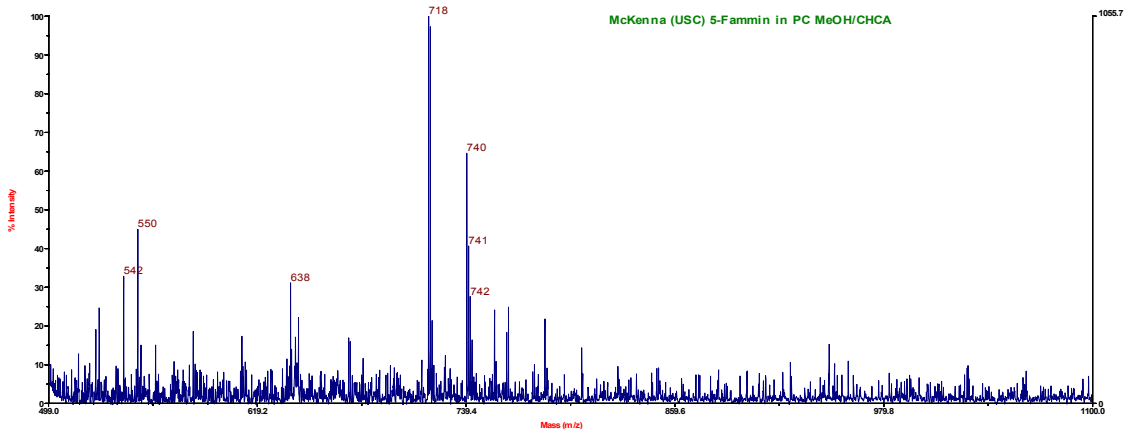
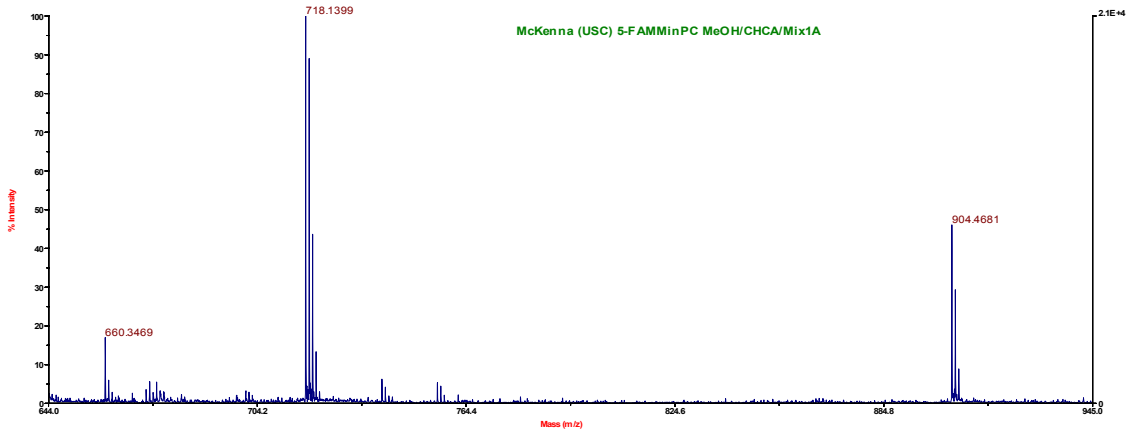


Figure A72. ^{31}P NMR (D_2O) spectrum of 5-FAM-MINPC, **7f1**.





Measured Mass 718.1399

Element	Low Limit	High Limit
C	28	38
H	20	40
N	0	5
O	11	16
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C35 H28 N O16	718.1403	-0.4	-0.5	22.5
C29 H29 N5 O15 P	718.1392	0.7	0.9	18.5
C31 H31 N2 O16 P	718.1406	-0.7	-0.9	18
C33 H26 N4 O15	718.1389	1.0	1.4	23
C36 H24 N5 O12	718.1416	-1.7	-2.4	27.5
C38 H26 N2 O13	718.1429	-3.0	-4.2	27
C34 H29 N3 O13 P	718.1433	-3.4	-4.7	22.5
C36 H32 O12 P2	718.1364	3.5	4.9	22

Figure A73. Mass spectrum of 5-FAM-MINPC, **7f1**.

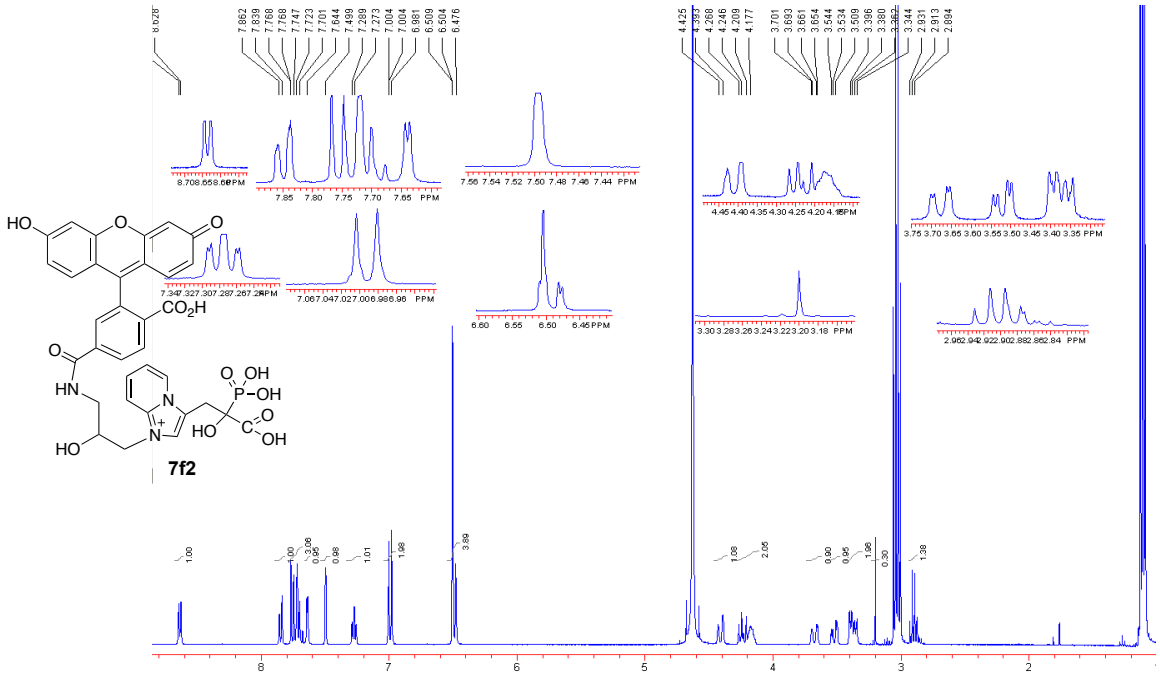


Figure A74. ^{1}H NMR (D_2O) spectrum of 6-FAM-MINPC, **7f2**.

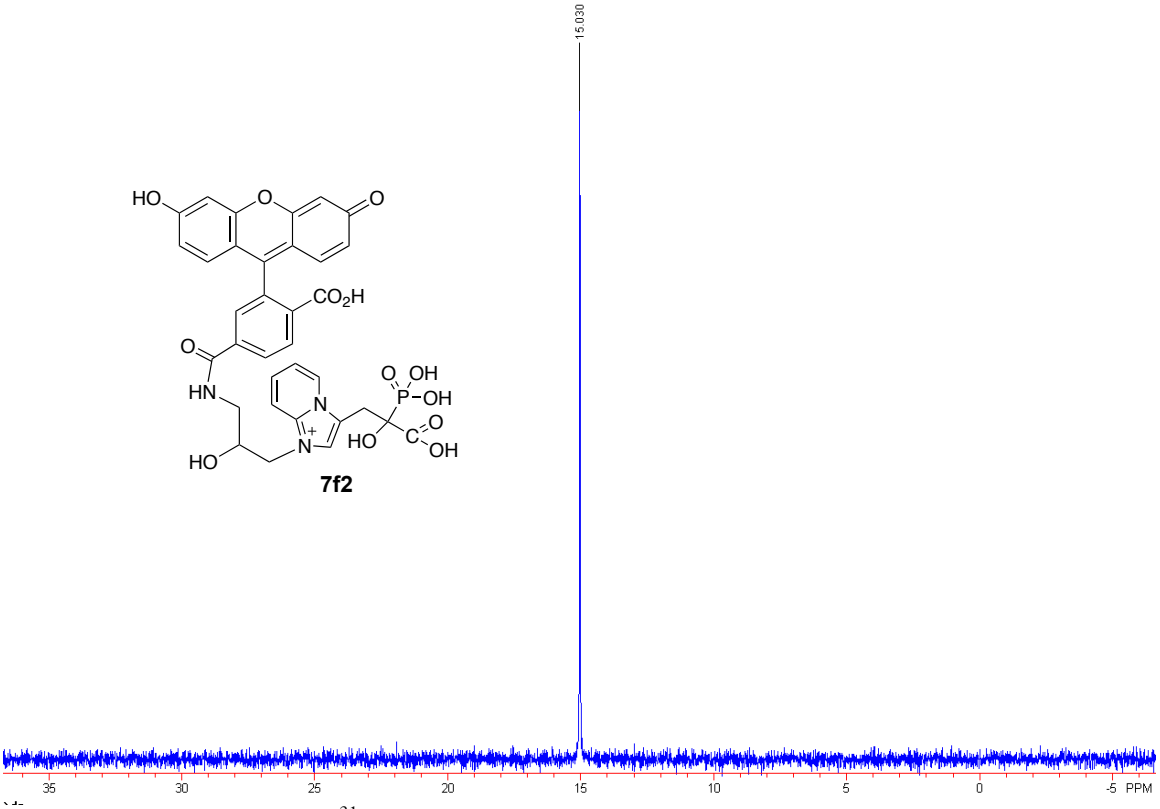
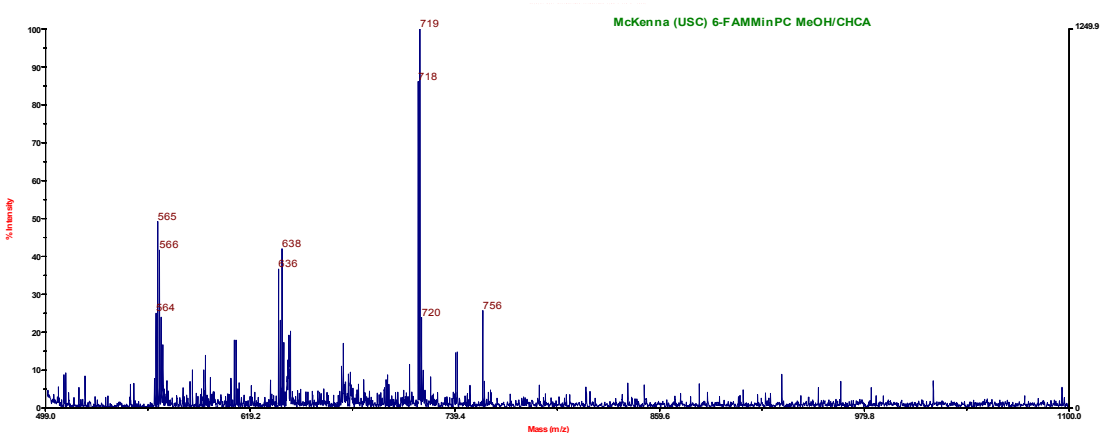
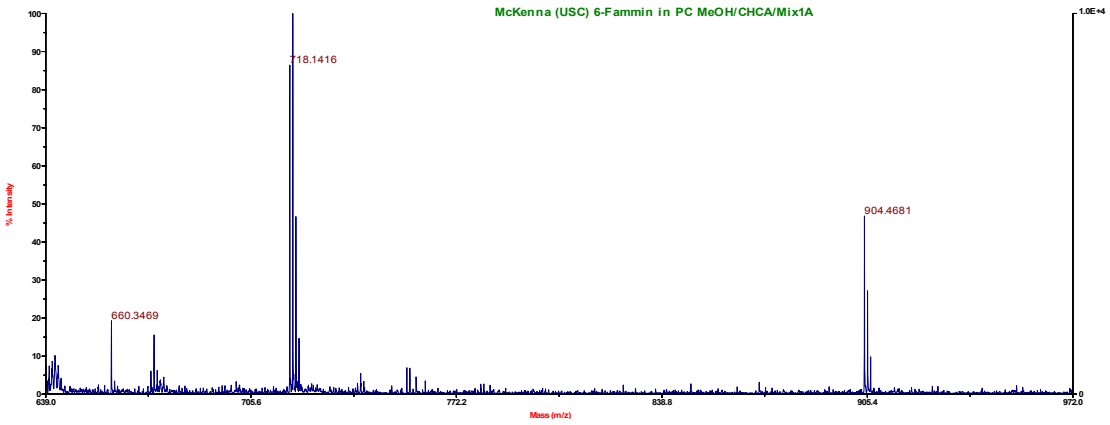


Figure A75. ^{31}P NMR (D_2O) spectrum of 6-FAM-MINPC, **7f2**.





Measured Mass 718.1416

Element	Low Limit	High Limit
C	28	38
H	20	40
N	0	5
O	11	16
P	0	2

Formula	Calculated Mass	mDaError	ppmError	RDB
C36 H24 N5 O12	718.1416	0.0	0.0	27.5
C31 H31 N2 O16 P	718.1406	1.0	1.4	18
C35 H28 N O16	718.1403	1.3	1.9	22.5
C38 H26 N2 O13	718.1429	-1.3	-1.9	27
C34 H29 N3 O13 P	718.1433	-1.7	-2.3	22.5
C30 H32 N4 O13 P2	718.1436	-2.0	-2.7	18
C29 H29 N5 O15 P	718.1392	2.4	3.3	18.5
C33 H26 N4 O15	718.1389	2.7	3.7	23
C36 H31 O14 P	718.1446	-3.0	-4.2	22
C32 H34 N O14 P2	718.1449	-3.3	-4.6	17.5

Figure A76. Mass spectrum of 6-FAM-MINPC, **7f2**.

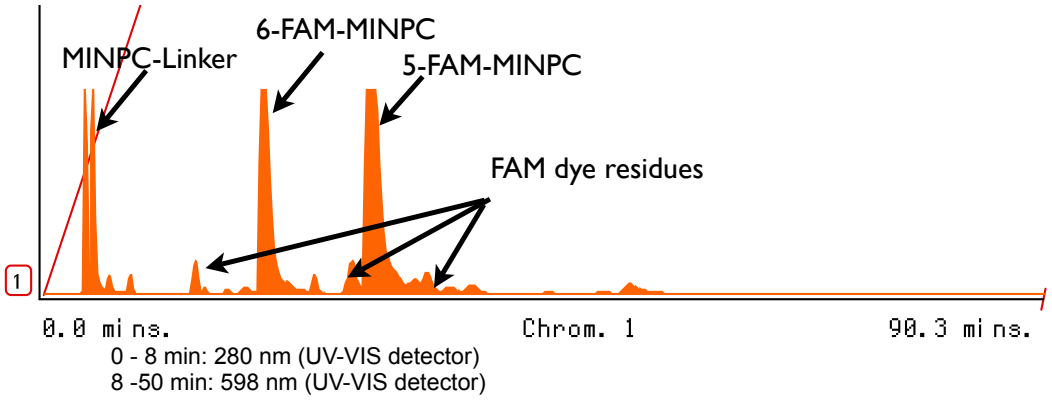
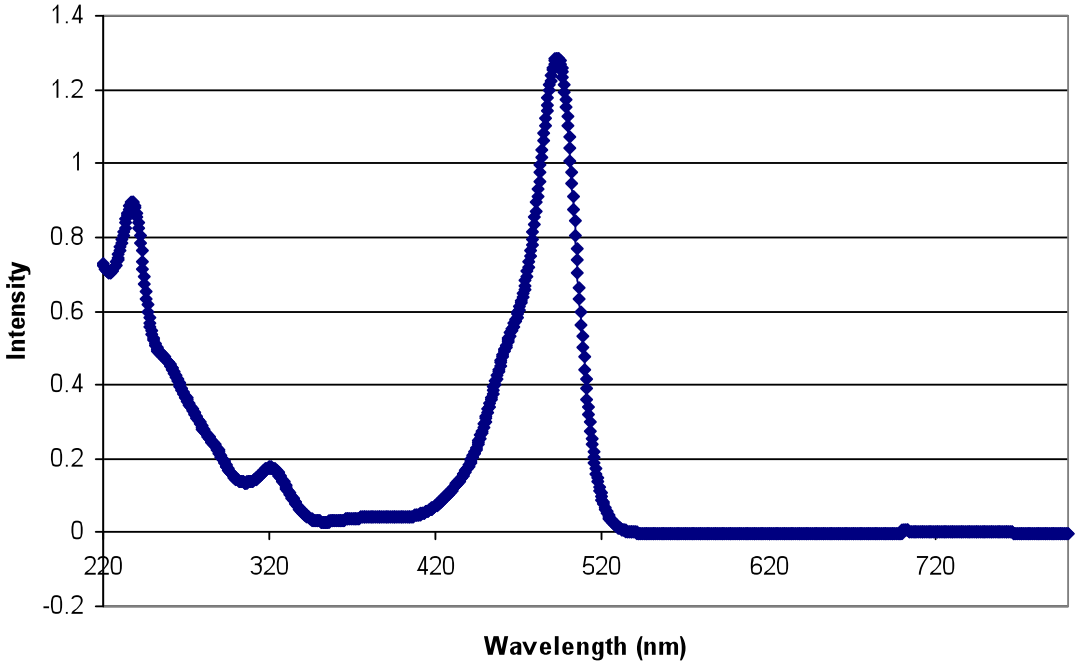


Figure A77. HPLC separation of 5-FAM-MINPC (**7f1**) and 6-FAM-MINPC (**7f2**).

Figure A78. UV-vis, fluorescence emission spectra of compounds **7a1 – 7f2**

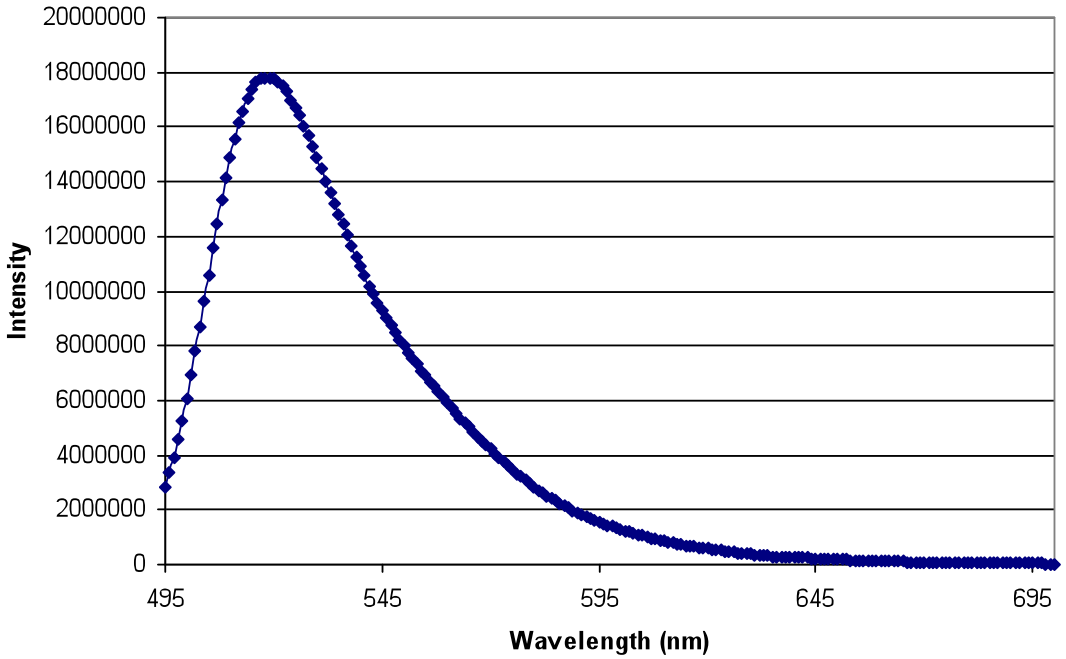
UV Absorption of 5(6)-FAM-RIS (7a1)

Sample in 0.1 M phosphate buffer, pH 7.2, with concentration at 17.6 μM (λmax = 493 nm). Recorded on DU 800 spectrometer. Extinction coefficient: 73,000$M^{-1}cm^{-1}$



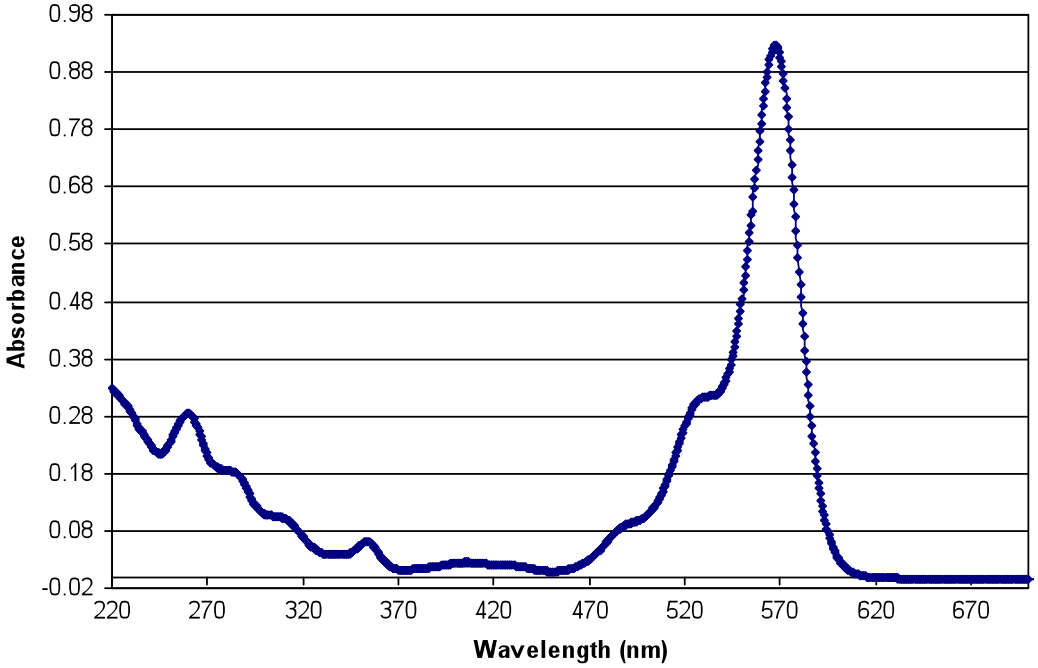
Emission Spectrum of 5(6)-FAM-RIS (7a1)

Sample in 0.1 M phosphate buffer, pH 7.2, with concentration at 1 μM. Recorded on Jobin Yvon Horiba Fluoromax-3 fluorometer (λmax = 519 nm).



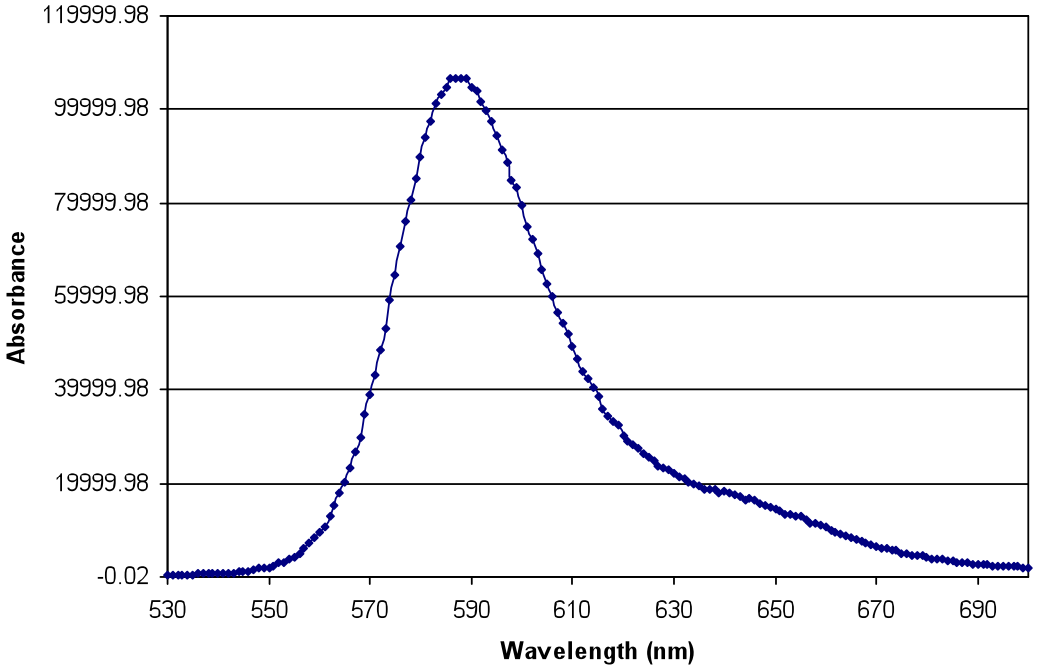
UV Absorption of 5(6)-RhR-RIS (7a4)

Sample in 0.1M phosphate buffer, pH 7.5, with concentration at 8.1 μM. Recorded on DU 800 spectrometer. λmax = 567.5 nm. Extinction coefficient: 114,850$M^{-1}cm^{-1}$.



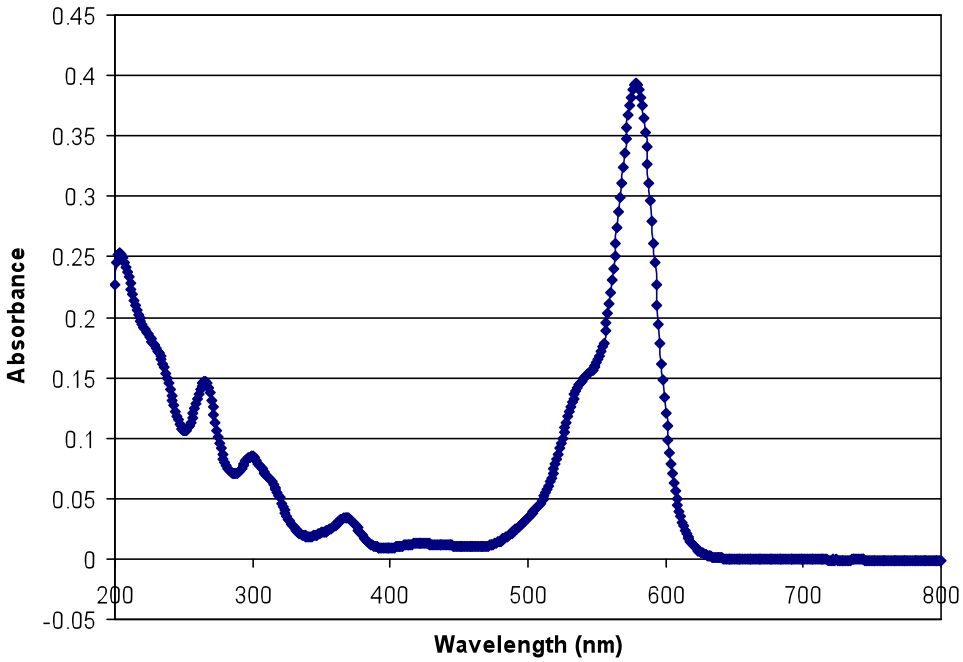
Emission Spectrum of 5(6)-RhR-RIS (7a4)

Sample in 0.1 M phosphate buffer, pH 7.5, with concentration at 1.05 μM. λmax = 589 nm. Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



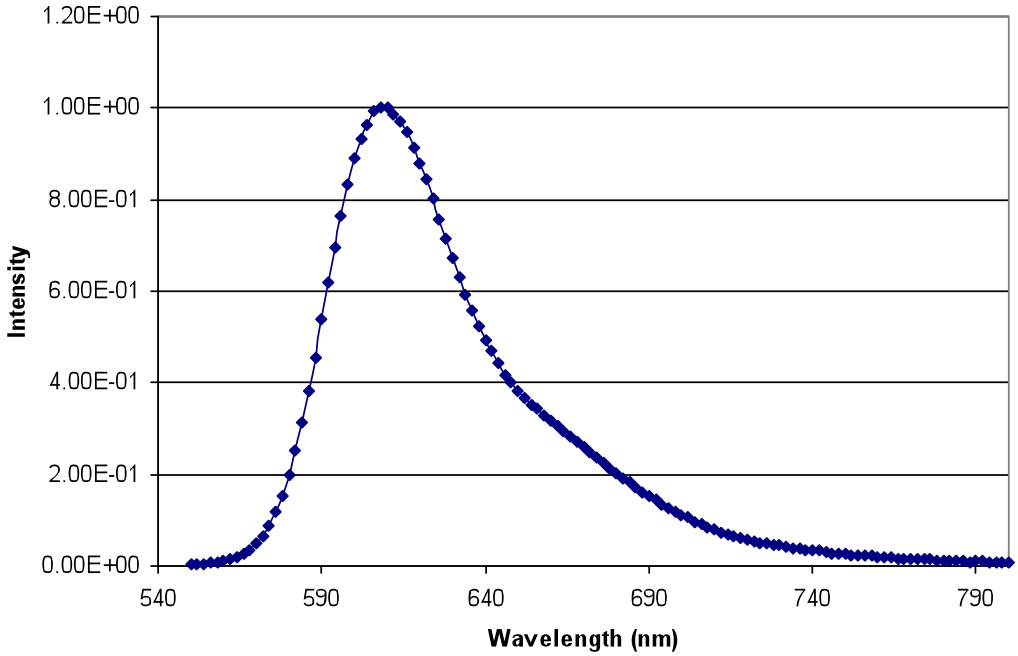
UV Absorption of ROX-RIS (7a5)

Sample in 0.1M phosphate buffer, pH 8, with concentration at 5.48 μM. Recorded on DU 800 spectrometer. Extinction coefficient: 72,000$M^{-1}cm^{-1}$



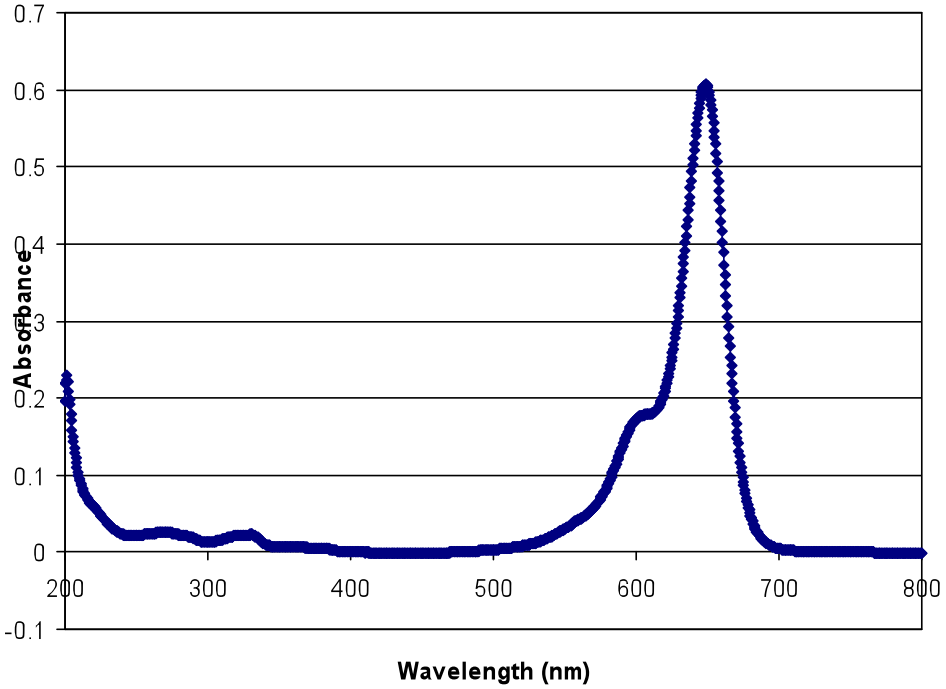
Emission Spectrum of ROX-RIS (7a5)

Sample in 0.1M phosphate buffer, pH 8, with concentration at 1.37 μM. Recorded on photon technology international quanta master model C-60SE spectrofluorimeter.



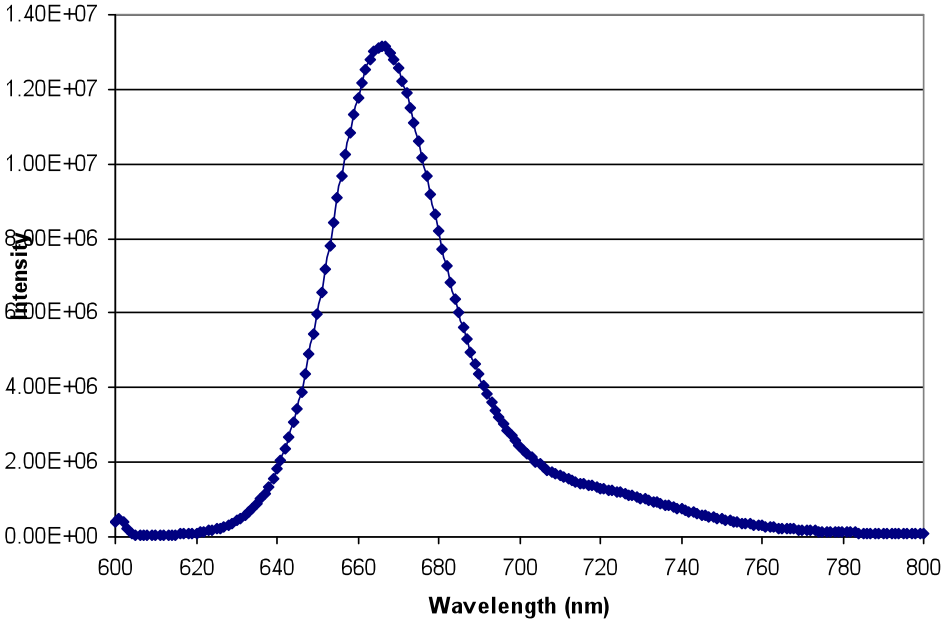
UV Absorption of AF647-RIS (7a6)

Sample in 0.1M phosphate buffer, pH 7.0, with concentration at 2.5 μM. Recorded on DU 800 spectrometer. Extinction coefficient: 240000$M^{-1}cm^{-1}$



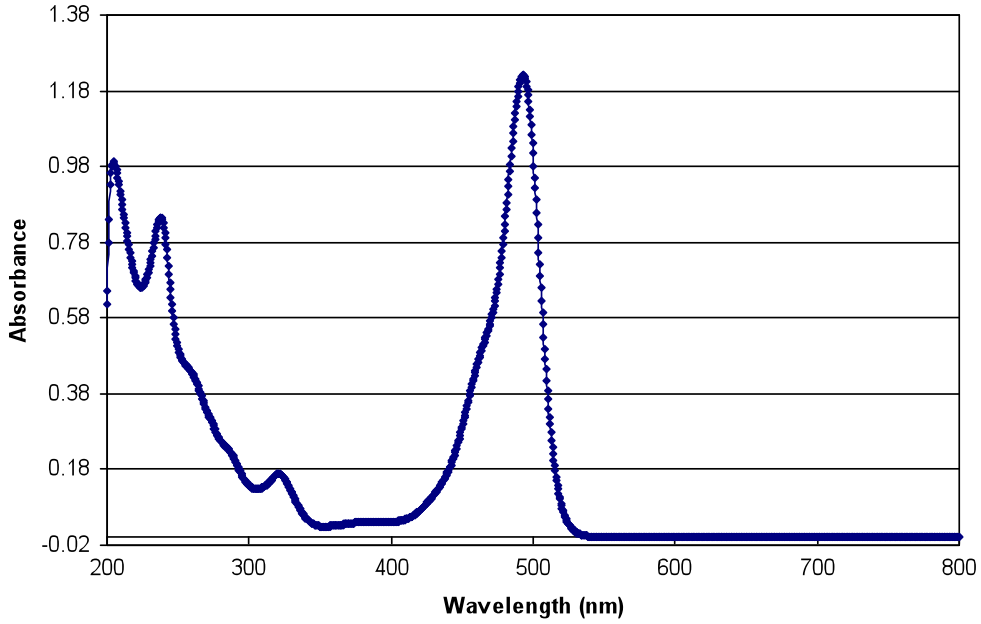
Emission Spectrum of AF647-RIS (7a6)

Sample in 0.1M phosphate buffer, pH 7.0, with concentration at 0.6 μM. Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



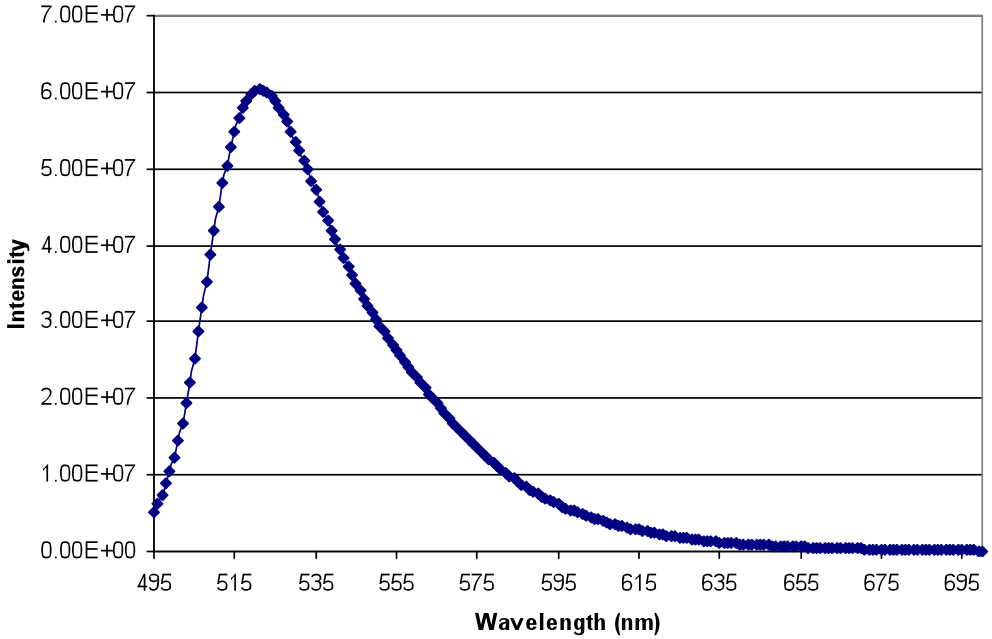
UV Absorption of 5(6)-FAM-RISPC (7b1)

Sample in 0.1M phosphate buffer, pH 7.2, with concentration at 16.7 μM. Recorded on DU 800 spectrometer. Extinction coefficient: 73,000$M^{-1}cm^{-1}$



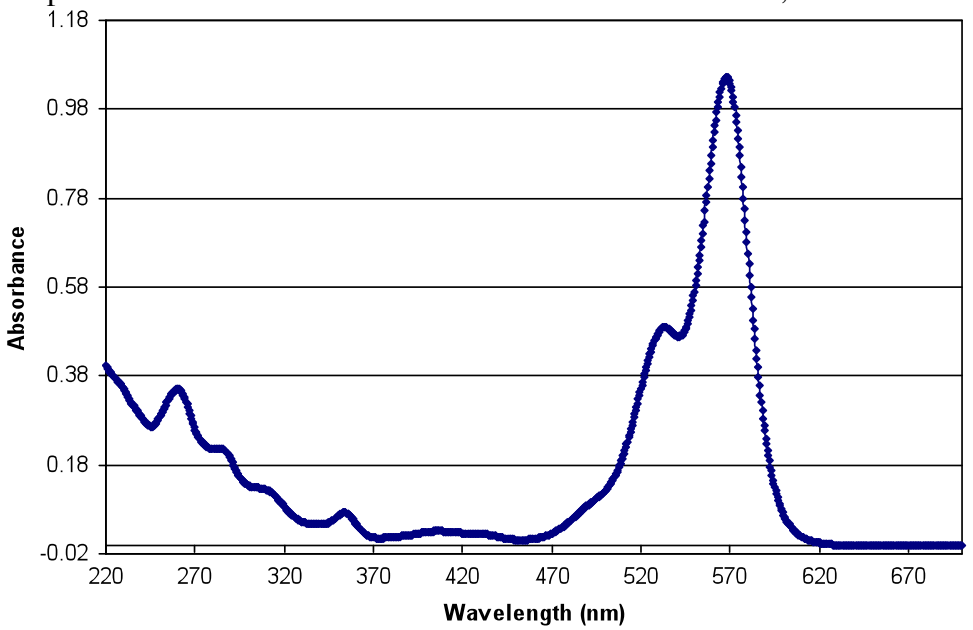
Emission Spectrum of 5(6)-FAMRISPC (7b1)

Sample in 0.1M phosphate buffer, pH 7.2, with concentration at 8.39 μM. Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



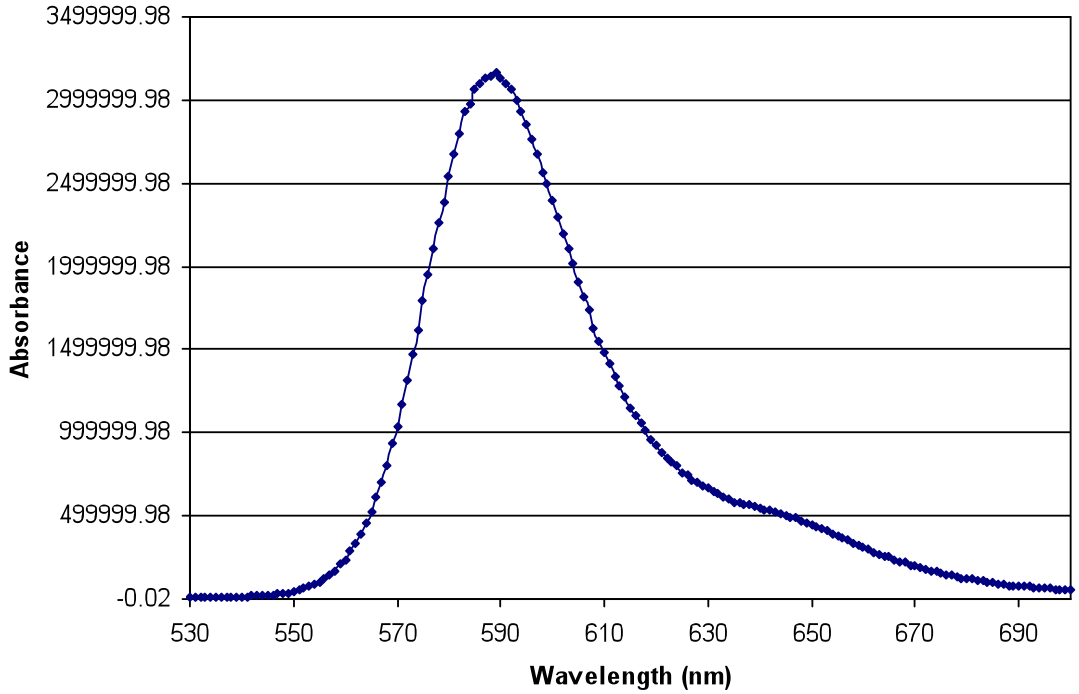
UV Absorption of 5(6)-RhR-RISPC (7b2)

Sample in 0.1M phosphate buffer, pH 7.5, with concentration at 9.16 µM. Recorded on DU 800 spectrometer. λmax = 568 nm. Extinction coefficient: 114,850$M^{-1}cm^{-1}$.



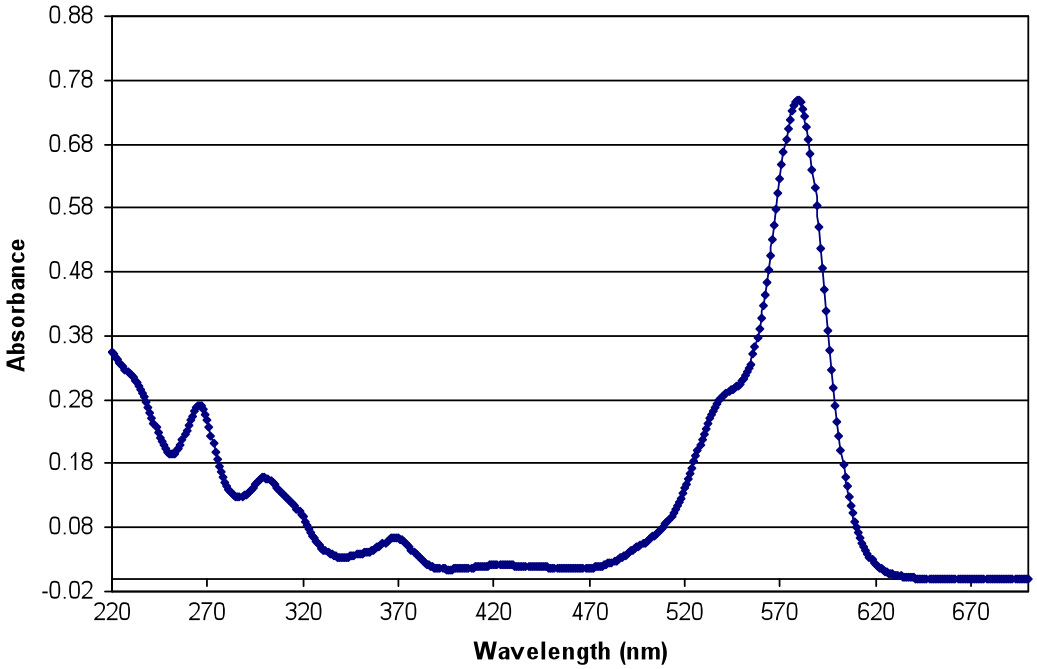
Emission Spectrum of 5(6)-RhR-RISPC (7b2)

Sample in 0.1 M phosphate buffer, pH 7.5, with concentration at 1.4 µM. λmax = 589 nm.Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



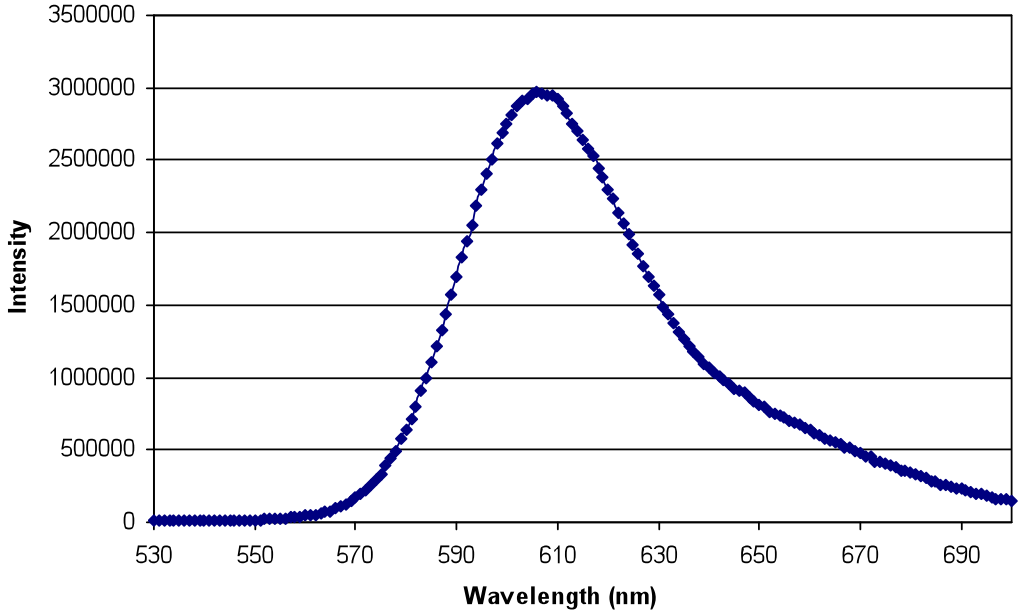
UV Absorption of ROX-RISPC (7b3)

Sample in 0.1M phosphate buffer, pH 8, with concentration at 10.4 μM. Recorded on DU 800 spectrometer. Extinction coefficient: 73,000$M^{-1}cm^{-1}$



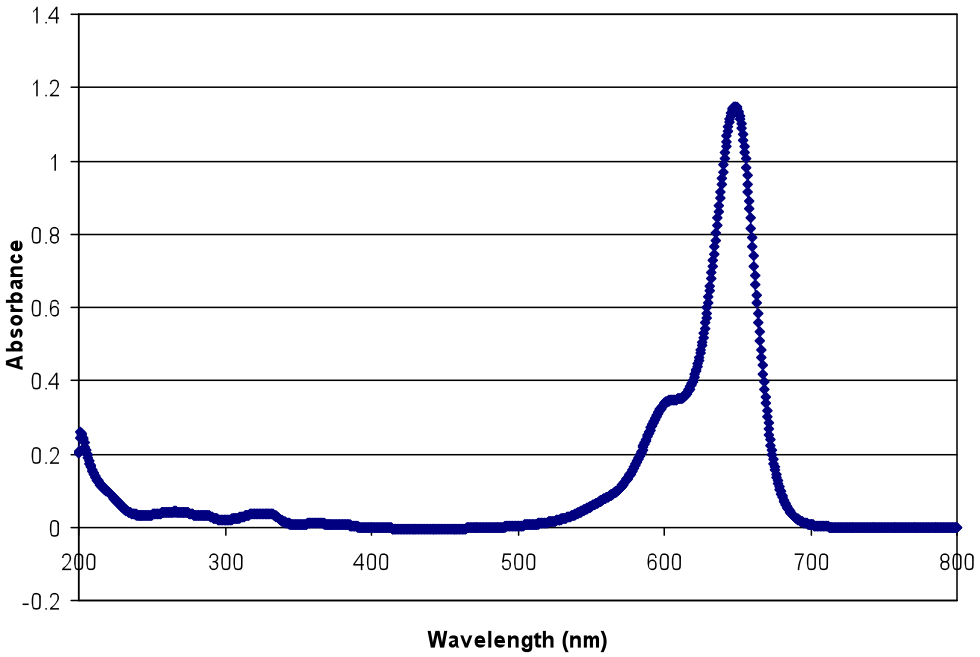
Emission Spectrum of ROX-RISPC (7b3)

Sample in 0.1M phosphate buffer, pH 8, with concentration at 2.0 μM. Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



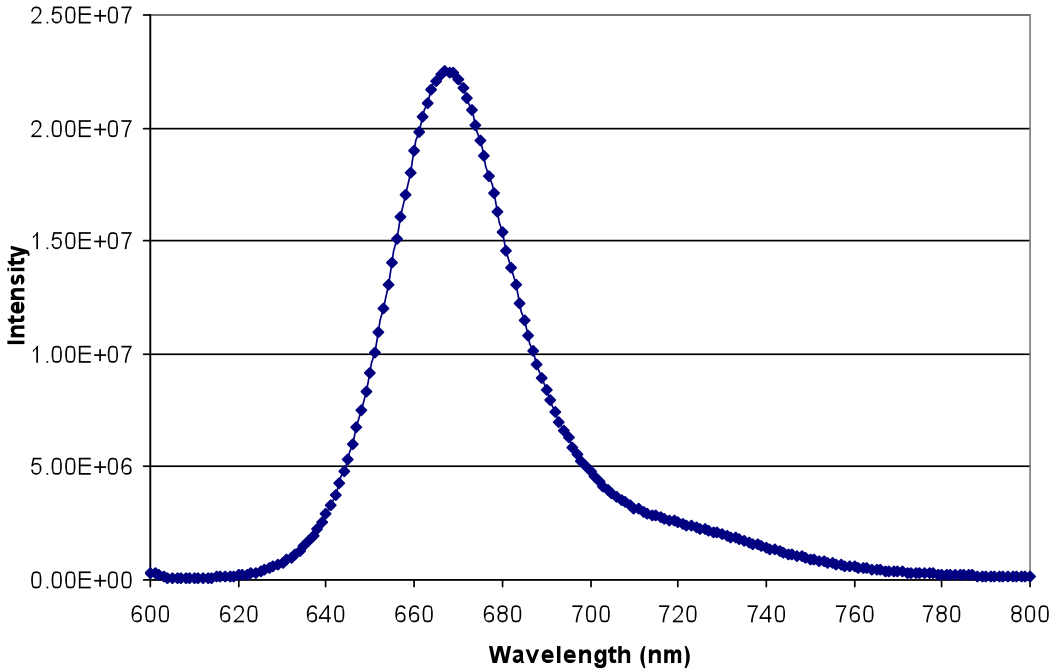
UV Absorption of AF647-RISPC (7b4)

Sample in 0.1M phosphate buffer, pH 7.0, with concentration at 4.8 μM. Recorded on DU 800 spectrometer. Extinction coefficient: 240000$M^{-1}cm^{-1}$



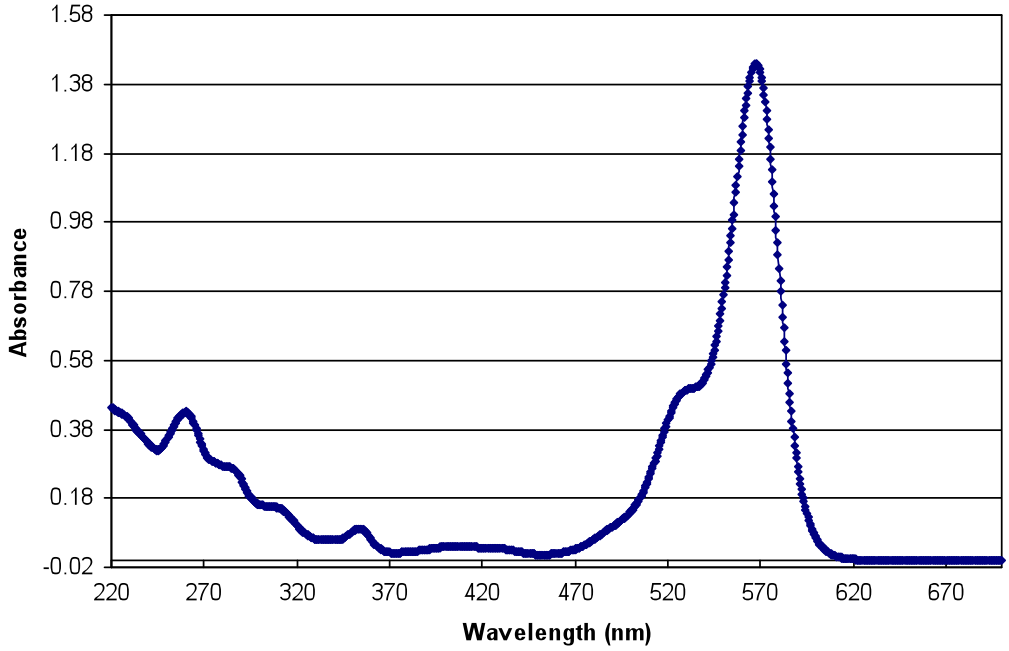
Emission Spectrum of AF647-RISPC (7b4)

Sample in 0.1M phosphate buffer, pH 7.0, with concentration at 1.2 μM. Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



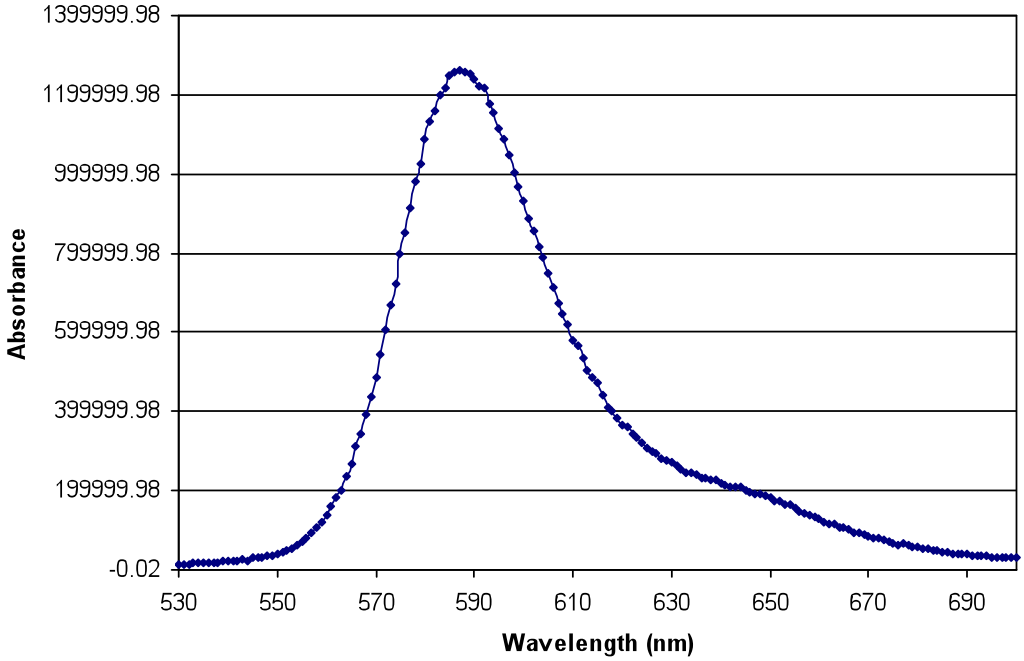
UV Absorption of 5(6)-RhR-dRIS (7c2)

Sample in 0.1M phosphate buffer, pH 7.5, with concentration at 12.5 μM. Recorded on DU 800 spectrometer. λmax = 567.5 nm. Extinction coefficient: 114,850$M^{-1}cm^{-1}$.



Emission Spectrum of 5(6)-RhR-dRIS (7c2)

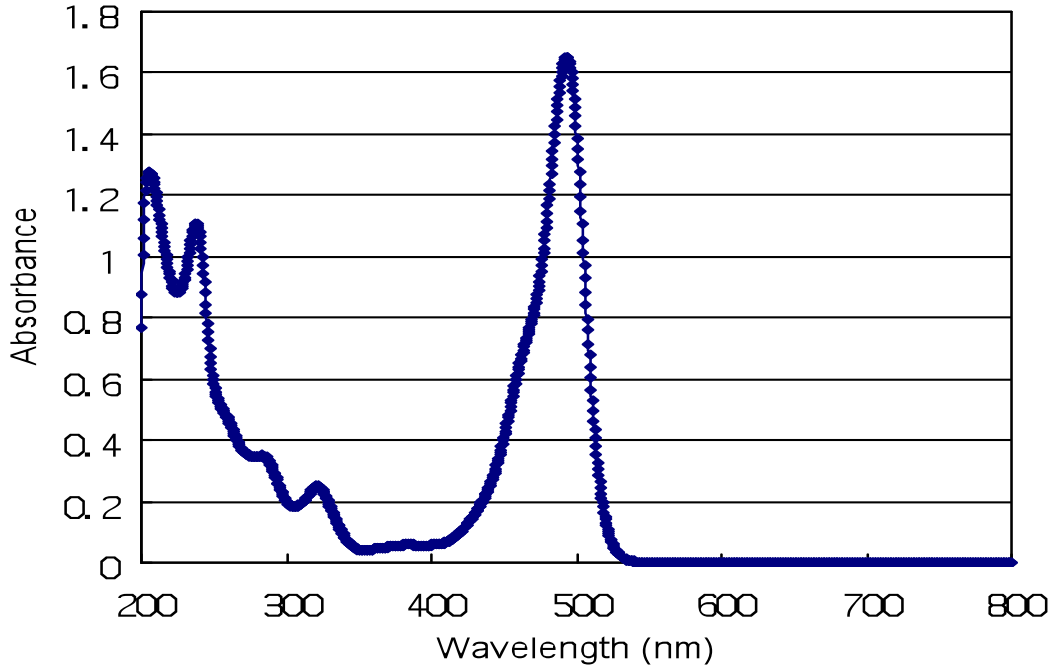
Sample in 0.1 M phosphate buffer, pH 7.5, with concentration at 3.1 μM. λmax = 589 nm.Recorded on Jobin Yvon Horiba Fluoromax-3 Fluorometer.



UV-VIS Spectrum of 5-FAM-ZOL (7d1)

In 0.1 M Phosphate buffer (pH 7.2), 22.6 μM, ε= 73,000 $M^{-1}cm^{-1}$

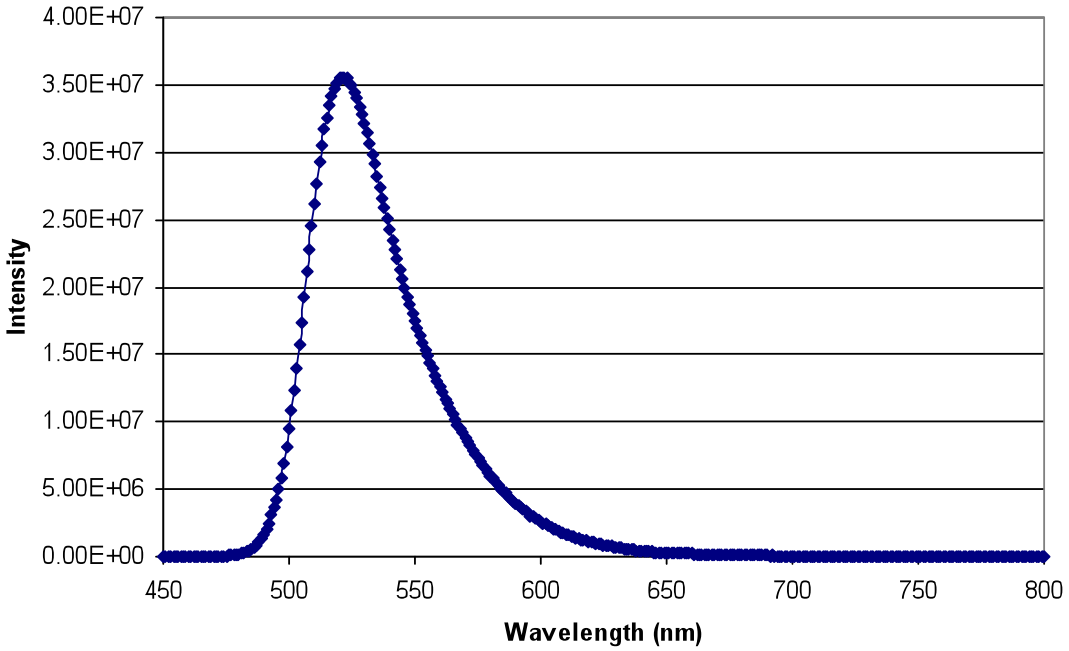
Recorded on DU 800 Spectrophotometer



Fluorescent Emission Spectrum of 5-FAM-ZOL (7d1)

In 0.1 M Phosphate buffer (pH 7.2), 5.66 μM

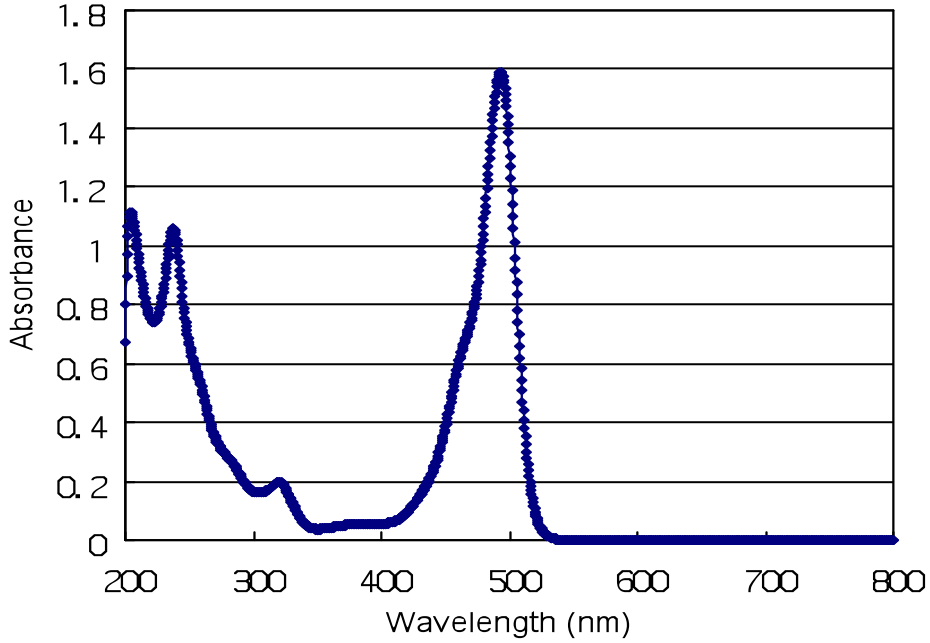
Recorded on Jobin Yvon Fluorolog Spectrometer



UV-VIS Spectrum of 6-FAM-ZOL (7d2)

In 0.1 M Phosphate buffer (pH 7.2), 21.8 μM, ε= 73,000 $M^{-1}cm^{-1}$

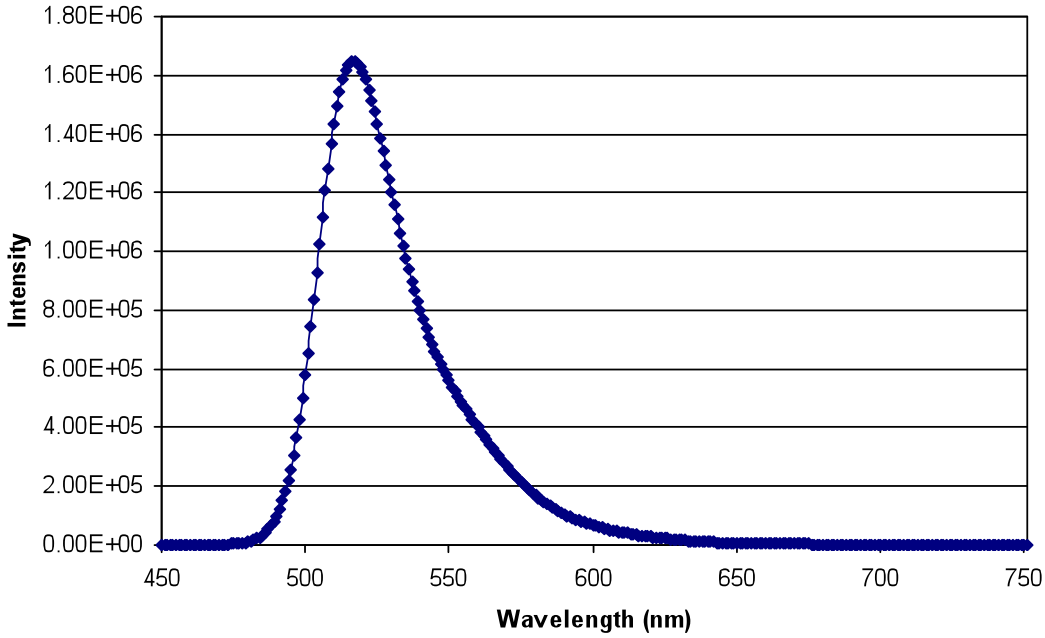
Recorded on DU 800 Spectrophotometer



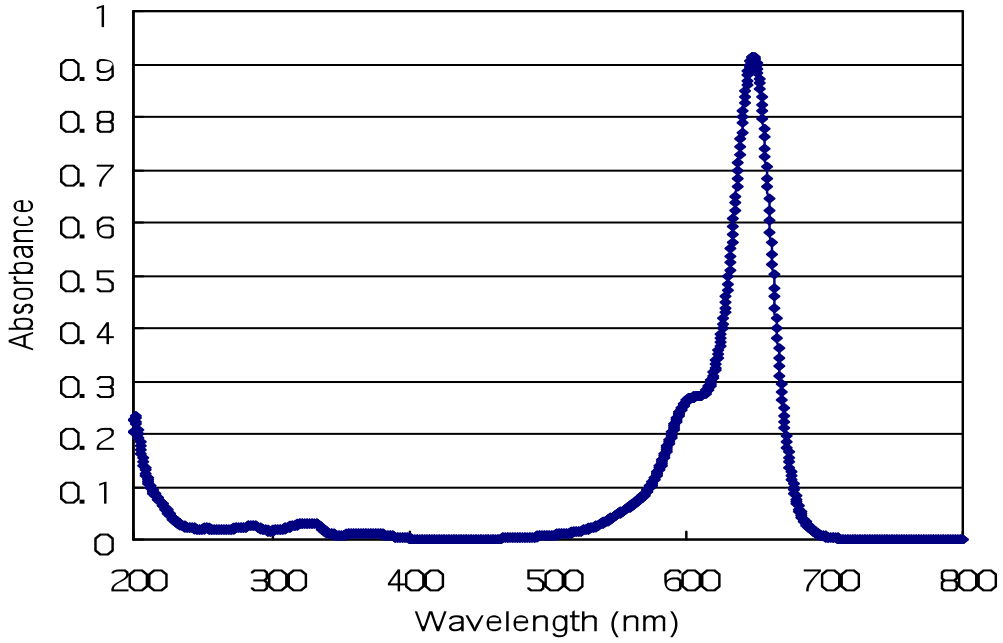
Fluorescent Emission Spectrum of 6-FAM-ZOL (7d2)

In 0.1 M Phosphate buffer (pH 7.2), 5.45 μM

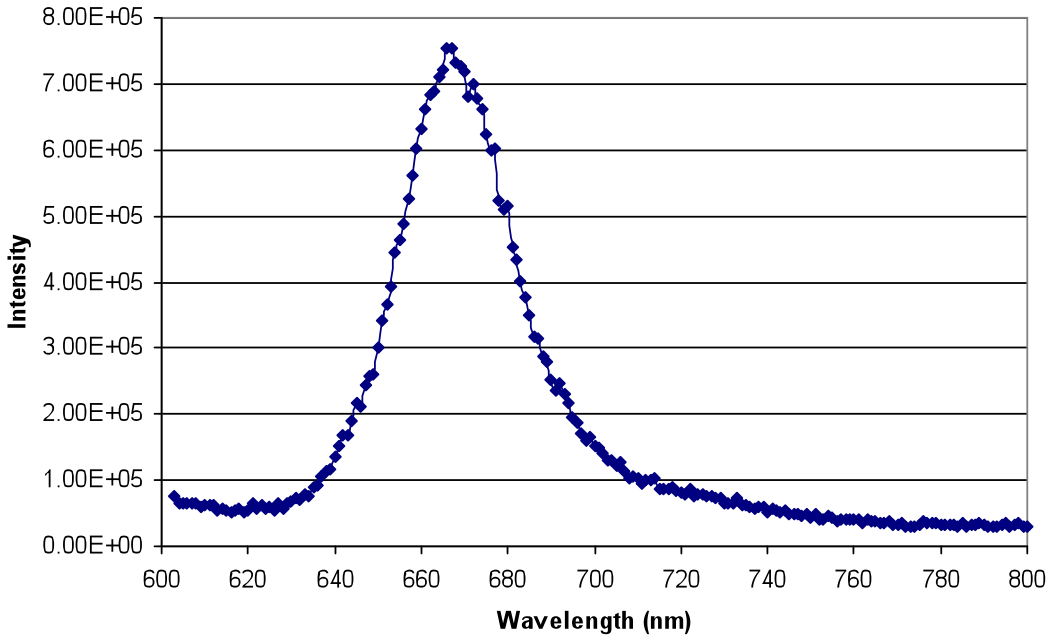
Recorded on Jobin Yvon Fluorolog Spectrometer



UV-VIS Spectrum of AF647-ZOL (7d3)
In 0.1 M Phosphate buffer (pH 7.0), 3.8 μM, ε = 240,000 $M^{-1}cm^{-1}$
Recorded on DU 800 Spectrophotometer



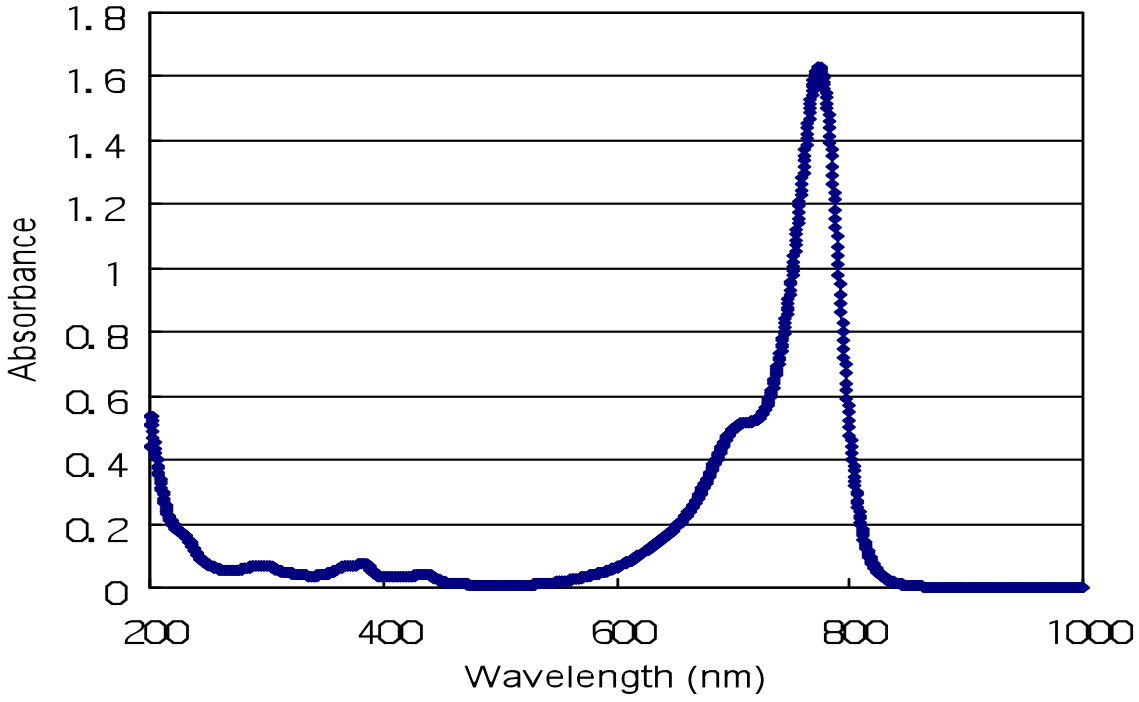
Fluorescent Emission Spectrum of AF647-ZOL (7d3)
In 0.1 M Phosphate buffer (pH 7.0), 0.95 μM
Recorded on Jobin Yvon Fluorolog Spectrometer



UV-VIS Spectrum of 800CW-ZOL (7d4)

In 1xPBS (pH 7.4), 6.79 μM, ε = 240,000 $M^{-1}cm^{-1}$

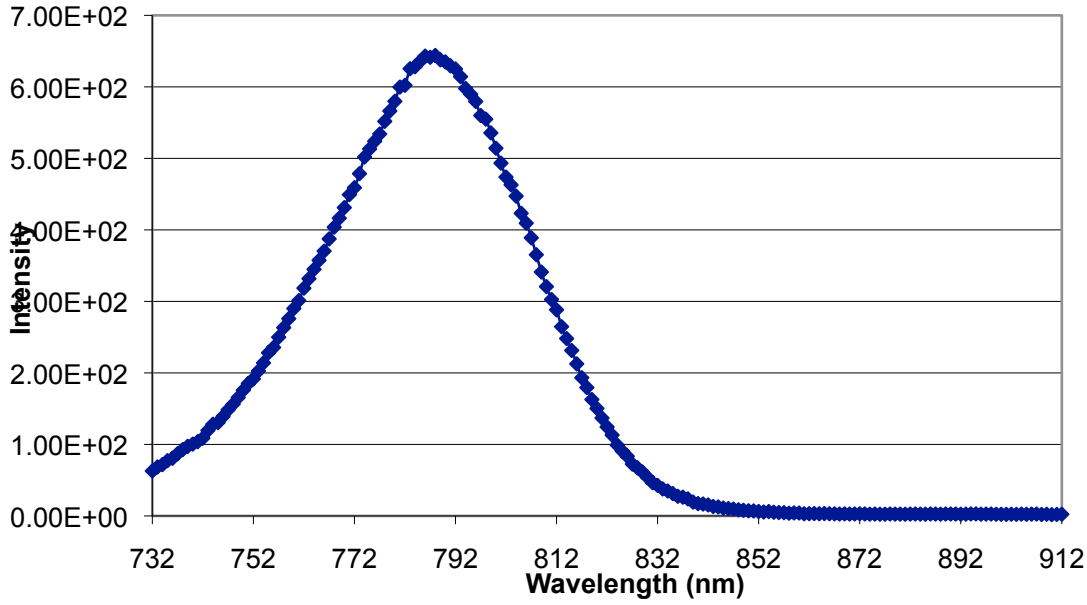
Recorded on DU 800 Spectrophotometer



Fluorescent Emission Spectrum of 800CW-ZOL (7d4)

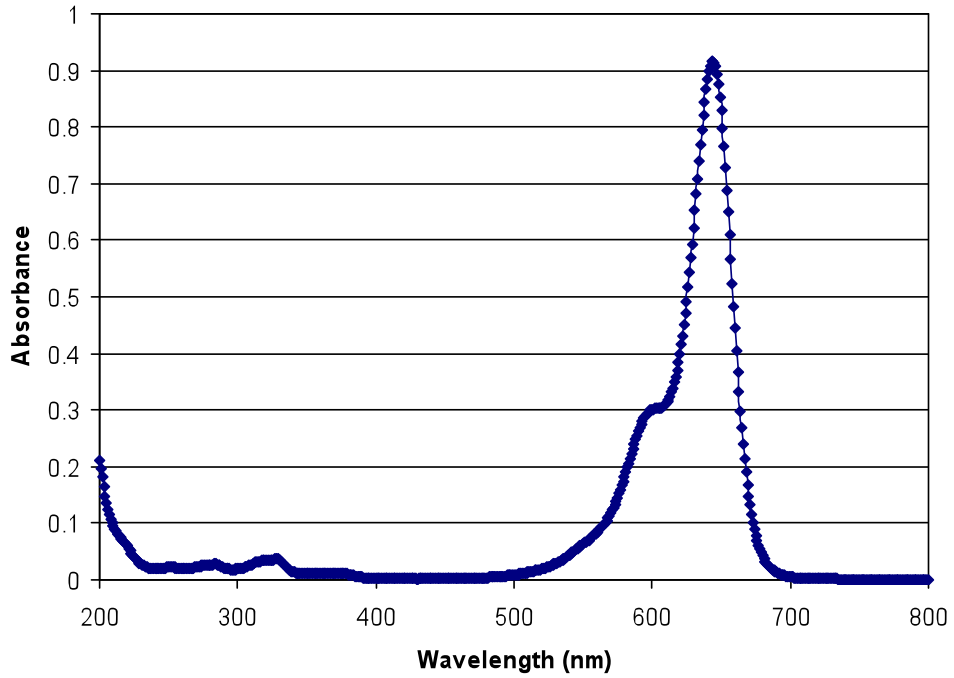
In 0.1 M Phosphate buffer (pH 7.0), 1.70 μM

Recorded on SHIMADZU spectrofluorophotometer RF-5301PC (corrected based on IRDye 800CW)



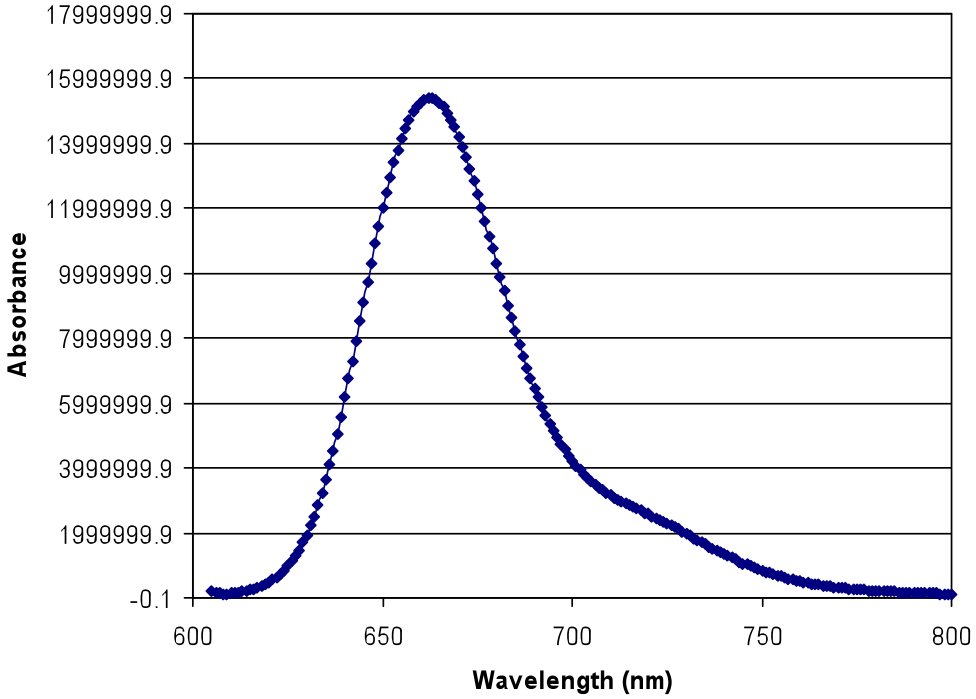
UV Absorption of Sulfo-Cy5-ZOL (7d5)

Sample in 1* PBS, pH 7.4, with concentration at 3.39 μM (λmax = 644 nm). Recorded on DU 800 spectrometer. Extinction coefficient: 240000$M^{-1}cm^{-1}$



Emission Spectrum of Sulfo-Cy5-ZOL (7d5)

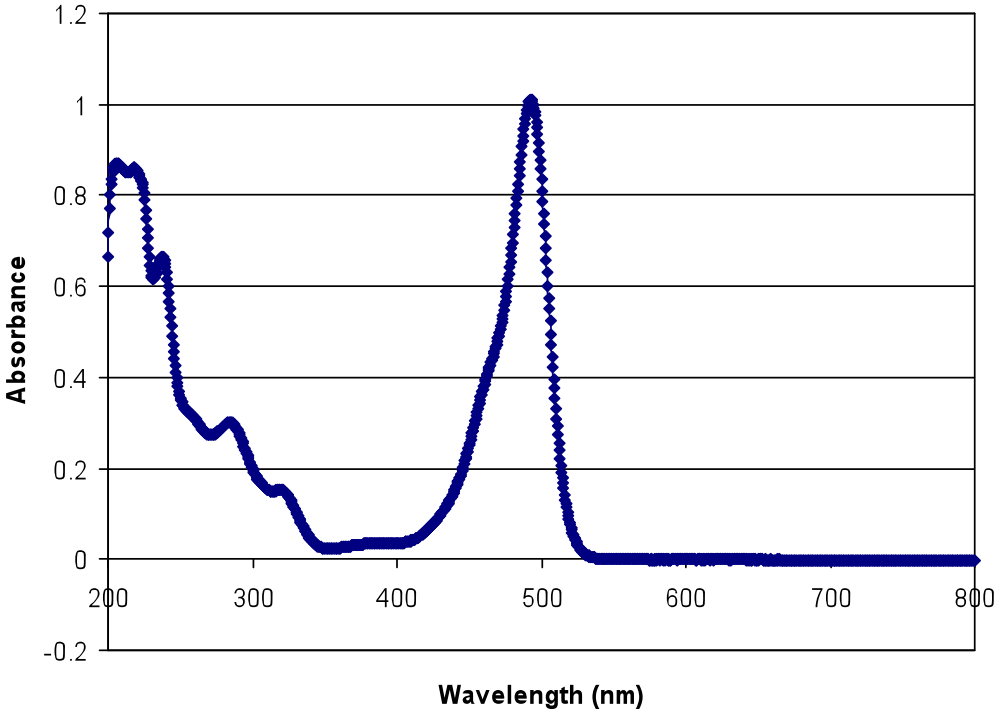
Sample in 0.1M phosphate buffer, pH 7.0, with concentration at 0.85 μM. Recorded on Jobin Yvon Nanolog spectrofluorimeter (λmax = 663 nm).



UV-VIS Spectrum of 5-FAM-MIN (7e1)

In 0.1 M Phosphate buffer (pH 7.2), 13.8 μM, ε = 73,000 $M^{-1}cm^{-1}$

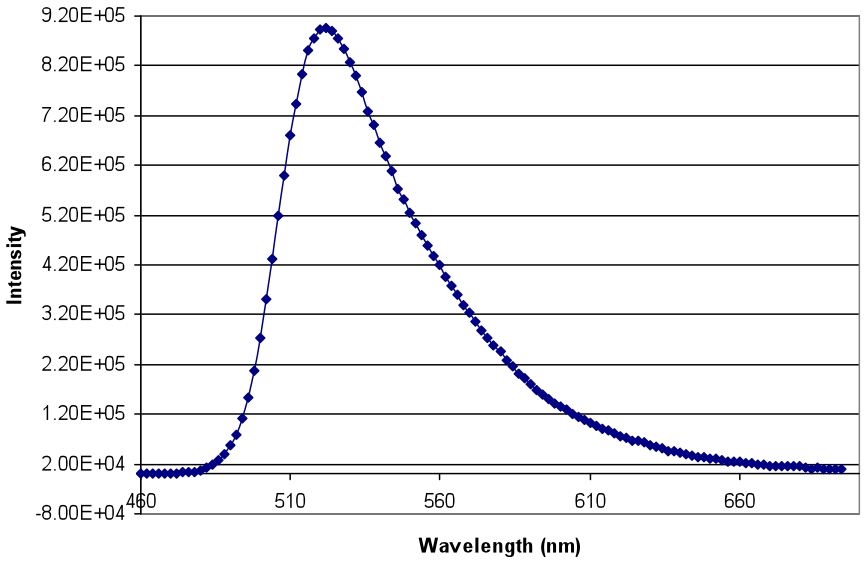
Recorded on DU 800 Spectrophotometer (λ = 493 nm)



Fluorescent Emission Spectrum of 5-FAM-MIN (7e1)

In 0.1 M Phosphate buffer (pH 7.2), 3.45 μM

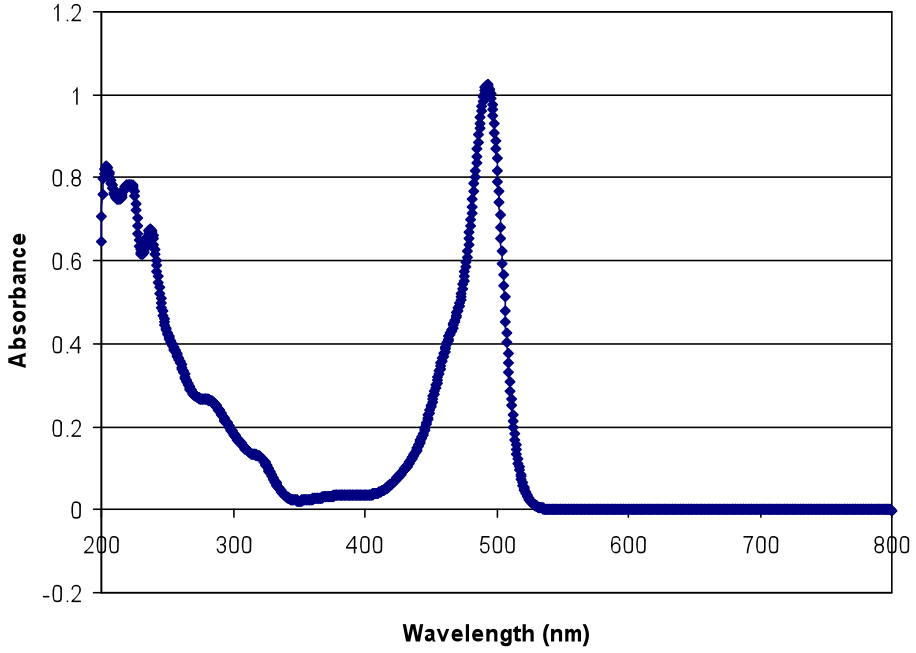
Recorded on PTI QuantaMaster model C-60SE Spectrometer equipped with a 928 PMT detector (λem = 522 nm)



UV-VIS Spectrum of 6-FAM-MIN (7e2)

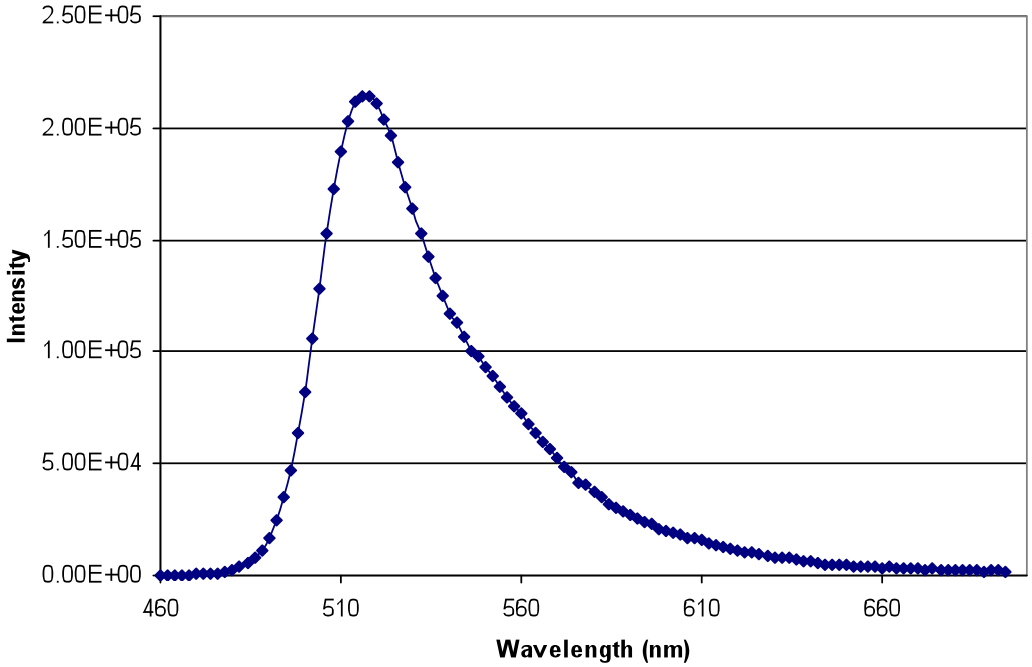
In 0.1 M Phosphate buffer (pH 7.2), 14.0 μM, ε = 73,000 $M^{-1}cm^{-1}$

Recorded on DU 800 Spectrophotometer (λ = 493 nm)



Fluorescent Emission Spectrum of 6-FAM-MIN (7e2)

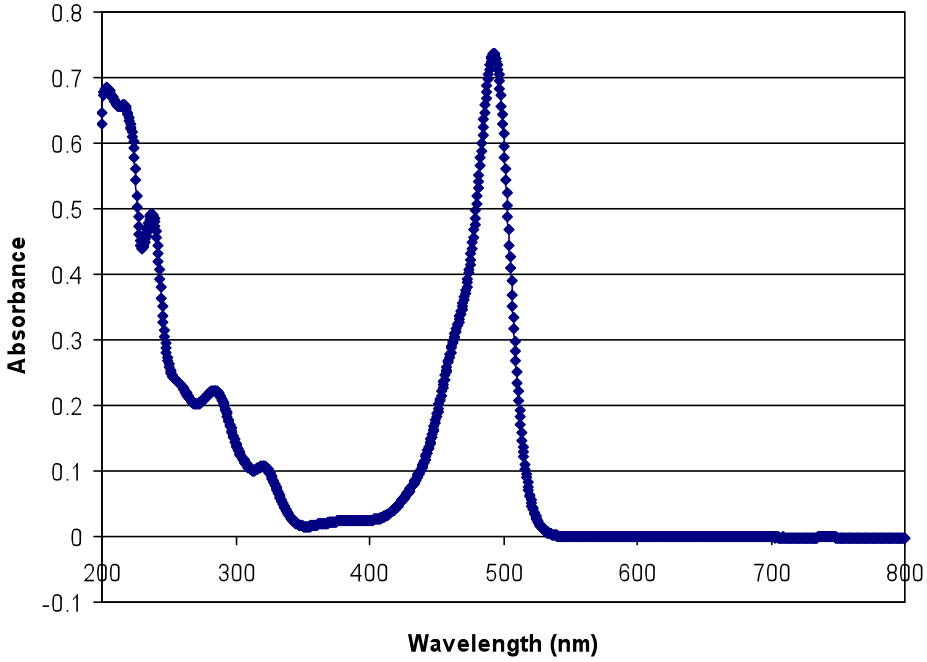
In 0.1 M Phosphate buffer (pH 7.2), 3.5 μM. Recorded on PTI QuantaMaster model C-60SE Spectrometer equipped with a 928 PMT detector (λem = 518 nm)



UV-VIS Spectrum of 5-FAM-MINPC (7f1)

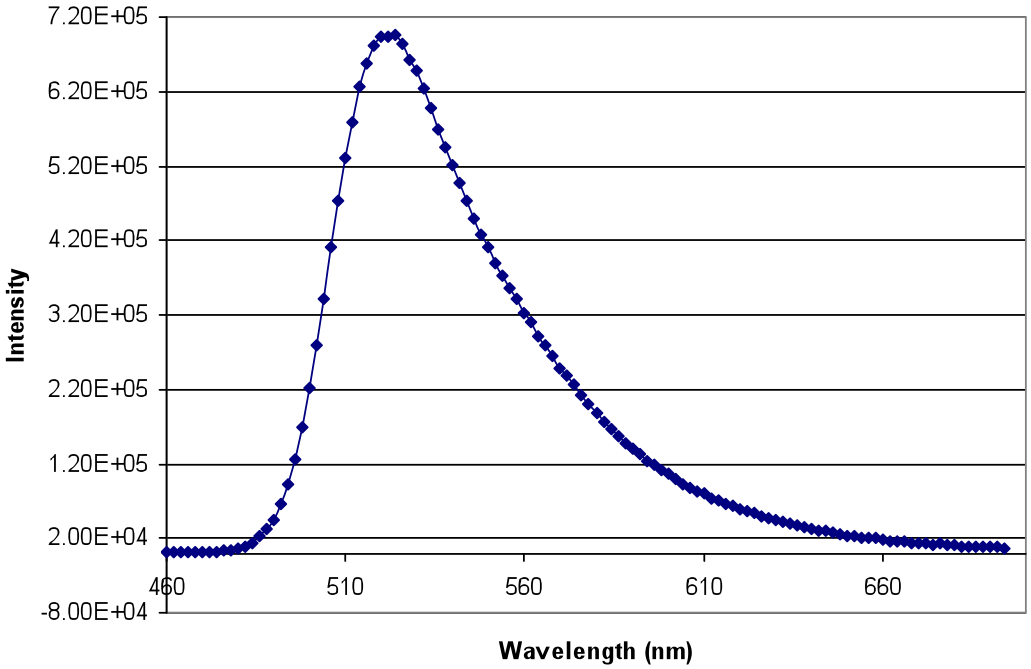
In 0.1 M Phosphate buffer (pH 7.2), 10.1 μM, ε = 73,000 $M^{-1}cm^{-1}$

Recorded on DU 800 Spectrophotometer (λ = 493 nm)



Fluorescent Emission Spectrum of 5-FAM-MINPC (7f1)

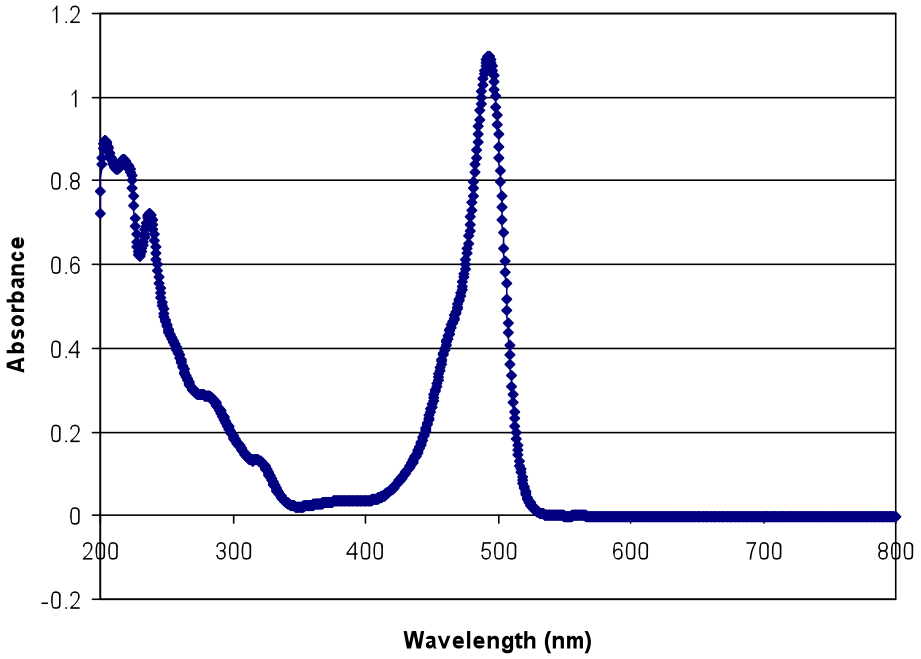
In 0.1 M Phosphate buffer (pH 7.2), 2.53 μM. Recorded on PTI QuantaMaster model C-60SE Spectrometer equipped with a 928 PMT detector (λem = 522 nm)



UV-VIS Spectrum of 6-FAM-MINPC (7f2)

In 0.1 M Phosphate buffer (pH 7.2), 15.1 μM, ε = 73,000 $M^{-1}cm^{-1}$

Recorded on DU 800 Spectrophotometer (λ = 493 nm)



Fluorescent Emission Spectrum of 6-FAM-MINPC (7f2)

In 0.1 M Phosphate buffer (pH 7.2), 3.78 μM. Recorded on PTI QuantaMaster model C-60SE Spectrometer equipped with a 928 PMT detector (λem = 517 nm)