Supporting Information to Accompany

"Carboxylate-containing two-photon probe for simultaneous detection of extra- and intracellular pH values in colon cancer tissue"

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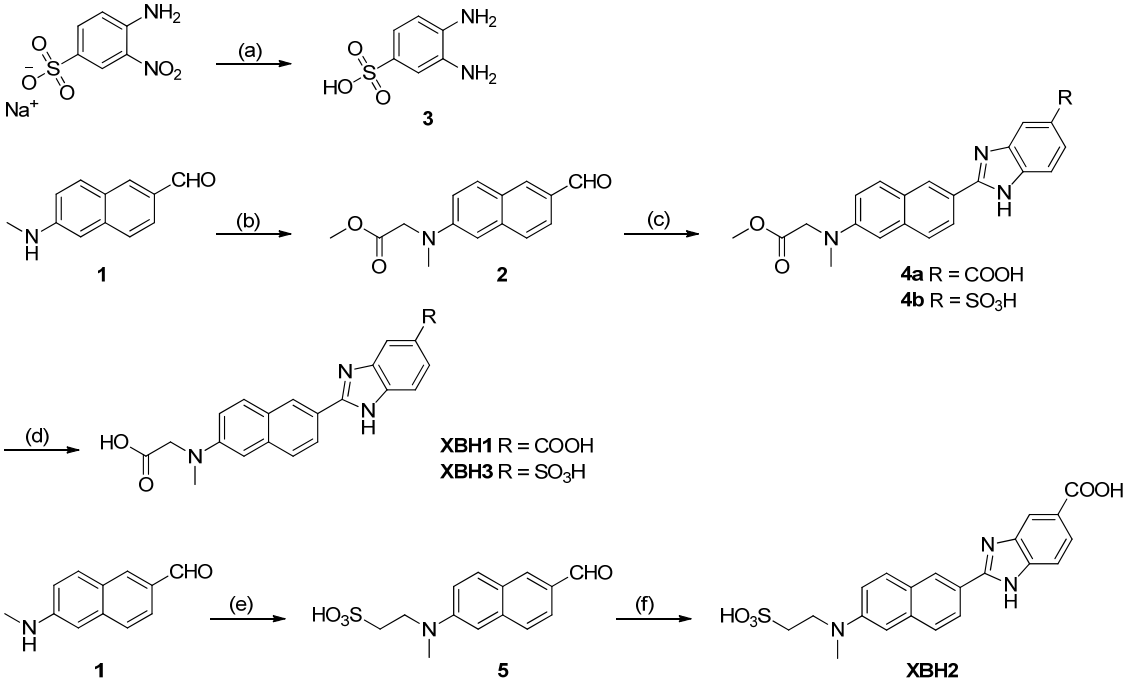

Scheme S1. Synthesis mechanism of **XBH1−3**. (a) tin(II) chloride, 12M HCl, H_2O, ethanol, (b) methyl bromoacetate, proton sponge, potassium iodide, acetonitrile, 80 °C, (c) **4a**: 3,4-diaminobenzoic acid, paratoluenefonic acid, DMF, 80 °C, **4b**: **3**, paratoluenefonic acid, DMF 80 °C, (d) 1M KOH, methanol/dioxane (1/1), (e) sodium 2-bromoethanesulfonate, proton sponge, potassium iodide, DMF, 80 °C, (f) **3**, paratoluenefonic acid, DMF 80 °C

1, 2 and 3b were prepared by the literature methods.[1,2]

Synthesis of 4a.

A mixture of **2** (257 mg, 1.00 mmol), **3a** (235 mg, 1.50 mmol), paratoluenefonic acid (38 mg, 0.20 mmol) and DMF (30 mL) in a two-necked flask was stirred at 80 °C for 16 h. after cooling in room temperature, the reaction mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2CO_3 and evaporated under vacuum. The product was purified by silica gel column chromatograph eluent with chloroform/methanol (5/1). Yellow powder of **4a** was obtained in 38 % yield (150 mg, 0.38 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.49 (br, s, 1H), 8.62 (s, 1H), 8.21 (m, 2H), 7.86 (m, 2H), 7.79 (d, 1H, J = 6.2Hz), 7.61 (d, 1H, J = 6.2Hz), 7.22 (d, 1H, 7.0Hz), 6.99 (s, 1H), 4.40 (s, 2H), 3.65 (s, 3H), 3.12 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.4, 169.8, 159.4, 154.5, 148.5, 136.1, 134.6, 130.1, 127.2, 126.7, 126.4, 124.9, 124.0, 123.6, 123.4, 116.9, 106.1, 53.8, 52.4 ppm.

Synthesis of 4b.

A mixture of **2** (500 mg, 1.94 mmol), **3b** (654 mg, 2.91 mmol), paratoluenesulfonic acid (74 mg, 0.39 mmol) and DMF (40 mL) in a two-neck flask was stirred at 80 °C for 24 h. after cooling, the reaction mixture was filtered. The product was purified by silica gel chromatograph eluent with chloroform/methanol (5/1). Yellow powder of **4b** was obtained in 19 % yield (92 mg, 0.37 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ 14.93 (br, s, 1H), 8.60 (s, 1H), 8.00 (d, 1H, J = 7.0 Hz), 7.92 (m, 3H), 7.75 (m, 2H), 7.31 (d, 1H, J = 7.0 Hz), 7.05 (s, 1H), 4.45 (s, 2H), 3.65 (s, 3H), 3.14 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 179.3, 171.4, 153.4, 148.4, 143.6, 143.1, 135.9, 134.9, 130.1, 127.2, 126.4, 124.8, 123.8, 120.8, 119.5, 118.4, 116.8, 106.2, 53.8, 52.5, 49.4 ppm.

Synthesis of XBH1.

To a solution of **4a** (30 mg, 0.08 mmol) in dioxane/methanol (1/1, 5 mL) was added 1M KOH (8.64 mg, 0.15 mmol). The reaction mixture was stirred at room temperature for 5 h. The solvent was evaporated and water (5 mL) added to the reaction mixture. The aqueous part of mixture was acidified with 1 N HCl until pH = 4. The product was obtained by filteration and wash with water followed by diethyl ether. Yellow powder of **XBH1** was collected in 35 % yield (10 mg, 0.03 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.68 (br, s, 1H), 8.57 (s, 1H), 8.18 (s, 1H), 8.13 (d, 1H, J = 8.4 Hz), 7.82 (m, 3H), 7.63 (d, 1H, J = 8 Hz), 7.22 (d, 1H, J = 9.2 Hz), 6.96 (s, 1H), 4.28 (s, 2H), 3.10 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 172.9, 168.8, 155.0, 149.5, 137.7, 136.8, 130.6, 130.4, 127.7, 127.1, 126.7, 126.5, 125.8, 125.2, 125.1, 124.8, 122.7, 120.2, 117.4, 117.2, 115.4, 106.3, 102.5, 54.3, 30.6 ppm. HRMS (FAB$^+$): m/z calcd for $[C_{21}H_{18}N_3O_4]^+$: 376.1292, found : 376.1292

Synthesis of 5.

A mixture of **1** (500 mg, 2.70 mmol), sodium 2-bromoehtnaesulfonate (570 mg, 2.70 mmol), proton sponge (1.6 g, 5.40 mmol), potassium iodide (224 mg, 1.35 mmol) and DMF (40 mL)

in a two-necked flask was stirred at 80 °C for 24 h. after cooling in room temperature, the reaction mixture was filtered after stirring with chloroform. The filtrate was evaporated under vacuum. The product was purified by silica gel column chromatograph eluent with dichloromethane/methanol (5/1). Yellow powder of **5** was obtained in 9 % yield (80 mg, 0.24 mmol). ^{1}H NMR (400 MHz, D_2O): δ 9.81 (s, 1H), 8.26 (s, 1H), 7.92 (d, 1H, *J* = 9.6 Hz), 7.72 (m, 2H), 7.29 (dd, 1H, *J* = 8.8Hz, J = 2.4Hz), 7.03 (d, 1H, *J* = 2.4 Hz), 3.90 (t, 2H, *J* = 7.6 Hz), 3.34 (s, 3H), 3.19 (t, 2H, J = 7.6 Hz)

Synthesis of XBH2

A mixture of **5** (40 mg, 0.13 mmol), **3a** (29 mg, 0.19 mmol), paratoluenefonic acid (5 mg, 0.03 mmol) and DMF (10 mL) in a two-necked flask was stirred at 80 °C for 16 h. after cooling in room temperature, the reaction mixture was filtered. Yellow powder of **XBH2** was obtained in 21 % yield (12 mg, 0.03 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.14 (br, s, 1H), 8.54 (s, 1H), 8.16 (s, 1H), 8.09 (s, 1H), 7.81 (m, 3H), 7.67 (s, 1H), 7.78 (s, 1H), 7.97 (s, 1H), 3.74 (m, 2H), 3.04 (s, 3H), 2.71 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.6, 154.9, 148.3, 140.0, 136.4, 130.3, 127.0, 126.9, 125.9, 124.9, 124.8, 124.0 123.1, 117.1, 105.7, 49.5, 48.1, 37.9 ppm. HRMS (FAB$^+$) : m/z calcd for $[C_{21}H_{20}N_3O_5S]^+$: 426.1118, found : 426.1120.

Synthesis of XBH3.

To a solution of **4b** (91.8 mg, 0.22mmol) in dioxane/methanol (1/1, 10 mL) was added 1 M KOH (18.12 mg, 0.32 mmol). The reaction mixture was stirred at room temperature for 5 h. The solvent was evaporated and water (10 mL) added to the reaction mixture. The aqueous part of mixture was acidified with 1 N HCl until pH = 4. The product was obtained by filteration and wash with water followed by diethyl ether. Yellow powder of **XBH3** was collected in 34 % yield (30 mg, 0.07 mmol). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.70 (br, s, 1H), 8.58 (s, 1H), 8.01 (d, 1H, *J* = 8.4 Hz), 7.93 (s, 1H), 7.87 (m, 2H), 7.76 (d, 1H, *J* = 8.4 Hz), 7.72 (d, 1H, *J* = 8.8 Hz), 7.26 (d, 1H, *J* = 9.2 Hz), 7.00 (s, 1H), 4.30 (s, 2H), 3.11 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 172.7 151.8 150.5 146.9 137.8 133.8 133.0 131.2 131.1 129.7 129.6 128.2 126.1 126.0 124.6 124.5 124.4 118.0 117.1 114.3 111.8 54.2 ppm. HRMS (FAB$^+$) : m/z calcd for $[C_{20}H_{18}N_3O_5S]^+$: 412.0962, found : 412.0964

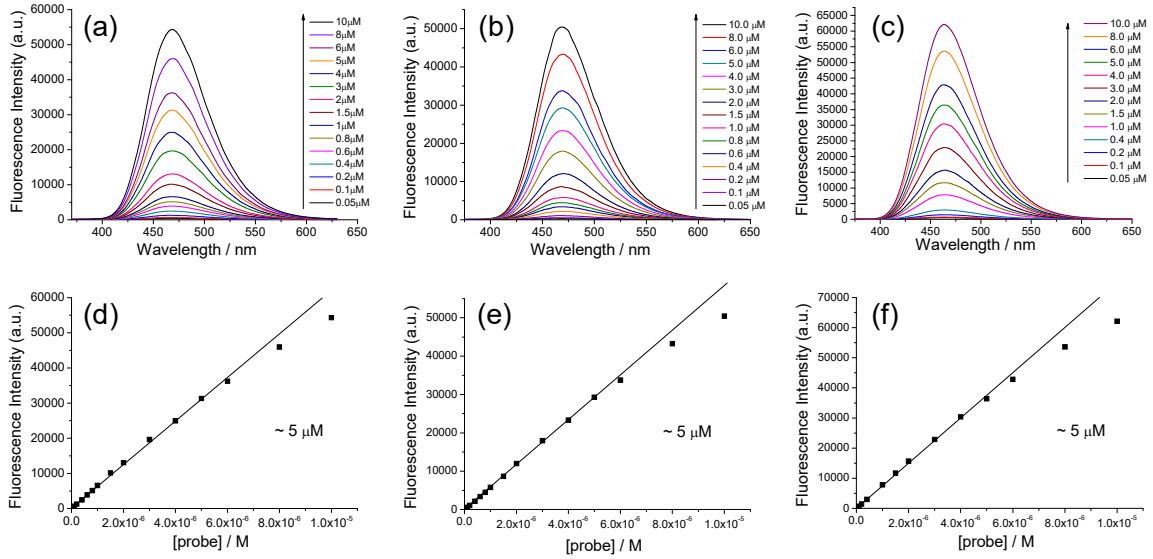

Figure S1. (a-c) One-photon fluorescence spectra of (a) **XBH1**, (b) **XBH2**, and (c) **XBH3**. (d-f) Plot of fluorescence intensity against (d) **XBH1**, (e) **XBH2**, and (f) **XBH3** concentration in universal buffer solutions. The excitation wavelength was 395 nm.

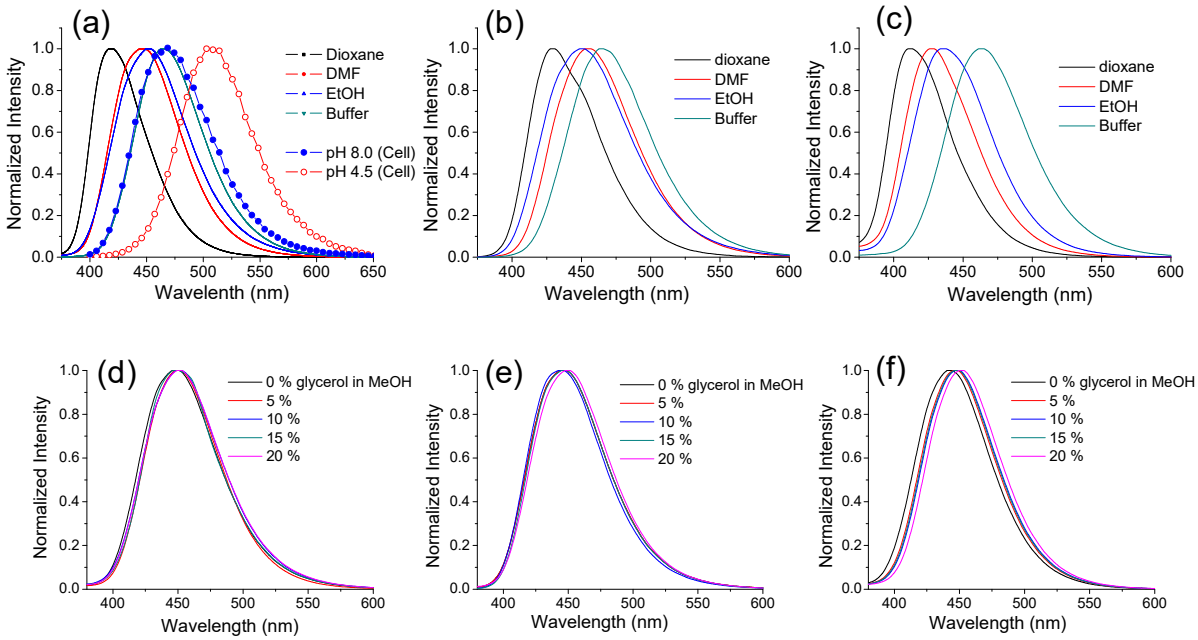

Figure S2. Fluorescence spectrum of (a, d) **XBH1**, (b, e) **XBH2**, and (c, f) **XBH3** in (a-c) various solvents and HeLa cell at pH 4.5 and 8.0. (d-f) Fluorescence spectrum upon viscosity changes (methanol-glycerol system). The excitation wavelength were 395 nm (OP) and 750 nm (TP), respectively.

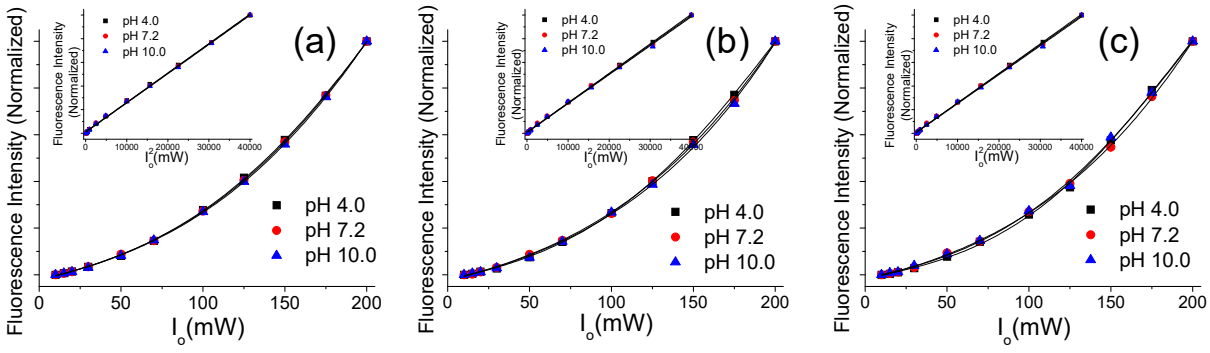

Figure S3. Dependence of output fluorescence intensity (I_{out}) of (a) **XBH1**, (b) **XBH2**, and (c) **XBH3** in universal buffer solutions at pH 4.0, 7.2, and 10 on the input laser power (I_{in}). The insert shows the linear dependence of I_{out} on I_{in}^2 (Excitation wavelength = 750 nm, 80 MHz, τ = 100 fs).

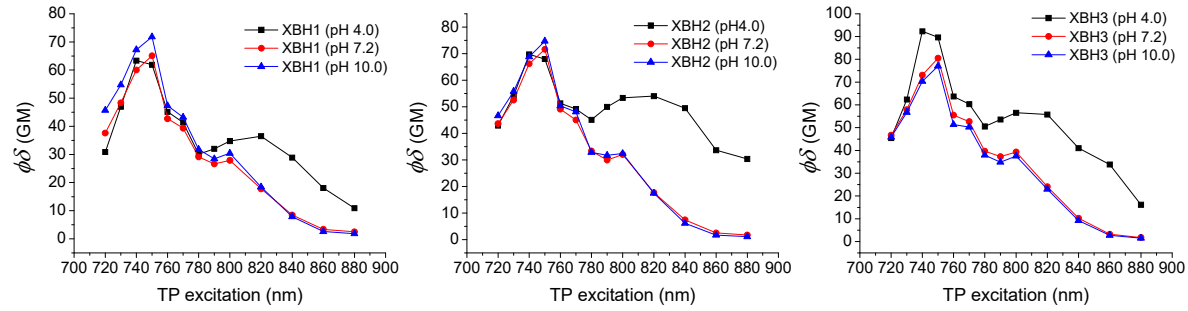

Figure S4. Two-photon action (δΦ) spectra of (a) **XBH1**, (b) **XBH2**, and (c) **XBH3** in universal buffer solutions at pH 4.0, 7.2, and 10, respectively.

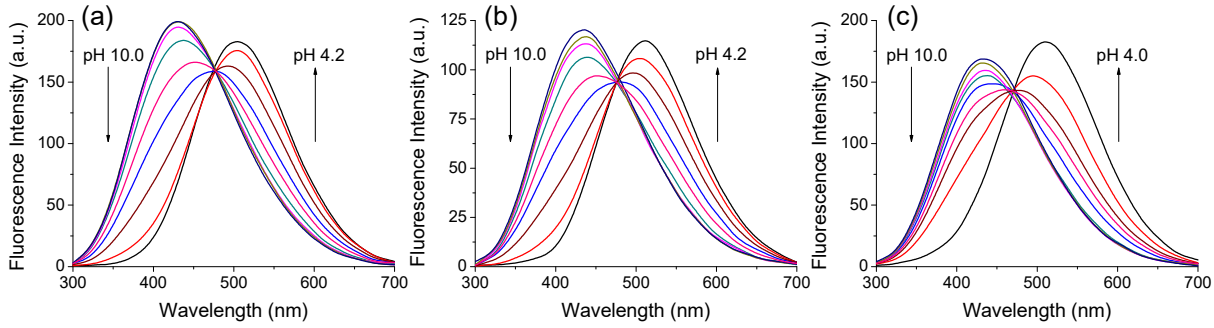

Figure S5. Two-photon fluorescence spectra of (a) **XBH1**, (b) **XBH2**, and (c) **XBH3** in a universal buffer at pH 4.0 to 10. Excitation wavelength was 750 nm.

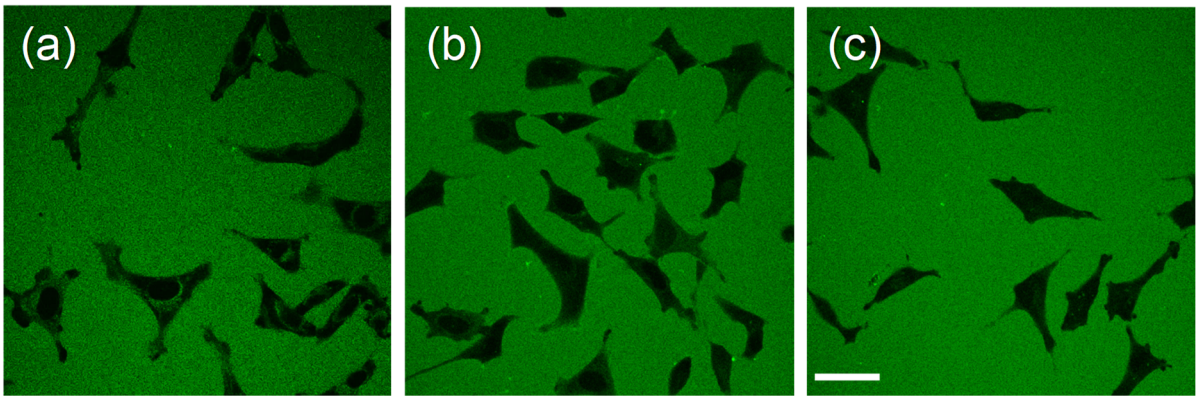

Figure S6. TPM images of HeLa cells incubated with 3 μM (a) **XBH1**, (b) **XBH2**, and (c) **XBH3** in normal growth medium for 10 min at room temperature. The excitation wavelength was 750 nm; the images were collected at 400–600 nm. The scale bar corresponds to 48 μm.

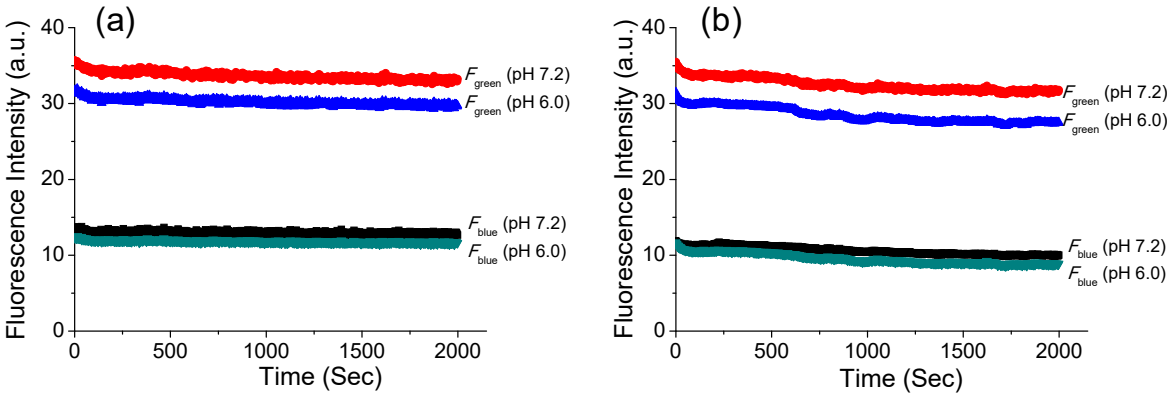

Figure S7. The relative TPEF intensity from (a) **XBH1** and (b) **XBH2** labeled extracellular region of HeLa cells as a function of time at different pHe. The digitized intensity was recorded with 2.00 sec intervals for the duration of one hour using *xyt* mode. The TPEF intensities were collected at F_{blue} (400-450 nm) and F_{green} (500-600 nm) with 2.2 mW average power at the focal point.

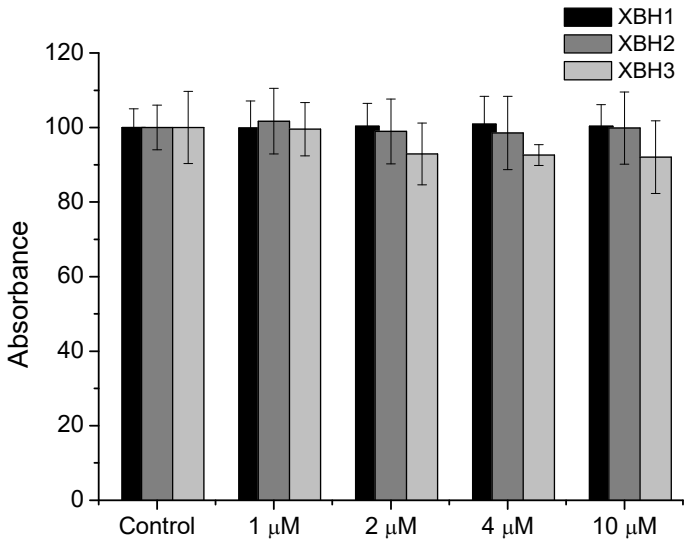

Figure S8. Viability of Hela cells in the presence of **XBH1**, **XBH2**, and **XBH3** as measured by using MTS assay. The cells were incubated with probe for 2 h.

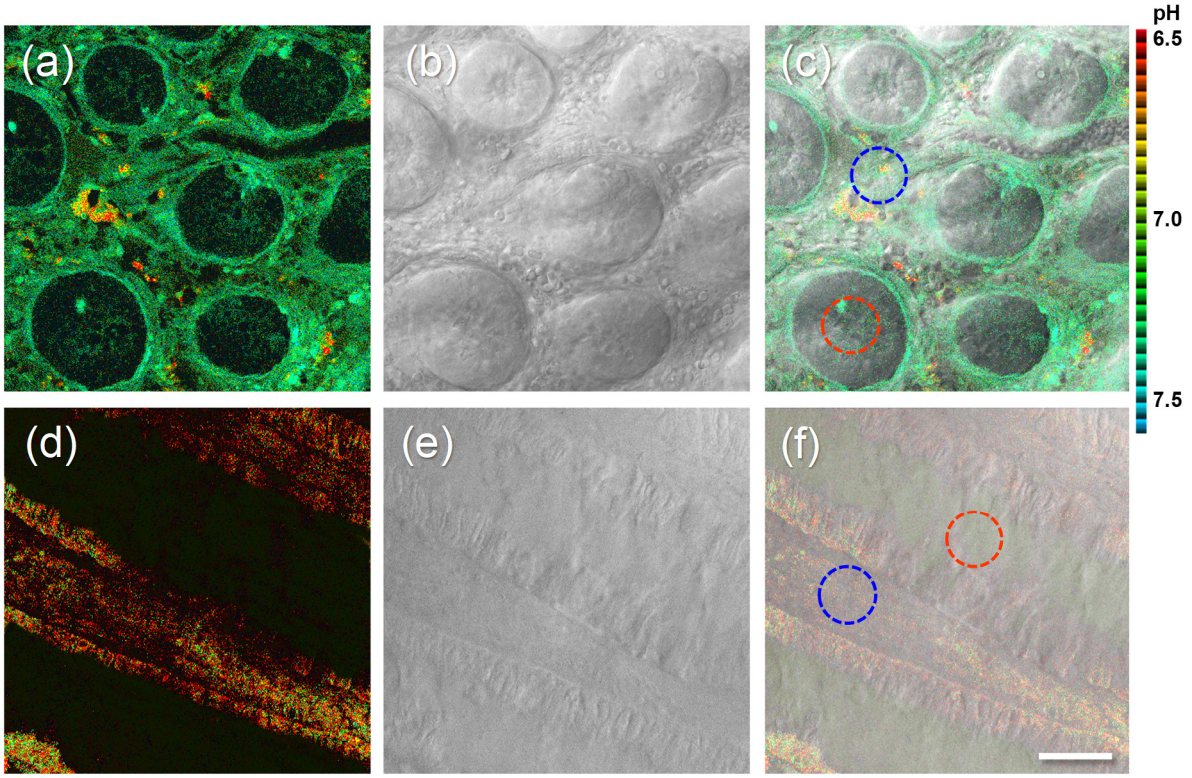

Figure S9. TPM images of human colon (a-c) normal and (d-f) cancer tissue. (a, d) Pseudocolored ratiometric TPM images (I_{green}/ I_{blue}) of colon (a) normal and (d) cancer tissue labeled with 10 μM **XBH2** for 30 min. (b, e) Bright field images. (c, f) Merged image of bright field image and ratiometric TPM image. Red circle in c and f shows the intestinal gland (intracellular region) where the cells are densely packed and blue circle indicates the surrounding media (extracellular region). Excitation wavelength is 750 nm. Scale bar: 60 μm.

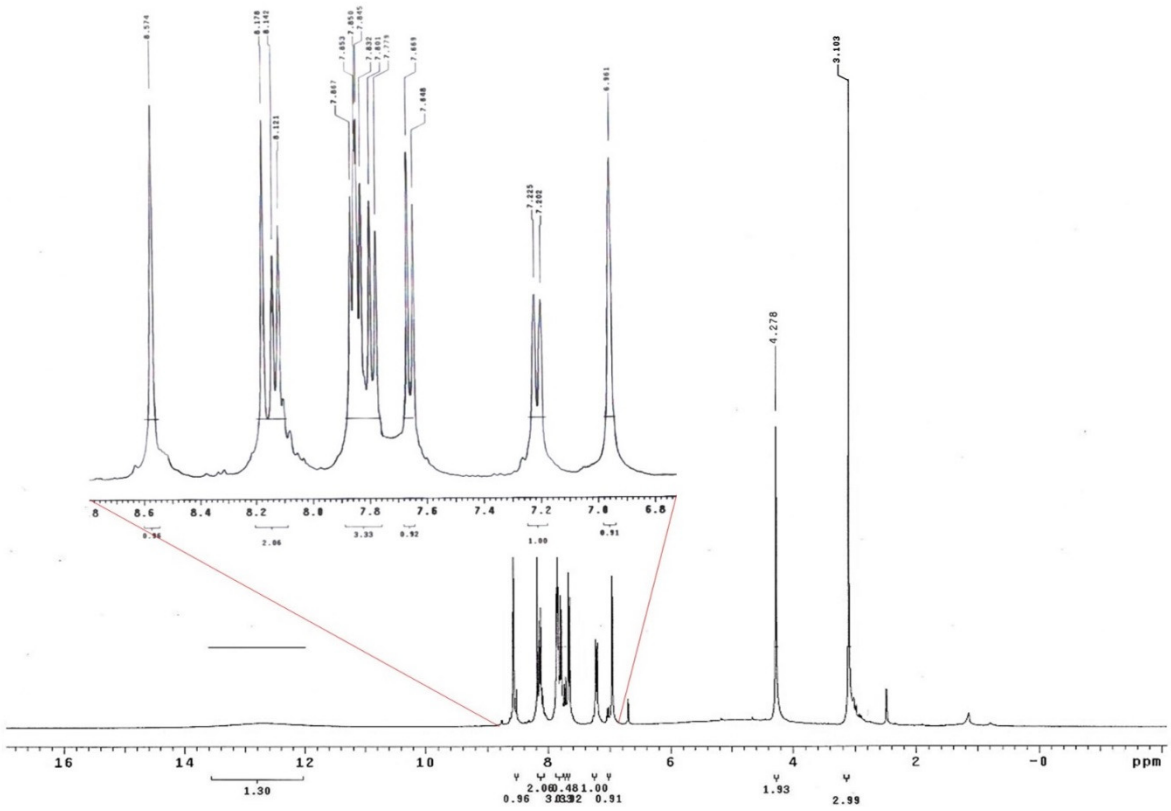

Figure S10. NMR spectrum (400 MHz) of **XBH1** in DMSO-d_6.

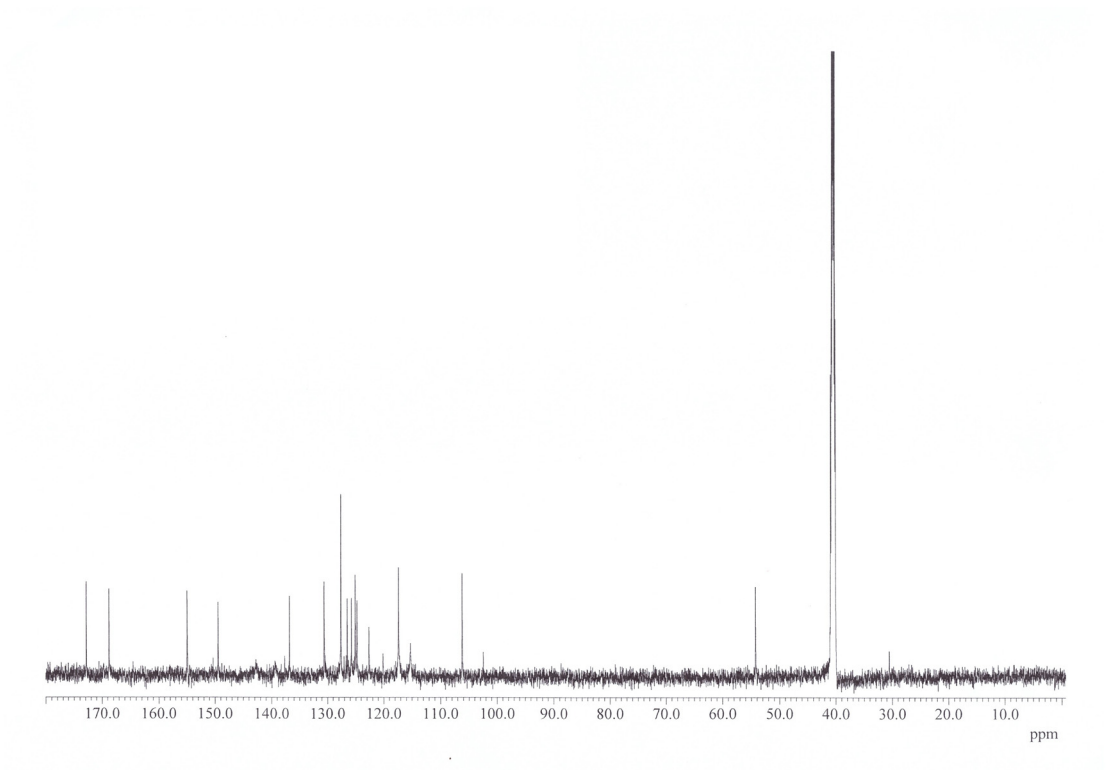

Figure S11.C-13 NMR spectrum (150 MHz) of **XBH1** in DMSO-d_6.

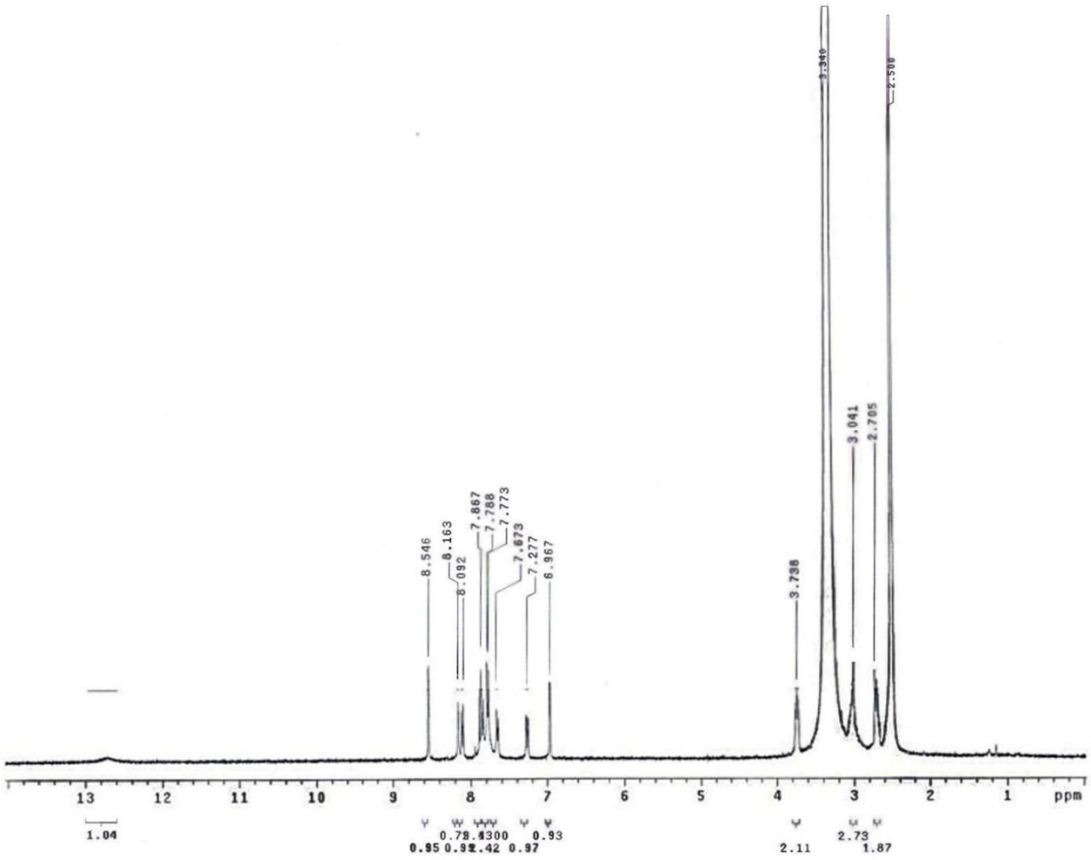

Figure S12. NMR spectrum (400 MHz) of **XBH2** in DMSO-d_6.

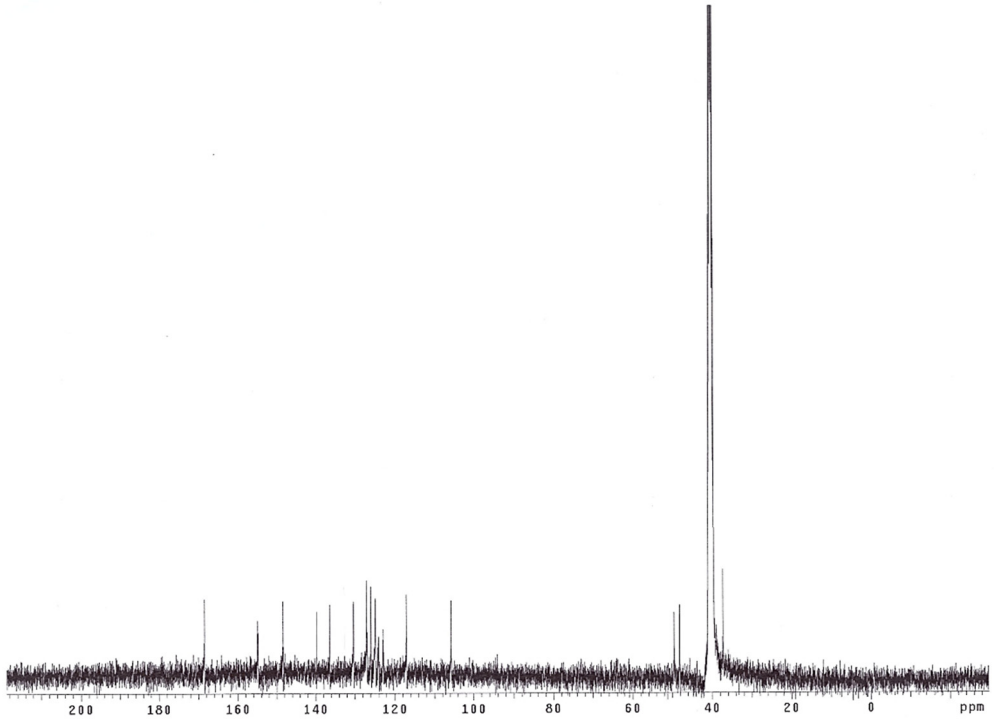

Figure S13. C-13 NMR spectrum (100 MHz) of **XBH2** in DMSO-d_6.

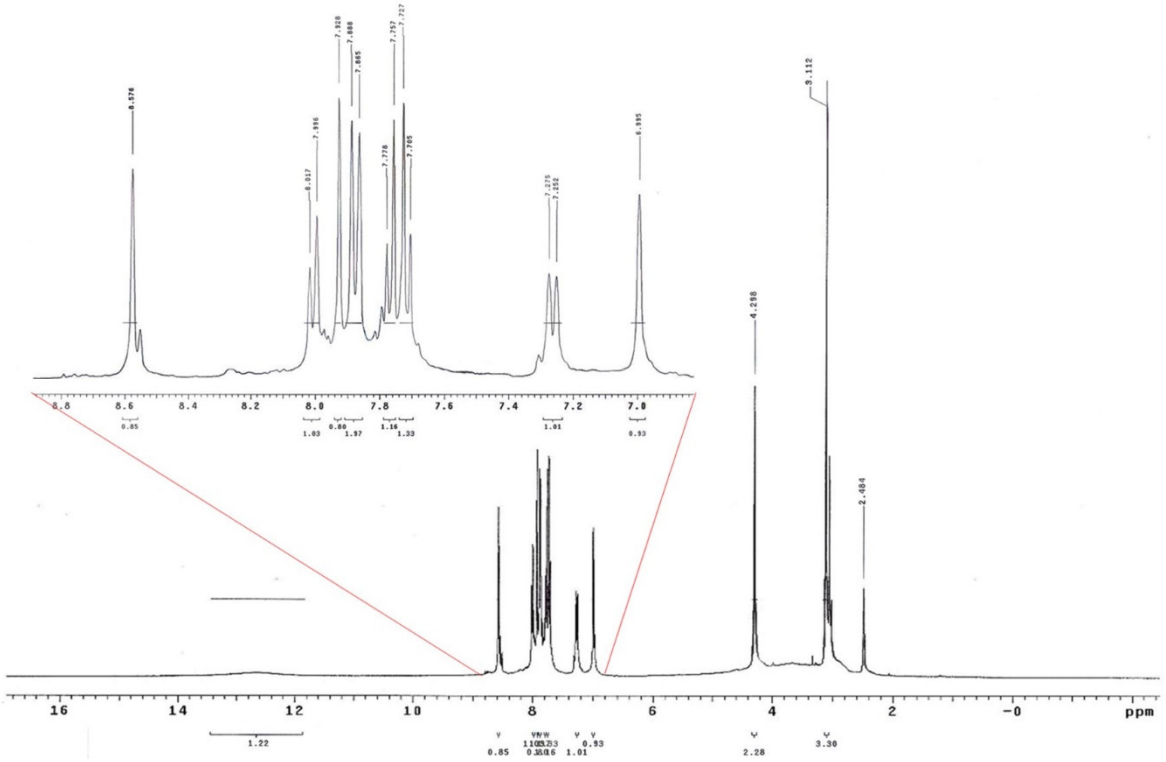

Figure S14. NMR spectrum (400 MHz) of **XBH3** in DMSO-d_6.

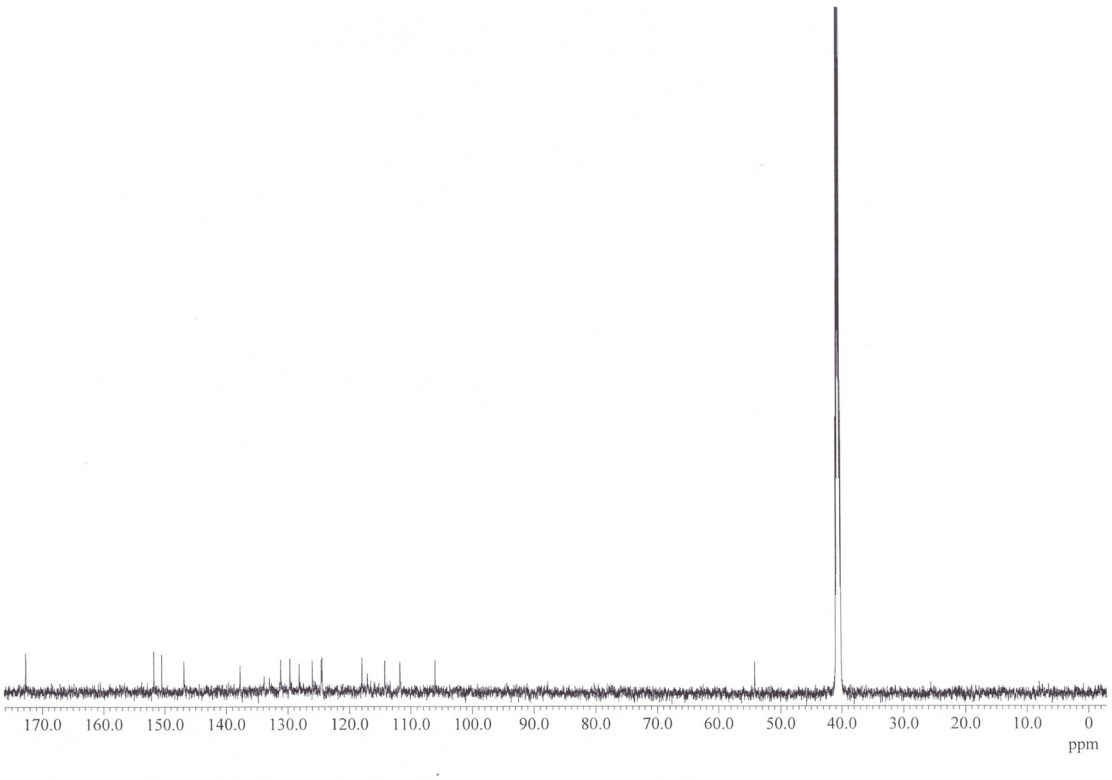

Figure S15. C-13 NMR spectrum (150 MHz) of **XBH3** in DMSO-d_6.

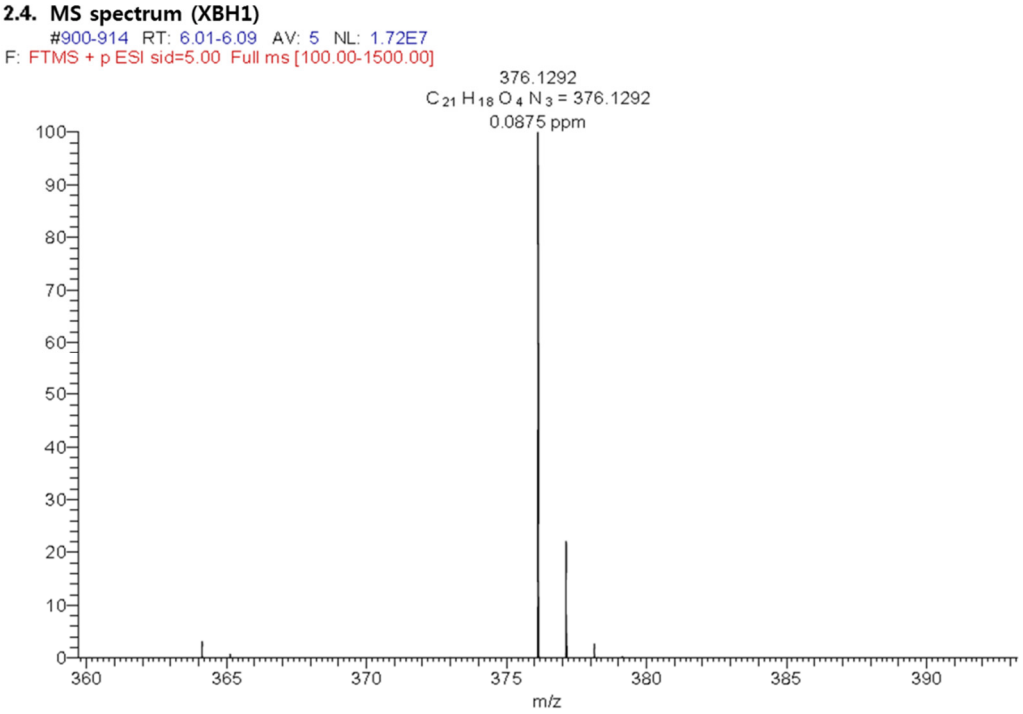

Figure S16. HRMS spectrum of **XBH1**.

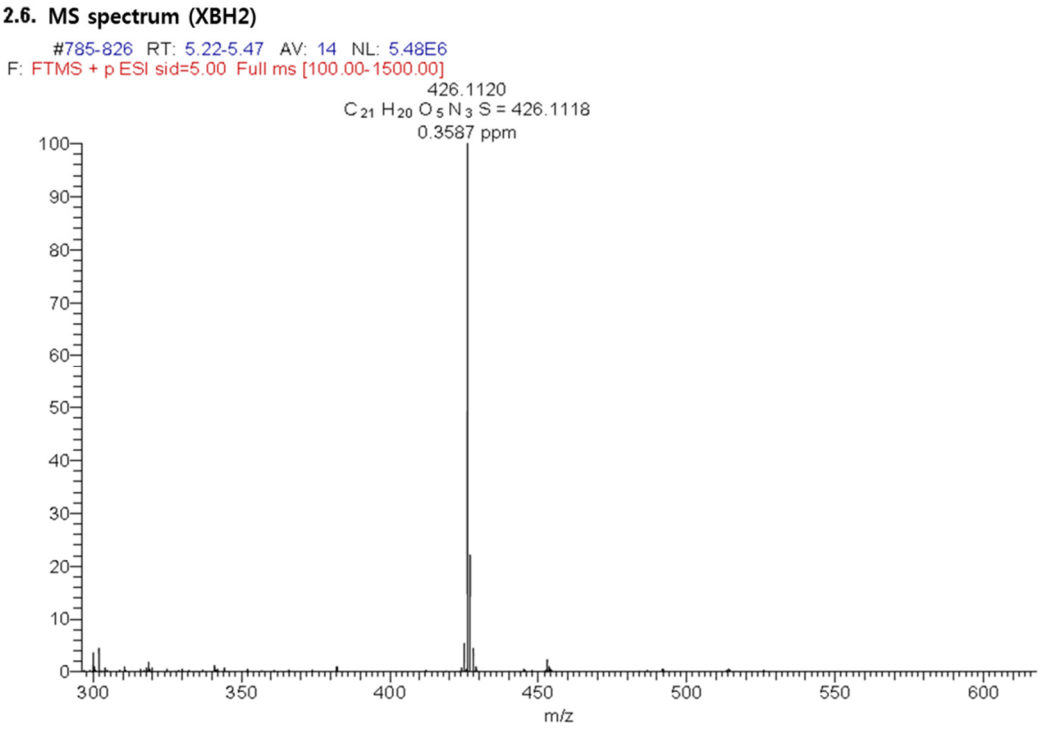

Figure S17. HRMS spectrum of **XBH2**.

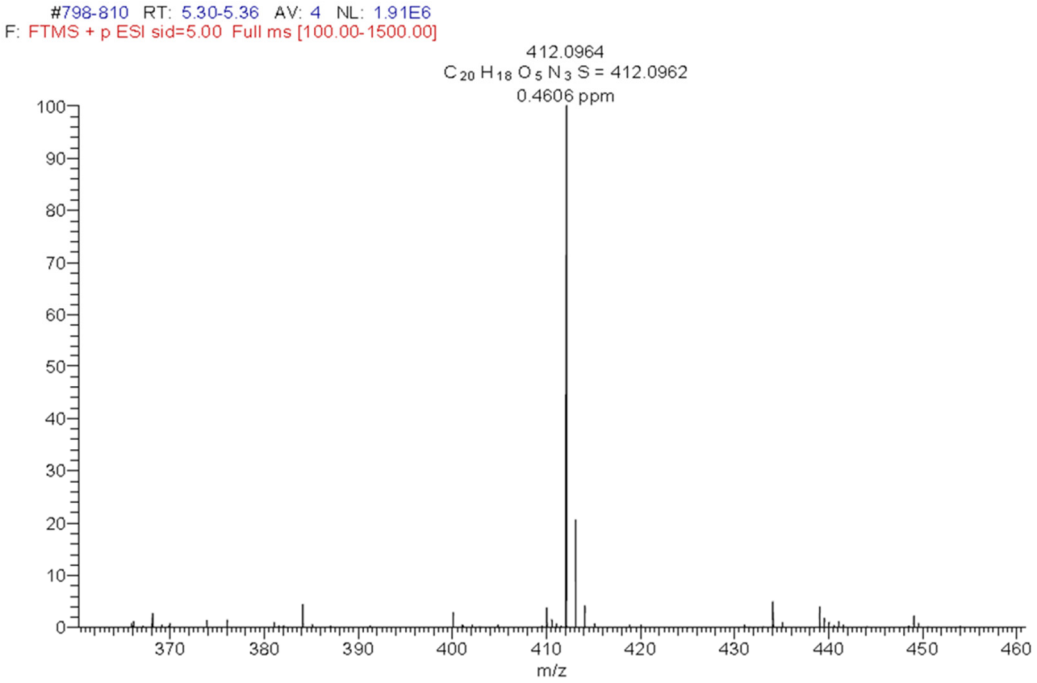

Figure S18. HRMS spectrum of **XBH3**.

References

(1) Masanta, G.; Lim, C. S.; Kim, H. J.; Han, J. H.; Kim, H. M.; Cho, B. R., *J. Am. Chem. Soc.* **2011,** *133* (15), 5698–5700.

(2) Masuya, A.; Iki, N.; Kabuto, C.; Ohba, Y.; Yamauchi, S.; Hoshino, H., *Eur. J. Inorg. Chem.* **2010,** *2010* (22), 3458–3465.