† Electronic Supplementary Information (ESI)

Long-term single cell bioluminescence imaging with C-3 position protected coelenterazine analogues

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Supplemental figures

Chemical stability of ethanolic FMZ analogues solutions

Solutions of FMZ and its analogues (50 μM) were prepared in ethanol and stored for various periods of time (0-5 days) at 25 °C in the dark. They were then subjected to HPLC equipped with a COSMOSIL $5C_{18}$-AR-II Packed Column (ID = 2.0 mm, column length = 100 mm) (Nacalai Tesque, INC., Kyoto, Japan) and the stability was evaluated by the percentage of the peak height. Eluent: (A) 0.1% aqueous trifluoroacetic acid (TFA) solution, (B) acetonitrile. The initial percentage of B was 35%. The percentage of B was linearly increased from 35% at 2 min to 65% at 6 min, followed by a decrease from 65% at 10 min to 35% at 16 min. The total measurement time for a sample was 20 min. The flow rate was set to 0.2 mL/min and chromatograms were recorded at 263 nm absorbance.

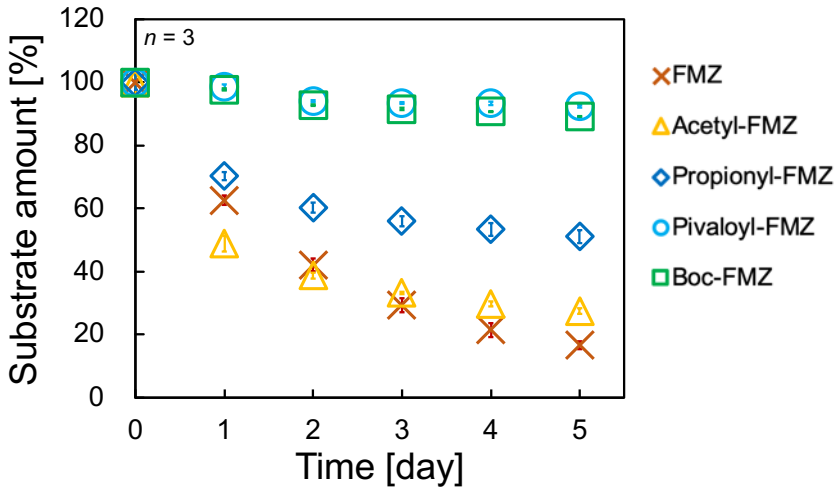


Fig. S1 Chemical stability of FMZ and its analogues in ethanol (50 μM) at 25 °C for various storage times (0-5 days). Error bars represent mean values ±1σ (n = 3).

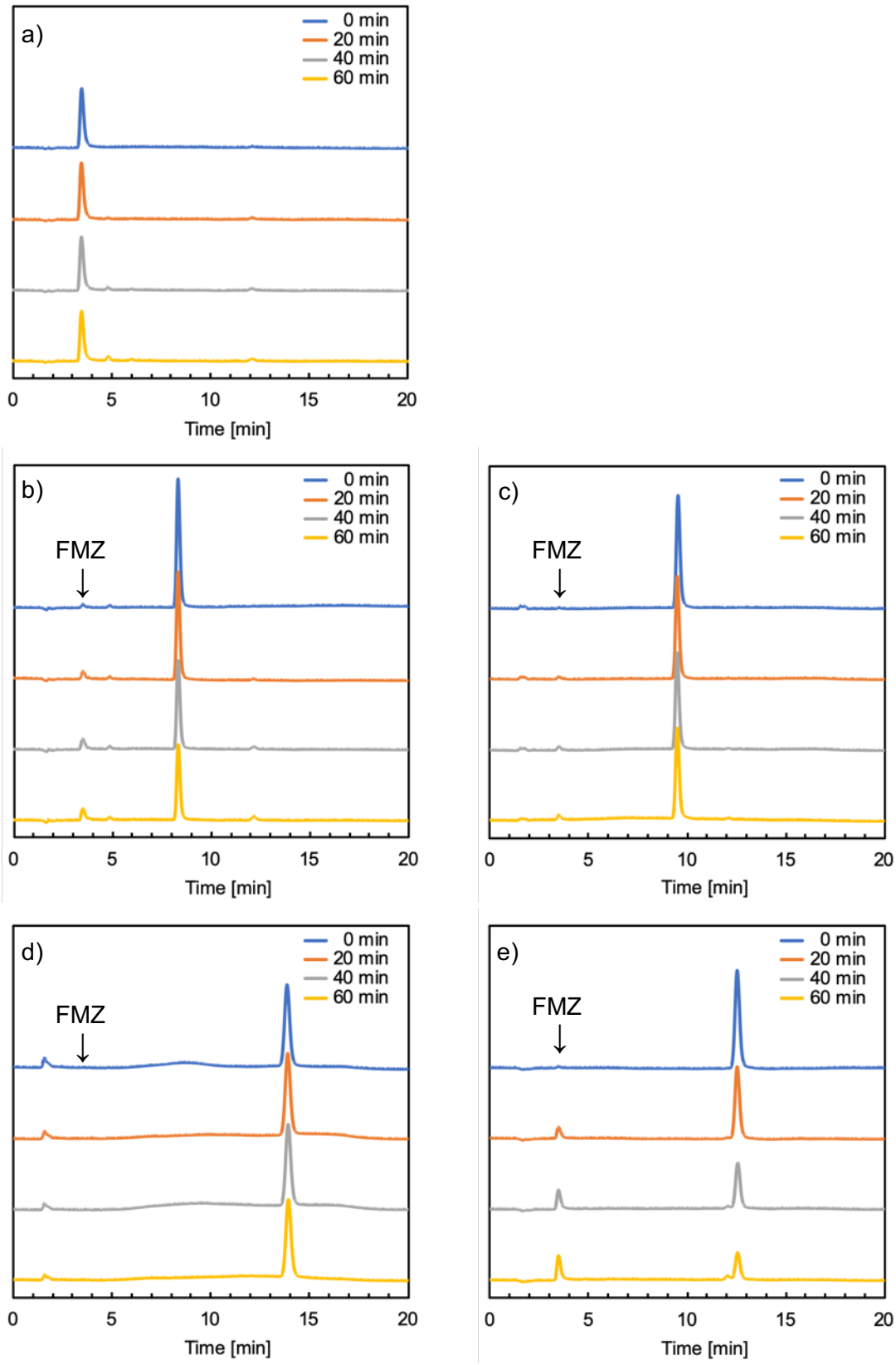


Fig. S2 Chromatograms of FMZ and its analogues in buffer solution (1 μM) kept at 25 °C for various times (0-60 min): (a) FMZ, (b) **Acetyl-FMZ,** (c) **Propionyl-FMZ,** (d) **Pivaloyl-FMZ**, (e) **Boc-FMZ**.

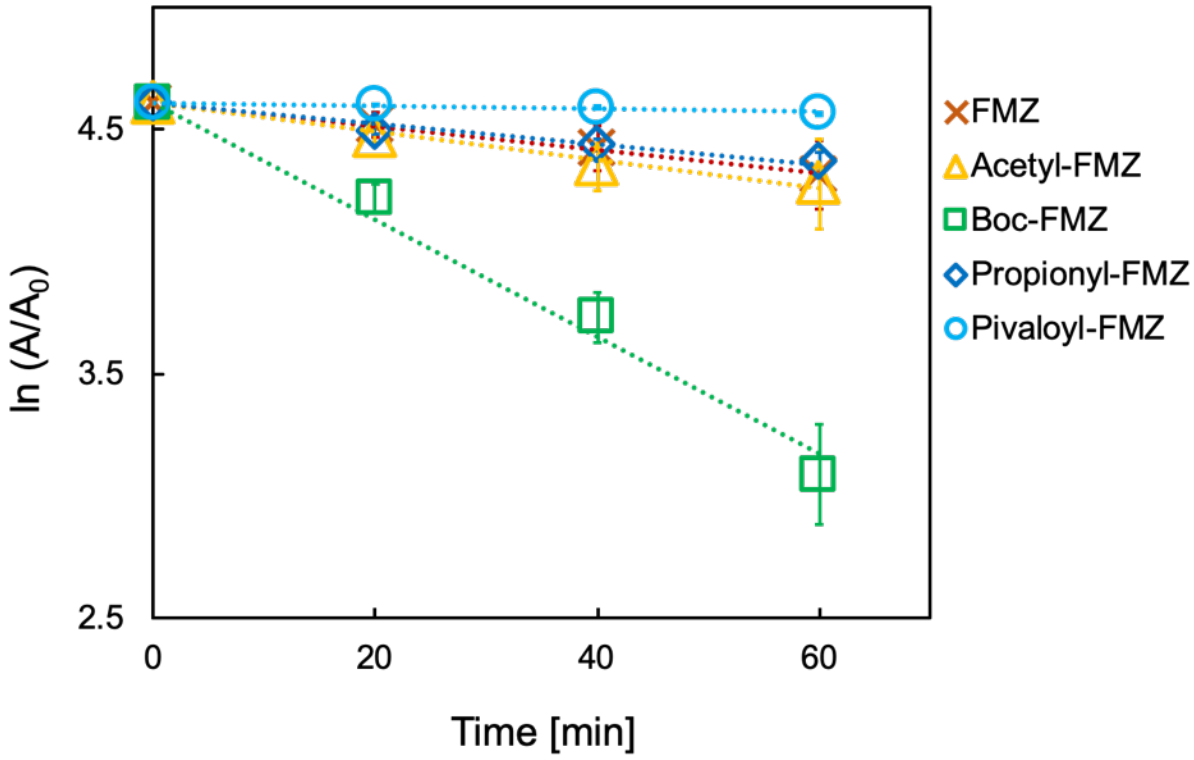


Fig. S3 Pseudo-first order kinetic plots of FMZ and its analogues calculated from the peak areas shown in Fig. S2. Error bars represent mean values ±1σ (n = 3). Chemical stability of FMZ, and cleavage rates of its analogues in HBSS buffer solution were evaluated with the apparent reaction rate constants (slope of linear regressions), summarized in Table 1 in the main text.

Deprotection assay by esterase

Solutions of FMZ and its analogues (25 µL, 4 µM) were prepared in Hanks' Balanced Salt solution (HBSS) - EtOH (96 / 4, v/v) and mixed with porcine liver esterase (25 µL, 20 nM, Sigma-Aldrich, USA) for 20 min at 25 °C in the dark on 96-well black well plates (3915, Corning Inc., New York, USA). Then, Nanoluc (Nluc) (50 µL, 5 nM) was added to the well plates and the BL intensity evaluated by the Tristar^2 Multimode Reader LB942 (Berthold Technologies, Bad Wildbad, Germany). The following conditions were used for luminescence acquisition: exposure time was 1 s, total measurement time was 10 min.

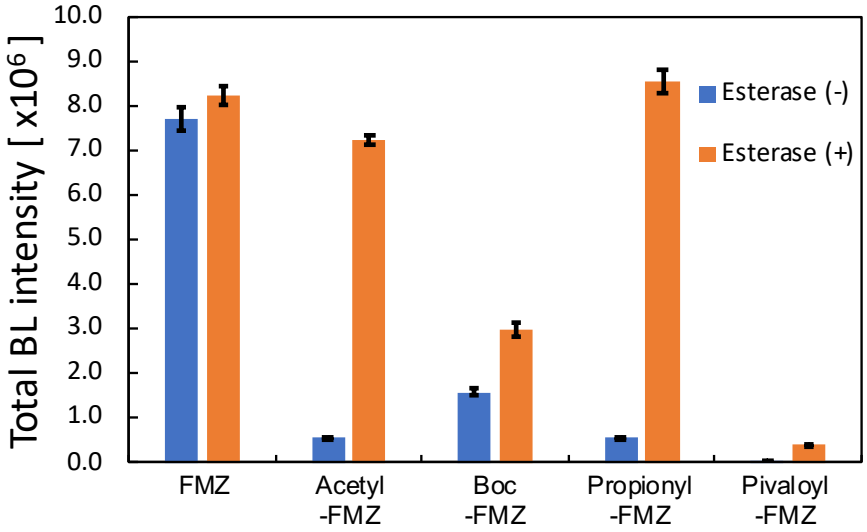


Fig. S4 Bioluminescence intensities of FMZ and protected FMZs with purified Nluc after 20 min incubation with or without esterase. Error bars represent mean values ±1σ (n = 3).

Determination of partition coefficients (Log P_{ow})

Log P_{ow} values (octanol-water partition coefficient) of all analogues were evaluated by HPLC. All samples were dissolved in ethanol and filtered through a 0.22 µm membrane filter. All samples were eluted to a COSMOSIL 5C_{18}-AR-II Packed Column (ID = 2.0 mm, column length = 100 mm) (Nacalai Tesque, INC.) with 55% (v/v) acetonitrile in 0.1% TFA aqueous solution at a flow of 0.2 mL/min and were detected at 263 nm absorbance. Thiourea was used as an unretained compound to determine the dead time (t_0). A series of standard compounds including acetanilide (log P_{ow} = 1.0), acetophenone (log P_{ow} = 1.7), benzene (log P_{ow} = 2.1), bromobenzene (log P_{ow} = 3.0), naphthalene (log P_{ow} = 3.6), phenanthrene (log P_{ow} = 4.5), fluoranthen (log P_{ow} = 5.1), triphenylamine (log P_{ow} = 5.7) and 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) (log P_{ow} = 6.5) were analyzed to obtain a calibration curve. The retention factor (k) is given by the following equation:

$$k = (t_R - t_0)/ t_0 \qquad (1)$$

where t_0 and t_R are the retention times of unretained compound (dead time) and the target analyte, respectively. The calculated k value is substituted to the Log P value with the following equation:

$$\log P_{OW} = a \log k + b \qquad (2)$$

where a and b are the linear regression coefficients from the calibration curve of log P_{OW} against log k values of reference substances.

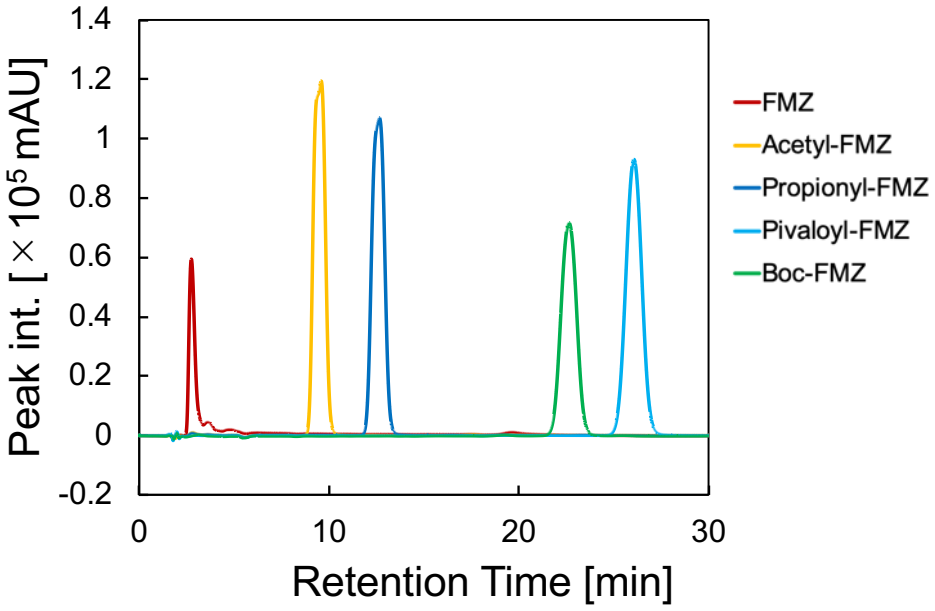


Fig. S5 Chromatograms of FMZ, **Acetyl-FMZ**, **Propionyl-FMZ**, **Pivaloyl-FMZ** and **Boc-FMZ**.

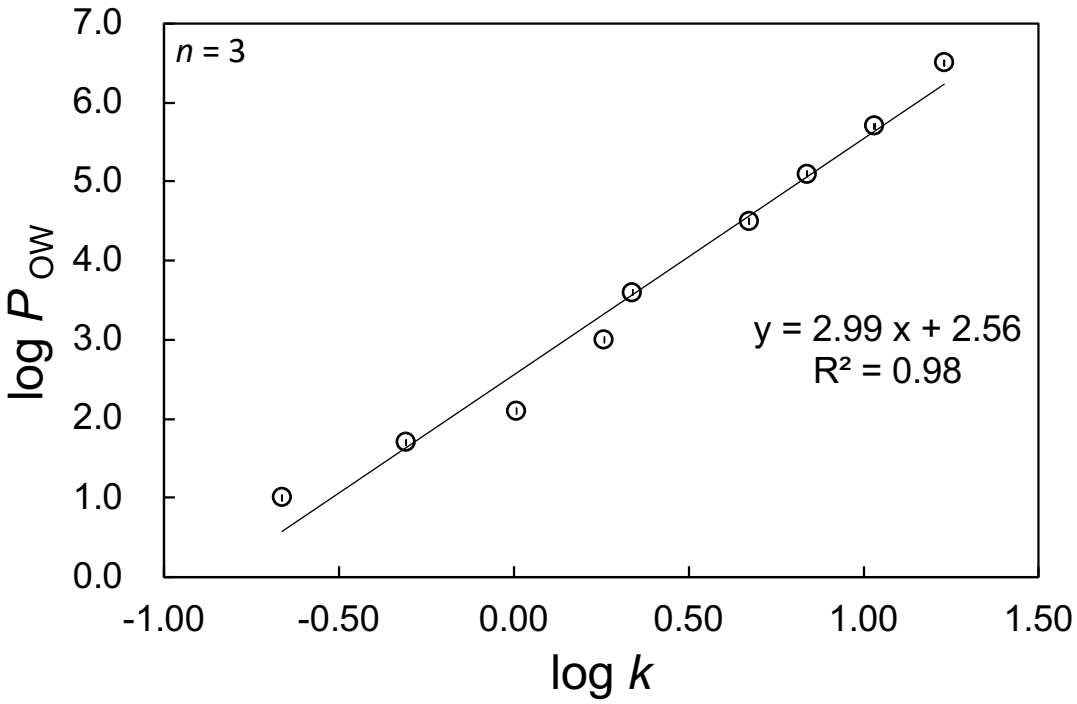


Fig. S6 Calibration curve generated by plotting log P_{OW} against log k; values obtained by HPLC of standards (acetanilide, acetophenone, benzene, bromobenzene, naphthalene, phenanthrene, fluoranthen, triphenylamine and DDT). Error bars represent mean values ±1σ (n = 3).

Cytotoxicity assay

The cytotoxicity of FMZ and its analogues on HEK293T cells was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded onto 96-well clear plates at a density of 4×10^3 cells per well and cultured for 24 h in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, USA) at 37 °C in 5% CO_2. The cultured cells were then exposed to FMZ and its analogues (1 µM) in HBSS - EtOH (99 / 1, v/v) at 37 °C for 48 h in 5% CO_2. Subsequently, MTT in PBS (-) was added to each well (finally diluted to 417 µg/mL). After an additional 4 h incubation, the medium was removed. Then, 200 µL of acidified isopropanol (0.04 mol/L HCl) was added to each well. The absorbance at 570 nm was measured with a hybrid multi-mode microplate reader (iMark Microplate Absorption Reader, BIO-RAD Laboratories Inc.). The relative percentage of cell survival was calculated based on the 100% arbitrary absorbance obtained for the blank control. Reported values represent the mean s.e.m. of 10 replicates on the same plate. Statistical analysis of cell viability was performed using Student's *t*-test.

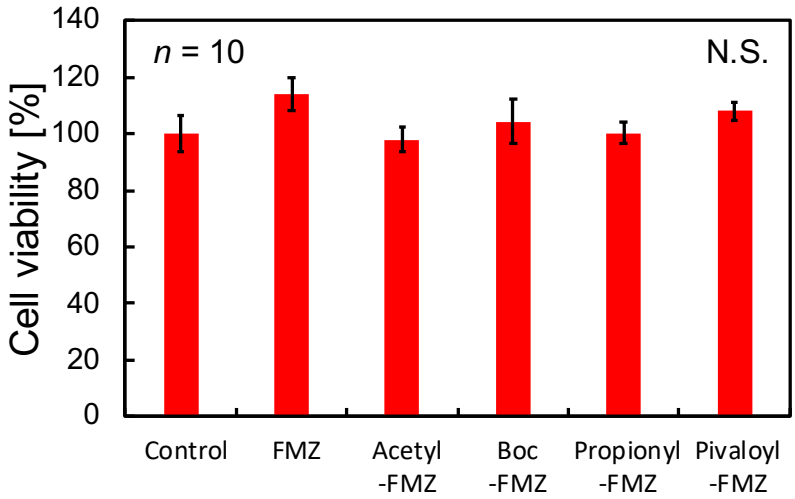


Fig. S7 Cell viability after incubation with each substrate for 48 h. Error bars represent means ± SEM (n = 10). *T*-tests were performed between control and FMZ or its analogues.

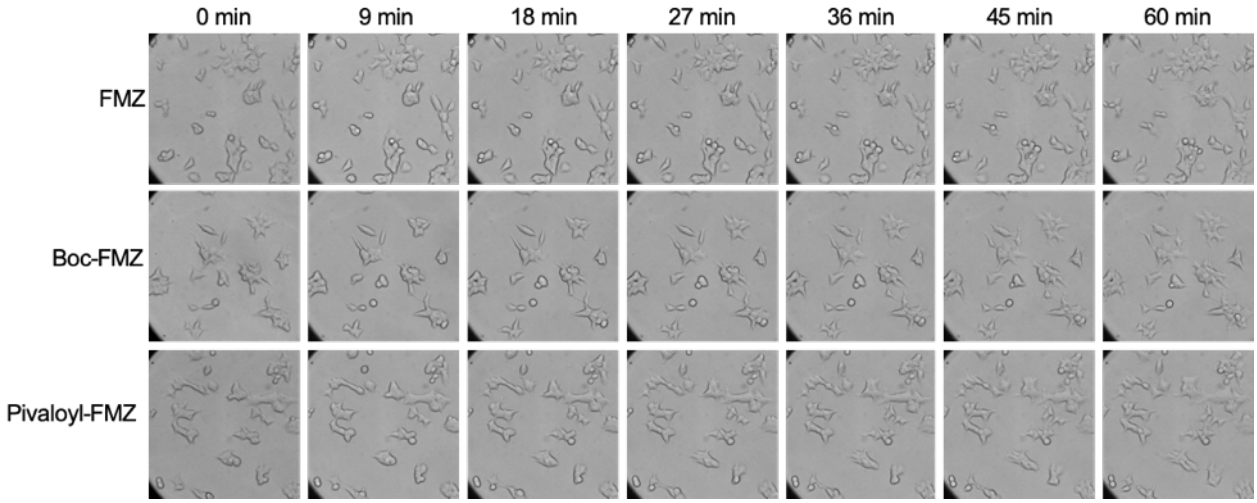


Fig. S8 Bright field image version of Fig. 5c of the main text showing HEK293T cells stably expressing Nluc during treatment with FMZ analogues.

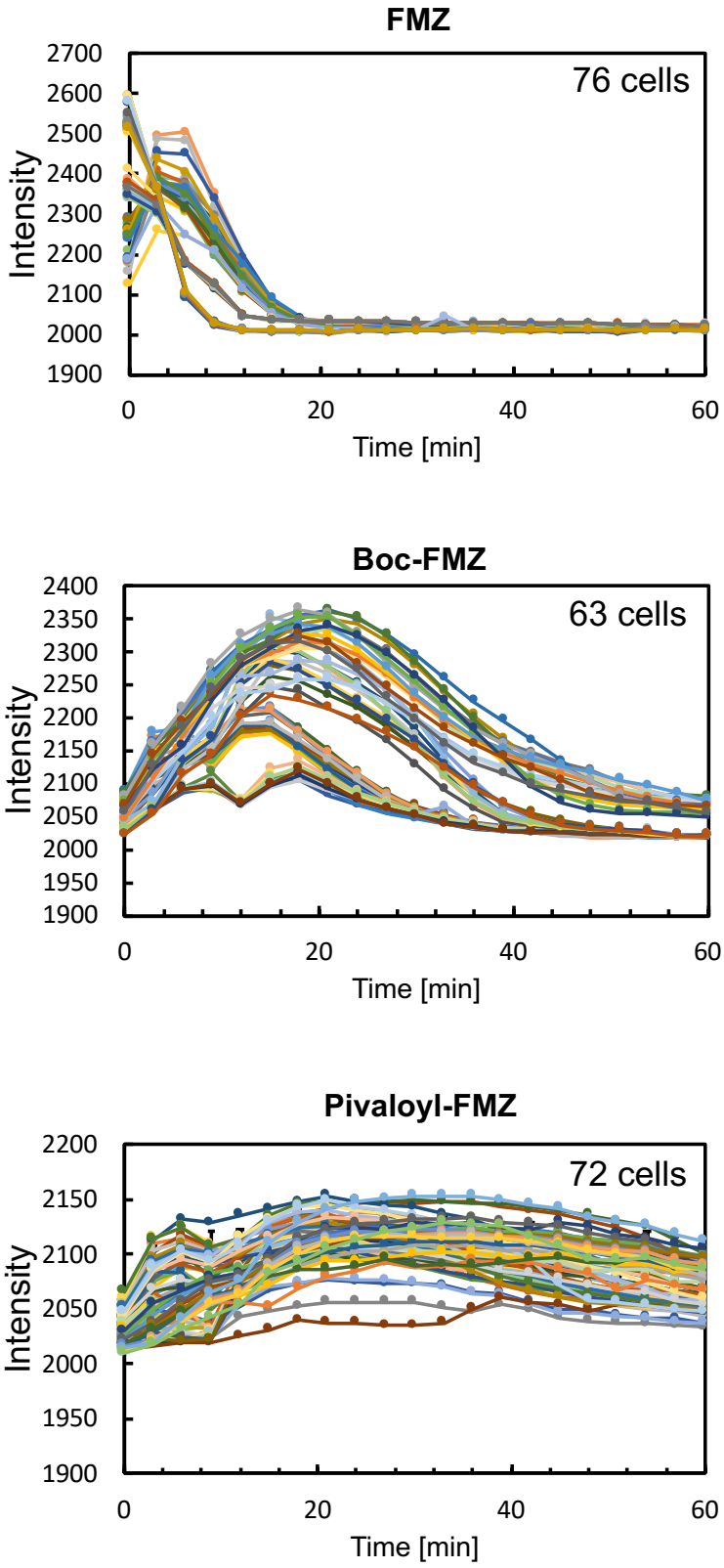


Fig. S9 Time-dependent BL intensity with FMZ and its analogues in individual HEK293T cells stably expressing Nluc extracted from Fig. 5c of the main text.

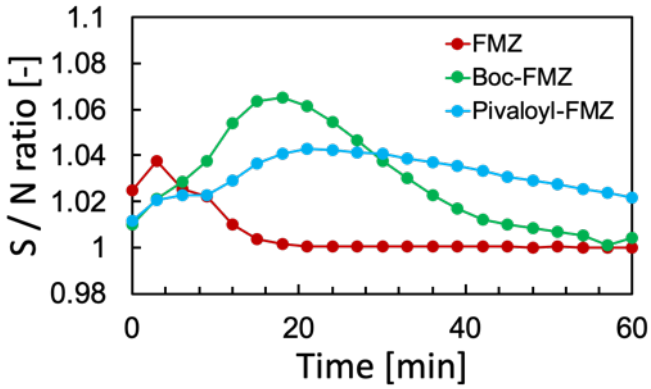
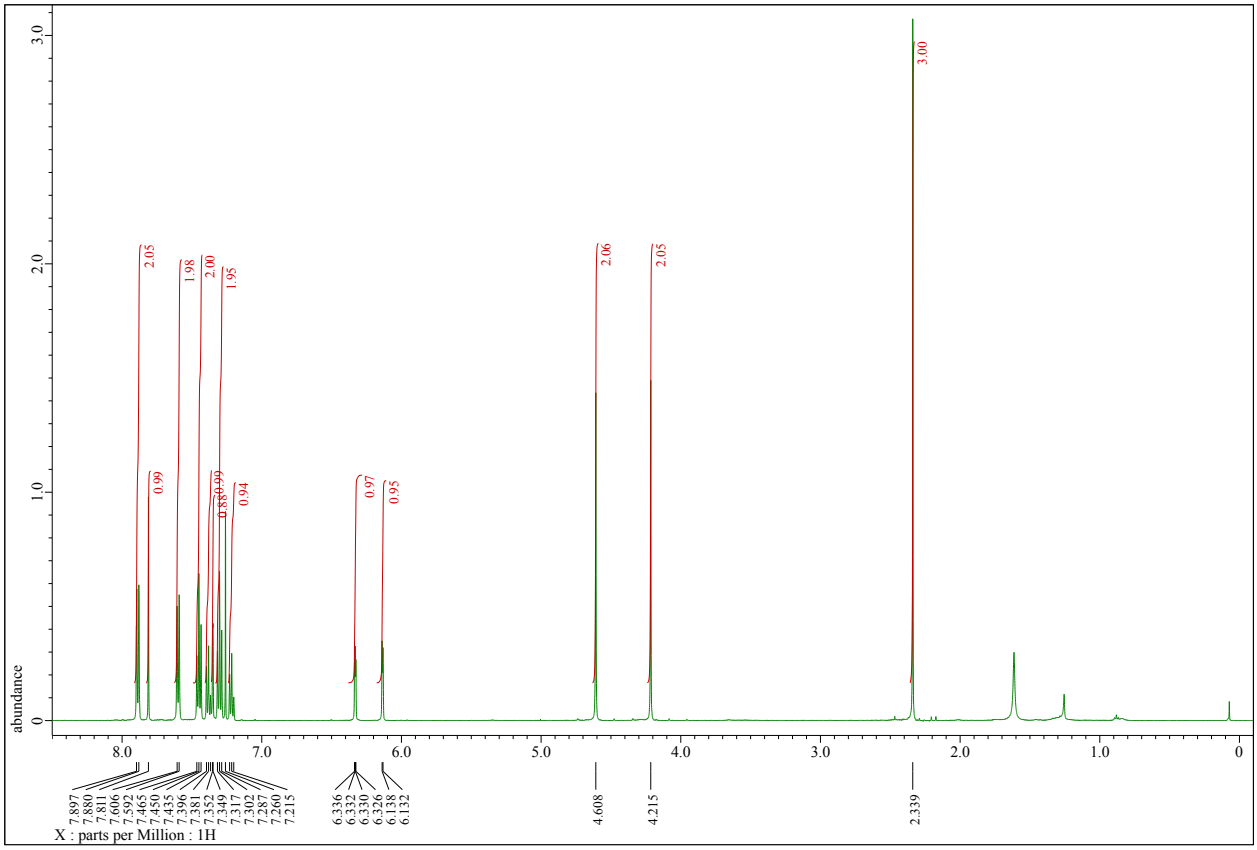
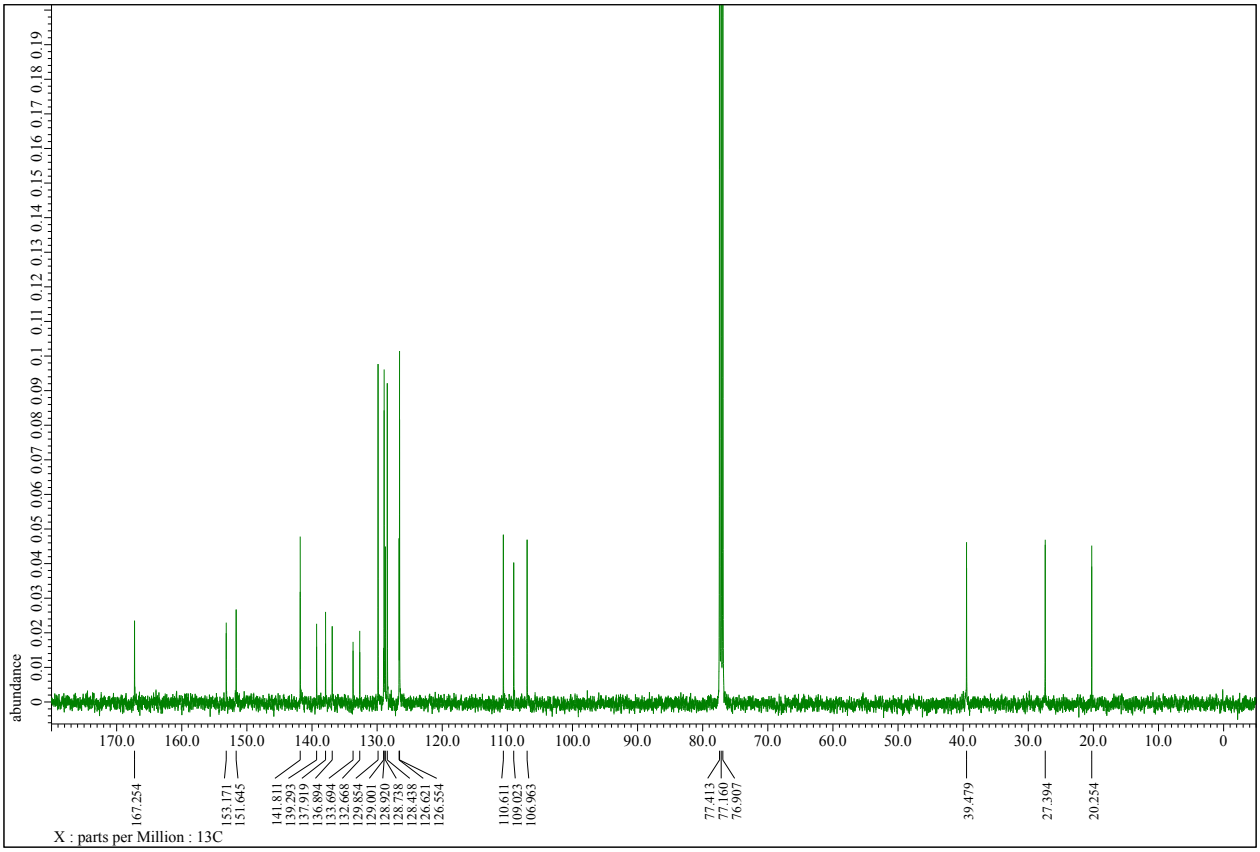


Fig. S10 Time courses of signal to noise ratio (S/N) of single cell imaging. S/N ratios were obtained from the intensities of cell BL/background from Fig. 5c of the main text.

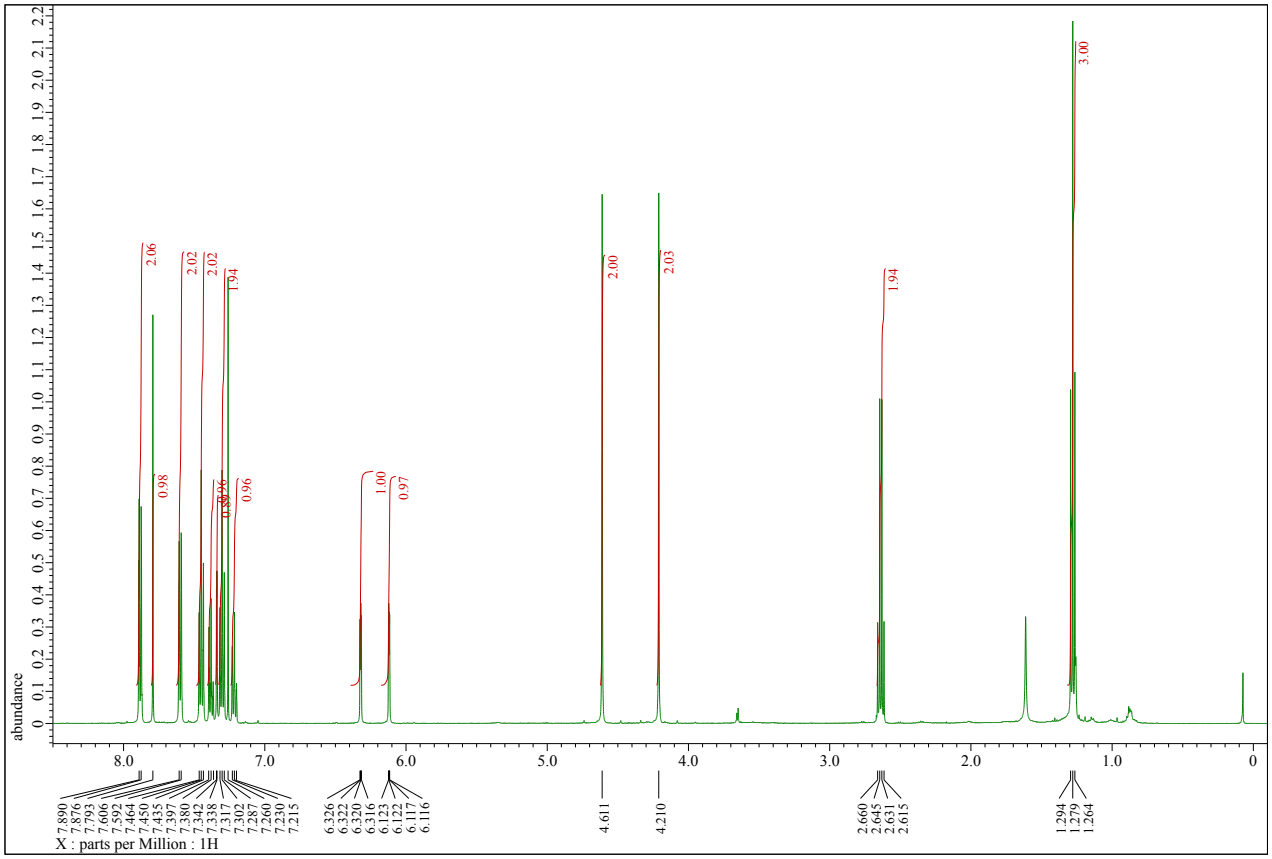
NMR spectra

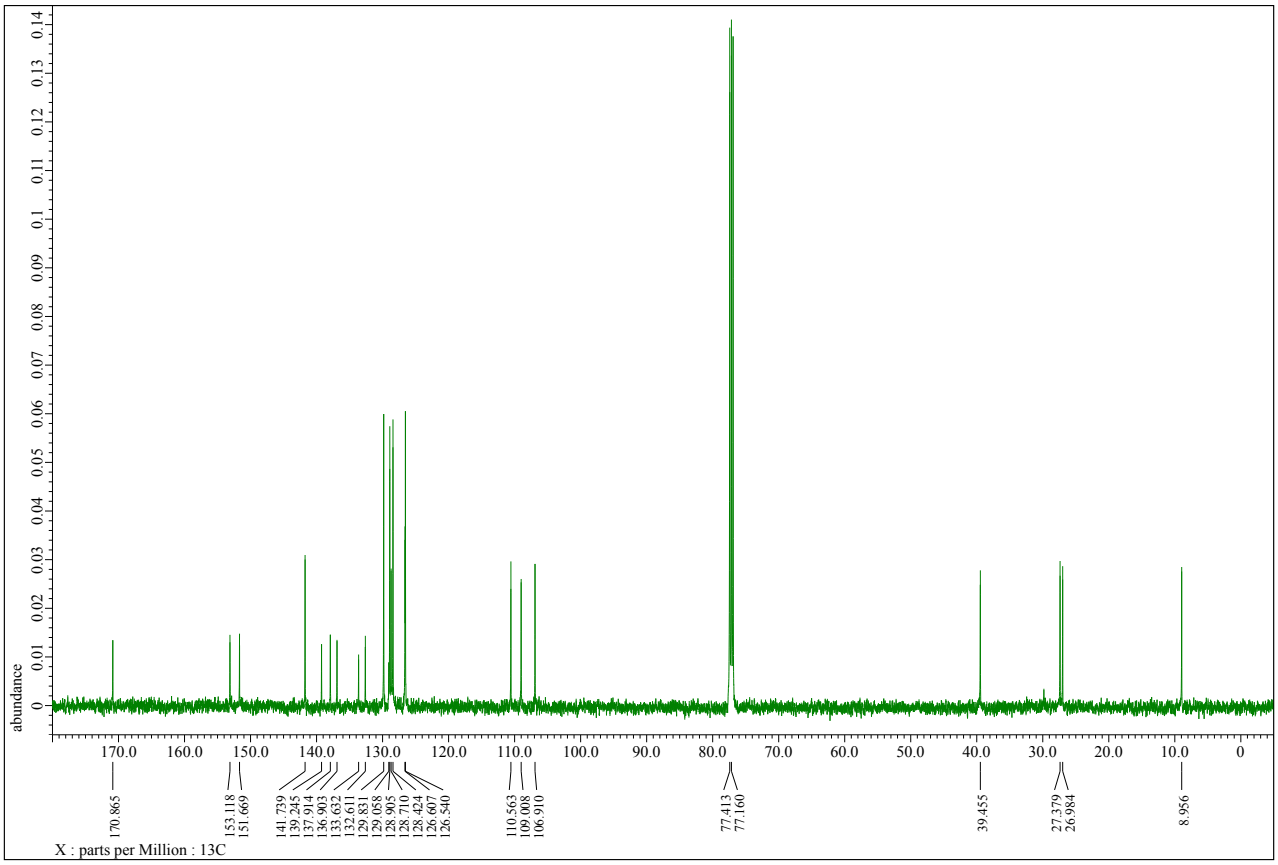
Acetyl-FMZ



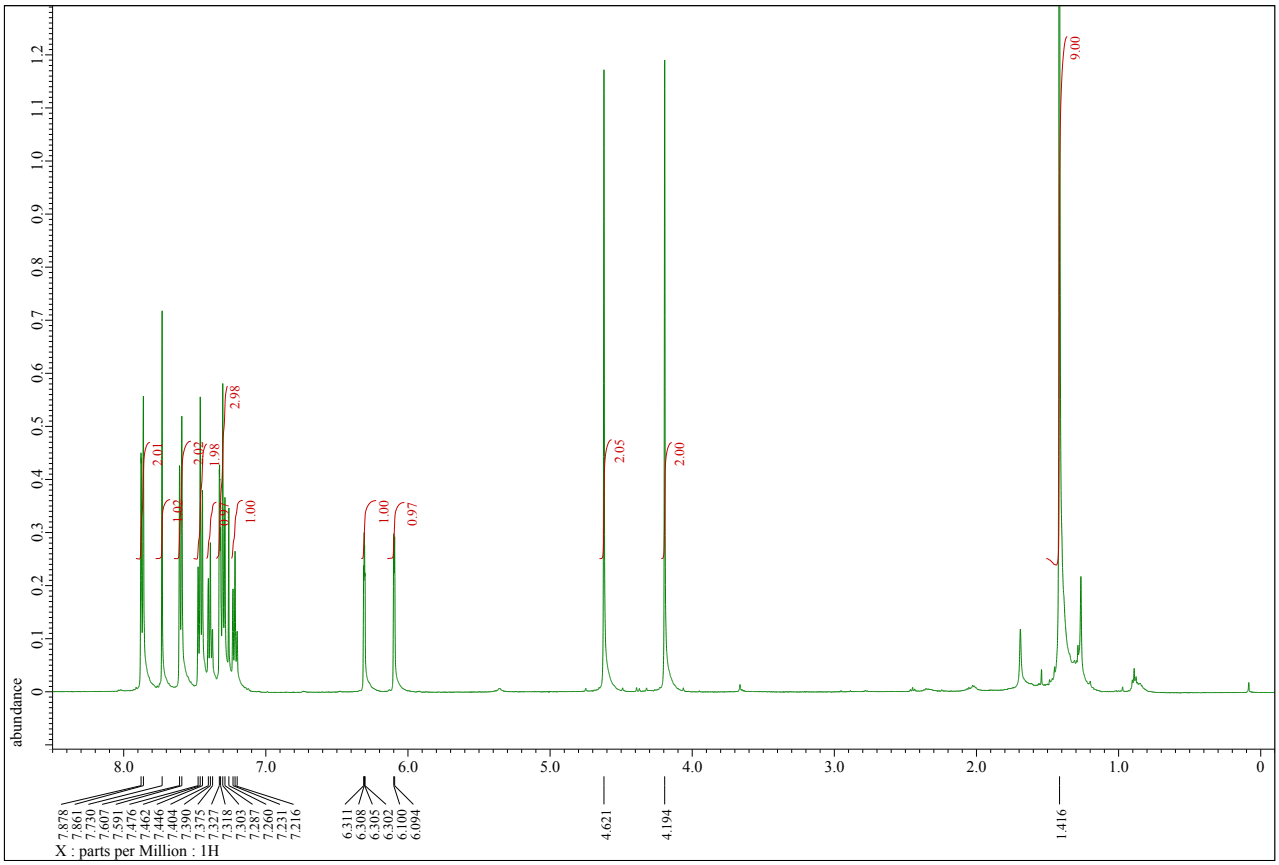


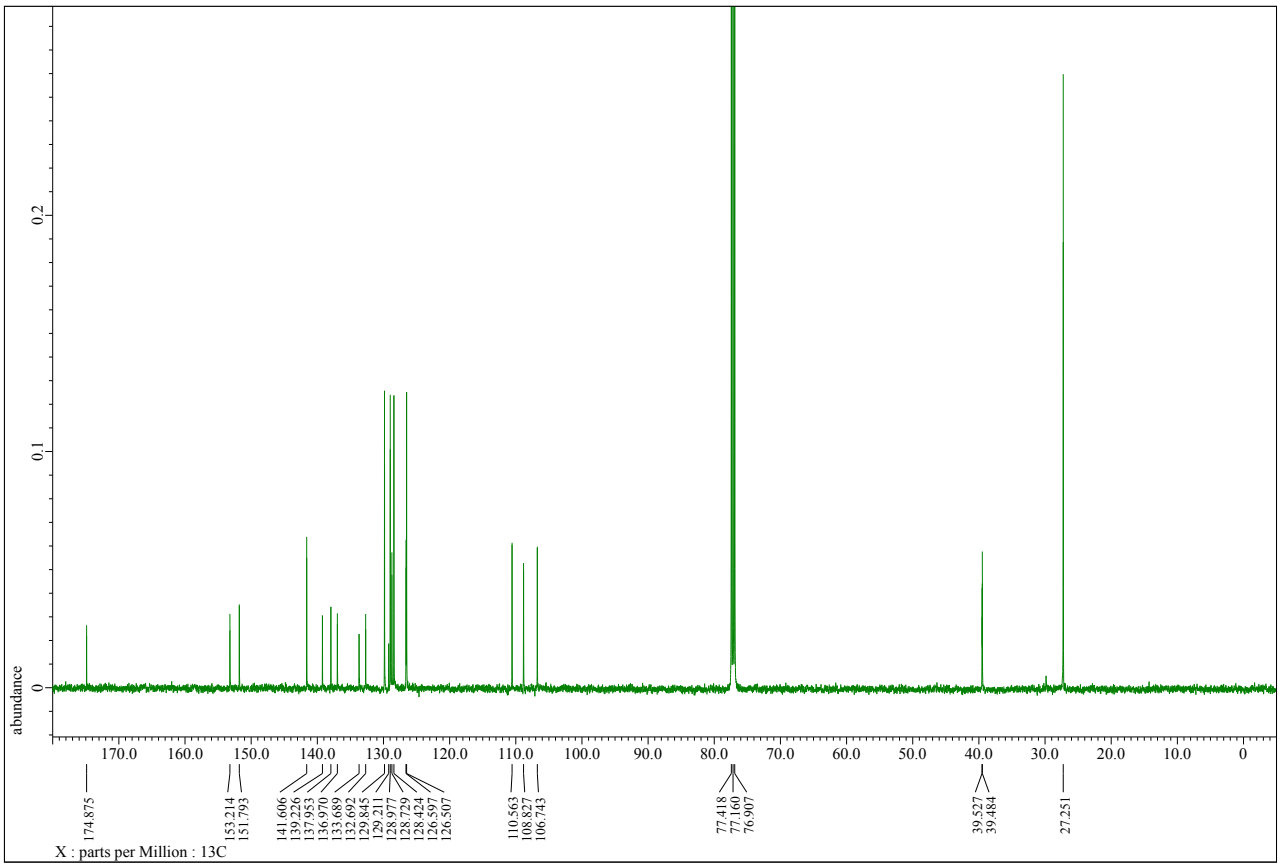
Propionyl-FMZ



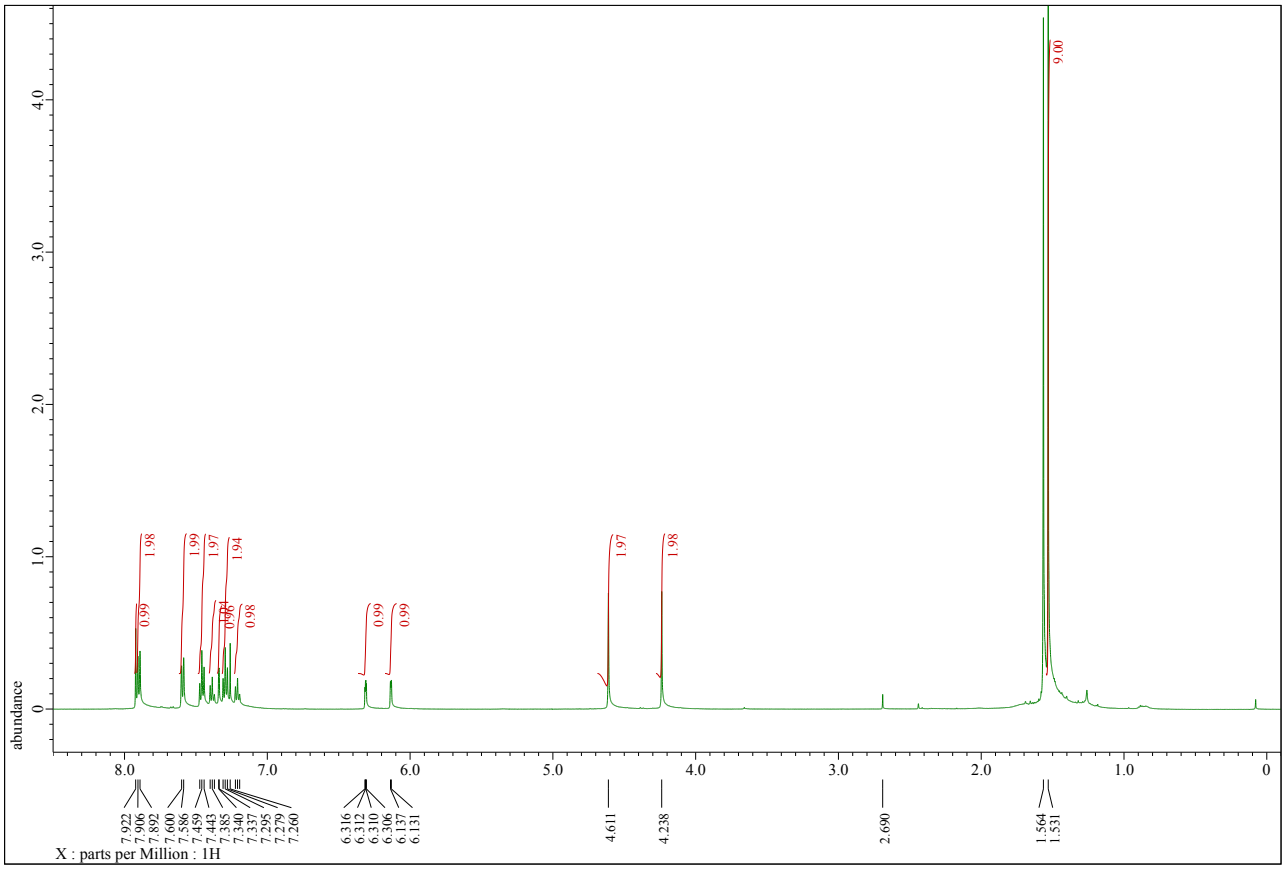


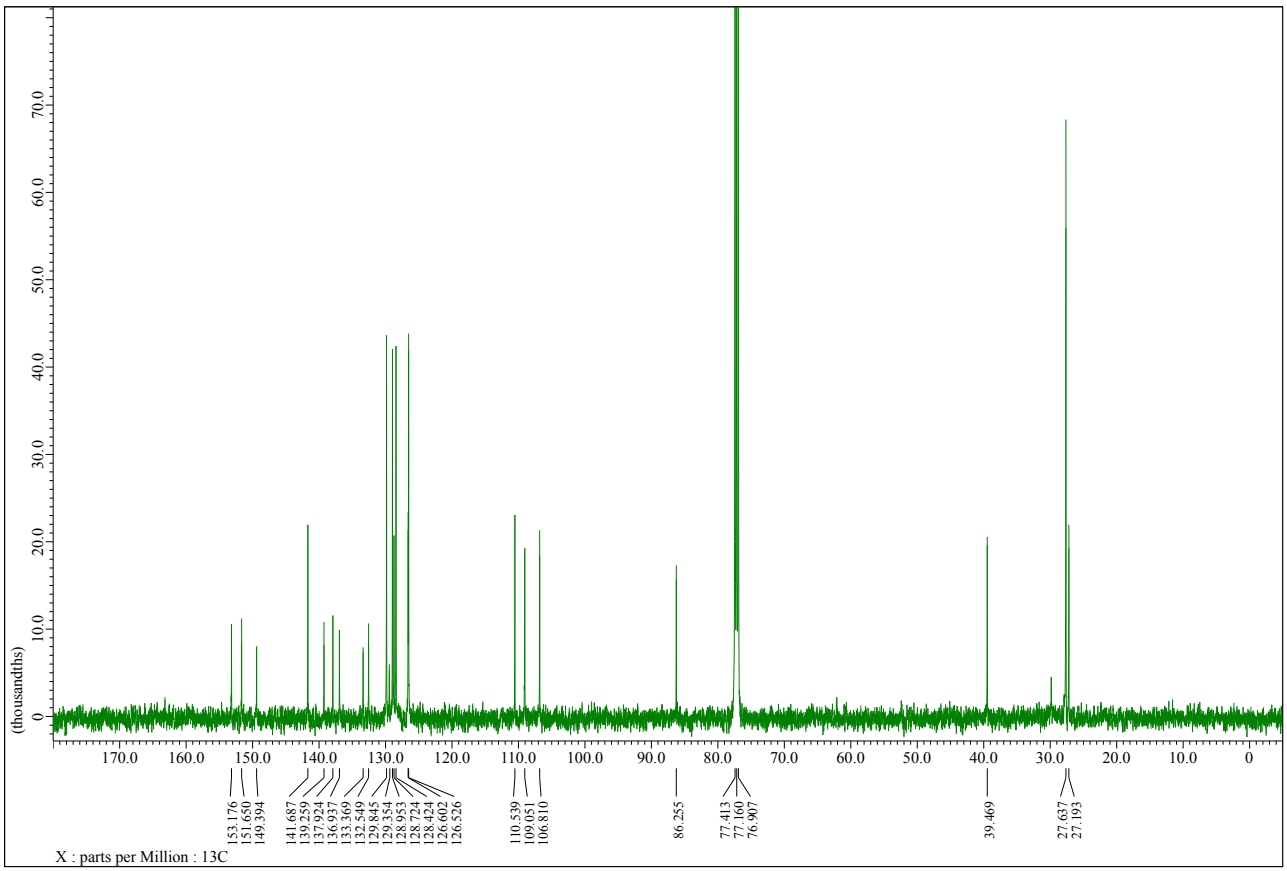
Pivaloyl-FMZ





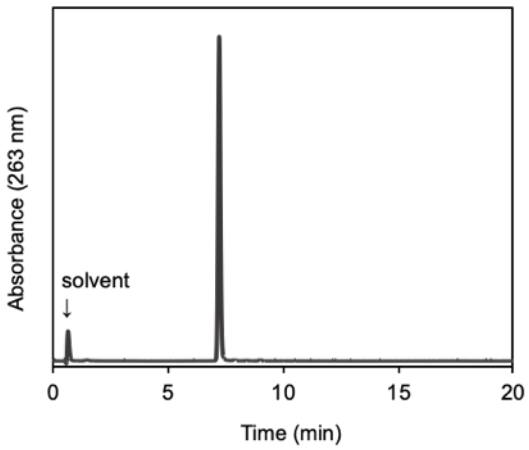
Boc-FMZ


abundance
X : parts per Million : 1H

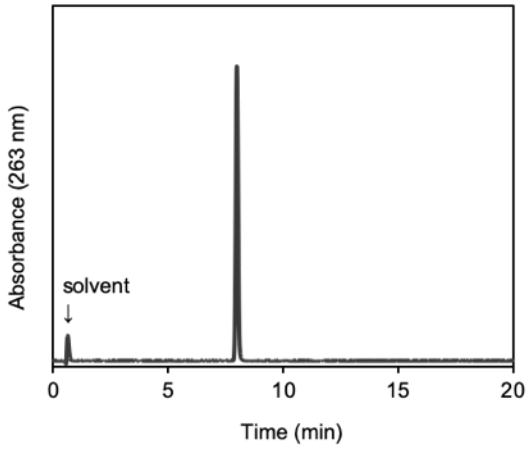

(thousandths)
X : parts per Million : 13C

Chromatograms

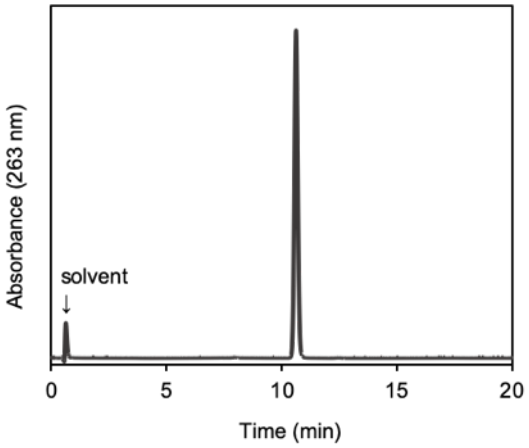
Acetyl-FMZ



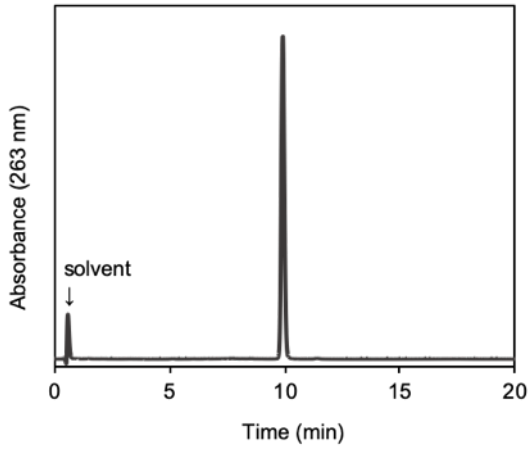
Propionyl-FMZ



Pivaloyl-FMZ

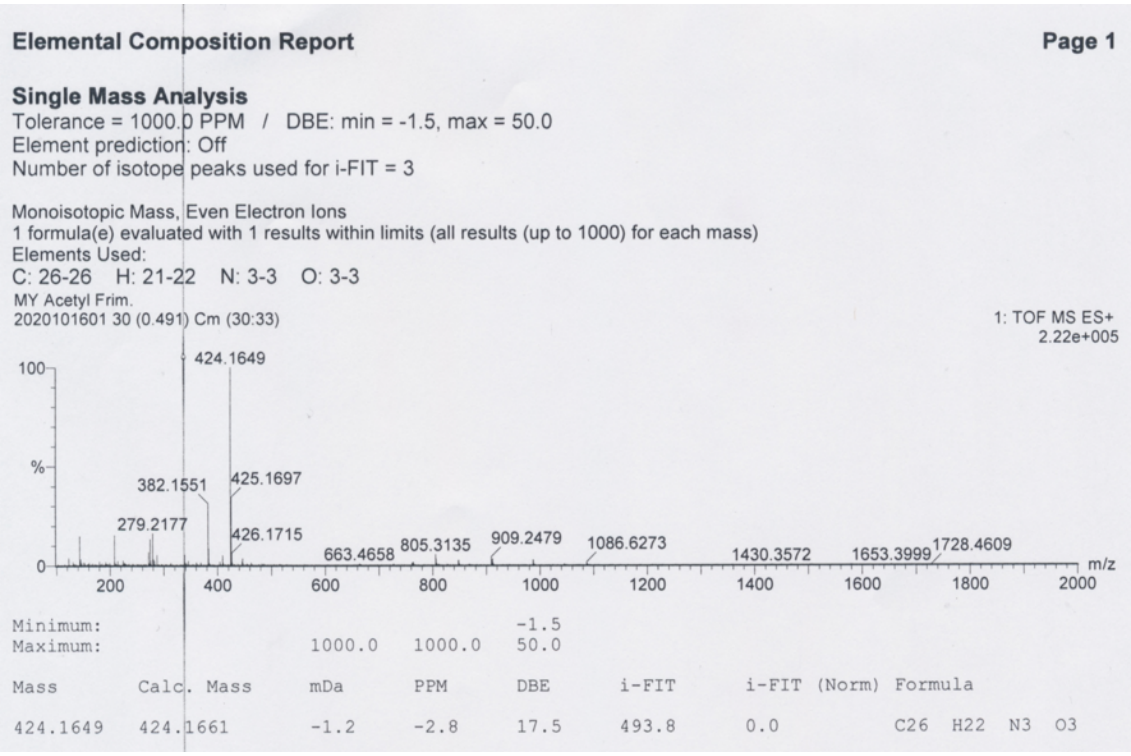


Boc-FMZ

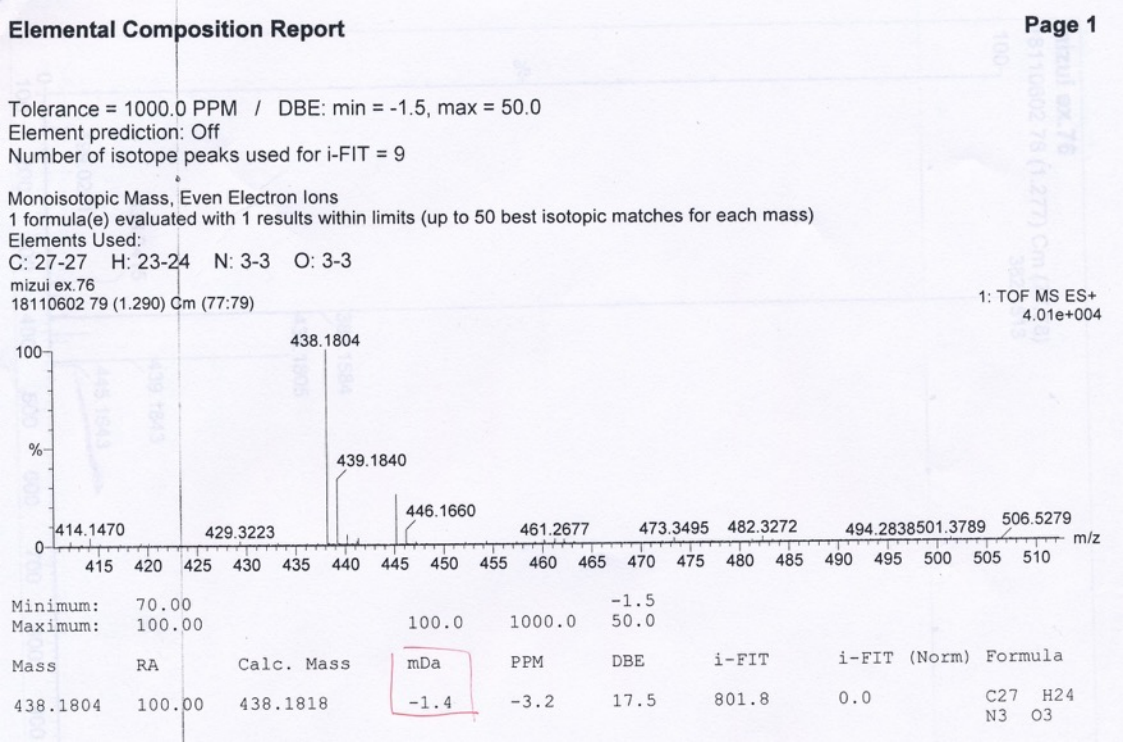


HRMS spectra

Acetyl-FMZ



Propionyl-FMZ



Pivaloyl-FMZ

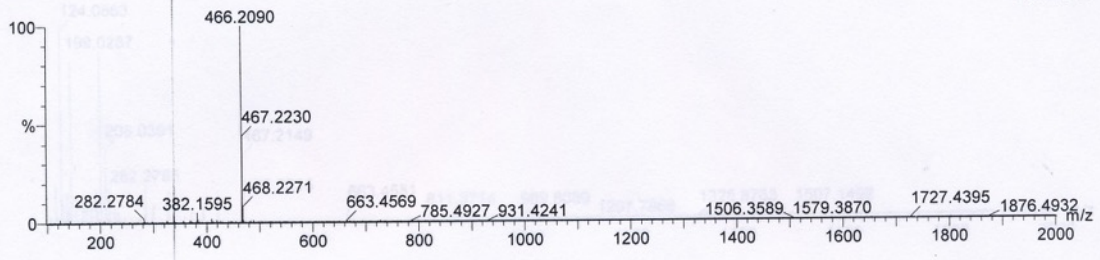
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 10000.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
1 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 29-29 H: 27-28 N: 3-3 O: 3-3

mizui Ex.77
18120701 27 (0.434) Cm (26:27)

1: TOF MS ES+
2.00e+005



Minimum:				-1.5			
Maximum:		1000.0	10000.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
466.2090	466.2131	-4.1	-8.8	17.5	349.5	0.0	C29 H28 N3 O3

Boc-FMZ

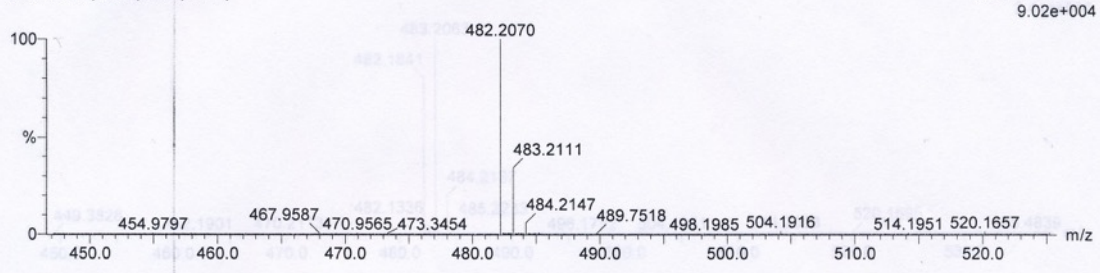
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 100.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
1 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 29-29 H: 27-28 N: 3-3 O: 4-4

0709mizui boc
180709 67 (1.093) Cm (67:72)

1: TOF MS ES+
9.02e+004



Minimum:				-1.5			
Maximum:		5000.0	100.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
482.2070	482.2080	-1.0	-2.1	17.5	388.6	0.0	C29 H28 N3 O4