Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Tetrazine-induced activation of a trimethyl lock as a click-to-release system for protected doxorubicin

Julia Friederich,[a] Chunfa Xu,[a,b] Patrick Raunft,[a] Hazel L. S. Fuchs,[a] and Mark Brönstrup[a,c,d*]

[a] Department of Chemical Biology, Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig.

[b] Institute of Pharmaceutical Science and Technology, College of Chemistry, Fuzhou University, Fuzhou 350108, China

[c] Institute for Organic Chemistry (IOC), Leibniz Universität Hannover, Schneiderberg 1B, 30167 Hannover, Germany.

[d] German Center for Infection Research (DZIF), Site Hannover-Braunschweig, Inhoffenstraße 7, 38124 Braunschweig, Germany.

General Remarks

Unless stated otherwise, all reagents were purchased from commercial suppliers and used without further purification. All solvents used for workup and purification were of HPLC grade. Anhydrous solvents were used for all reactions in which water was not also used as a solvent and in which the total amount of organic solvent did not exceed 100 mL. All anhydrous solvents were purchased from commercial suppliers. Moisture-sensitive reactions were performed under argon atmosphere in dried glassware. Reactions were monitored by TLC, LC-MS or NMR. The removal of organic solvents took place using rotary evaporators at 30 °C, the removal of water at 40 °C. For lyophilization of substances, the solutions in question were frozen with liquid nitrogen and freeze-dried on an Alpha 2-4 LSCbasic (CHRIST) instrument. Centrifugations were performed using a Universal 32 R centrifuge (HETTICH).

Column chromatographic purifications were carried out on silica gel (Si 60, 40 - 63 µm particle size) from the producer MERCK under elevated pressure (flash chromatography). The eluents used are listed after the indicated retention factors.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a BRUKER Avance III 500 with the probe head PABBO BB/^{19}F-^{1}H/D Z-GRD (500 MHz for ^{1}H, 125 MHz for ^{13}C) or a BRUKER Avance III HD 700 with the probe head CPTCI ^{1}H-^{13}C/^{15}N/D Z-GRD (700 MHz for ^{1}H, 176 MHz for ^{13}C) at room temperature. Samples were measured as solutions in deuterated solvents. Chemical shifts are reported in ppm relative to solvent signal. Multiplicity is indicated as follows:

s (singlet); bs (broad singlet); d (doublet); t (triplet); q (quartet); quin (quintet), m (multiplet); as well as combination of those e.g. dd (doublet of doublets), etc.

Low resolution mass spectrometry (LRMS) data were recorded using an AGILENT 1100 HPLC system equipped with DAD detector and connected to an AGILENT 6130 quadrupole mass detector with electrospray ionization (ESI) (ACN-H_2O + 0.05 % TFA)

High resolution mass spectrometry (HRMS) data were recorded using a DIONEX Dionex Ultimate 3000 HPLC system equipped with a DAD detector and a BRUKER maXis HD QTOF mass detector with electrospray ionization (ESI). Samples were directly injected via an Ultimate 3000RS autosampler (THERMO FISHER SCIENTIFIC). The mass-to-charge ratio *m/z* is being reported.

UV/Vis data were recorded using a PowerWave ™ microplate spectrophotometer (BioTek® Instruments, Inc.) with Gen5™ version 2.09.2 software. Samples were measured in 96 well plates (Greiner Bio-One GmbH, microplate, 96 Well, PS, F-bottom (Chimney Well) µCLEAR®, black, med. binding).

Synthesis of mono and di substituted tetrazines

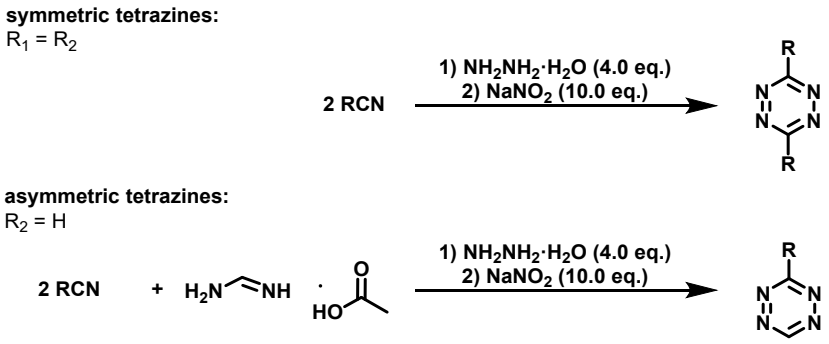


Figure S1: Pinner synthesis of symmetric (top) and asymmetric (bottom) tetrazines.

Determination of rate constants

Phenyl-vinyl ether / tetrazine model system:

Rate constants *k* for different tetrazines were measured under pseudo first order conditions with a 100- to 300-fold excess of phenylvinylether in DMSO with 10% water by following the exponential decay in UV absorbance of the tetrazine at 510 and 540 nm over 15 h in 1 min

intervals. Measurements were carried out at 25 °C shacking for 5 s before each measurement. Stock solutions of each tetrazine (2 mM) and of phenylvinylether (200, 300, 400, 500, 600 mM) in DMSO with 10% water were prepared. Equal volumes of the respective stock solutions were mixed leading to a final concentration of 1 mM of the respective tetrazine and final concentrations of 100, 150, 200, 250, 300 mM of phenylvinylether, corresponding to 100 to 300 equivalents.

Data were fit to a single-exponential equation. Each measurement was carried out in triplicates. The mean of the observed rates *k*' was plotted against the concentration of phenylvinylether and fit to a linear equation. The rate constant was obtained from the slope of the plot. All data processing was performed using GraphPad Prism 9 version 9.1.2.

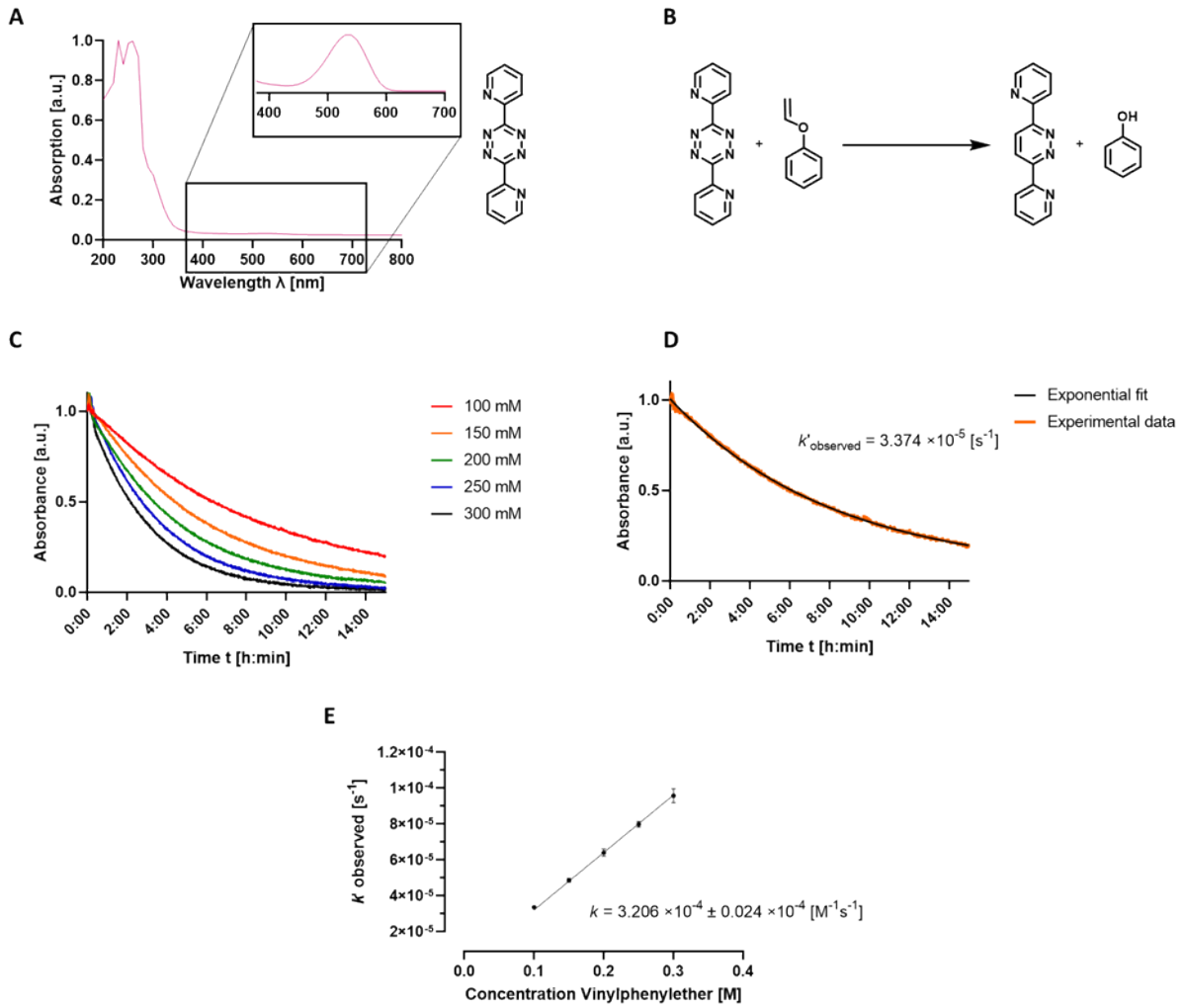


Figure S2: Determination of the rate constant *k* for the reaction between tetrazine **Tz4** and phenyl vinyl ether. A) Absorption spectrum of tetrazine **Tz4**; B) IEDDA reaction between **Tz4** and phenyl vinyl ether; C) Reactions were performed in DMSO with 10% H_2O. Absorbance at λ = 510 and 540 nm (only decay at 540 nm depicted) over time upon reaction of **Tz4** (final concentration 1 mM) with phenyl vinyl ether

for final vinyl concentrations of 100 mM, 150 mM, 200 mM, 250 mM, and 300 mM. All measurements were performed in triplicates; D) Determination of $k'_{observed}$ via an exponential fit. Data only shown for one replicate for clarity reasons; E) $k'_{observed}$ plotted against the concentration of phenyl vinyl ether. Final k determined by the slope of the resulting linear function.

Doxorubicin release in DMSO

TML-Doxo (2.25 mg, 2.91 µmol, 1.0 eq.) was dissolved in 700 µL DMSO-d_6 in a 5 mm NMR tube. The homogeneity of the magnetic field was adjusted by gradient shimming on the z-axis, and a reference ^{1}H spectrum was acquired at 37 °C. Tetrazine **Tz6** (5.77 mg, 29.1 µmol, 10 eq.) was added to the solution and the tube was immediately placed back in the NMR spectrometer (BRUKER Avance III HD 700 with the probe head CPTCI ^{1}H-^{13}C/^{15}N/D Z-GRD). ^{1}H spectra were acquired in 2 min 28 s time intervals over a period of 40 h. During the non-measuring times the sample was kept at 37 °C using a water bath. ^{1}H spectra were acquired with a relaxation delay of 5 s and 16 scans per experiment.

Data were processed and analyzed using TopSpin 3.6.4. Regions of interest were integrated and the values obtained were transferred to GraphPad Prism 9.1.2 (226) for further analysis.

The observed rates $k'_{observed}$ of the product formation and the depletion of the starting material were obtained by fitting the data to a single-exponential equation. In case of the intermediate, the $k'_{observed}$ refers only to the decrease of the intermediate starting from t = 3 h (the maximum of the intermediate), as the second reaction step was assumed to be rate determining step, thus simplifying the integrated rate law.

$$A \overset{k_1}{\rightarrow} B \overset{k_2}{\rightarrow} C$$

Rate laws:

$$\frac{d[A]}{dt} = -k_1[A]$$

$$\frac{d[B]}{dt} = k_1[A] - k_2[B]$$

$$\rightarrow \frac{d[B]}{dt} = k_1[A(0)] \cdot e^{-k_1 t} - k_2[B]$$

$$\frac{d[C]}{dt} = k_2[B]$$

Integrated rate laws:

$$[A(t)] = [A(0)] \cdot e^{-k_1 t}$$

$$[B(t)] = \frac{k_1}{k_2 - k_1}[A(0)] \cdot \left(e^{-k_1 t} - e^{-k_2 t}\right)$$

$$[C(t)] = [A(0)] \cdot \left[1 - \frac{k_2}{k_2 - k_1} \cdot e^{-k_1 t} + \frac{k_1}{k_2 - k_1} \cdot e^{-k_2 t}\right]$$

Assumption: second reaction step = rate determining step $k_1 \gg k_2$

$$[B(t)] \approx [A(0)] \cdot e^{-k_2 t}$$

$$[C(t)] \approx [A(0)] \cdot \left(1 - e^{-k_2 t}\right)$$

Equation S1: Assumptions and underlying mathematical description of the release process.

Doxorubicin release under physiological conditions

Reactions for the Doxorubicin release from the **TML-Doxo** construct were prepared in 400 µl volume with 10 µM **TML-Doxo** and 1 mM **Tz5** (100x excess). The reaction was performed in four different buffer conditions, (1) tris buffered saline (TBS, from TBS tablets, Medicago, 50 mM tris + 150 mM NaCl, pH 7.6), which is a buffer comparable to the phosphate buffered saline (PBS) with tris as the active buffer component instead of phosphate, to enable subsequent MS analysis. (2) 10% fetal bovine serum (FBS, gibco, Qualified, HI) was added to the TBS to simulate a protein component in the buffer. Further, (3) the cell culture medium RPMI 1640 (1x, gibco), and (4) RPMI supplemented with 10% FBS were used. RPMI with 10% FBS is a standard cell culture recipe and simulates in vivo conditions. The reaction was incubated for 15 h and conducted in duplicates. After incubation, 600 µl acetonitrile: methanol (1:1) supplemented with formic acid and internal standard caffeine (0.2% (V/V) and 10 ng/ml final concentration) were added to the reaction. After mixing, a brief centrifugation was performed at 30.000 g, 4°C. 800 µl of the clarified supernatant were transferred into a screw-top glass vial and measured using LC-MS/MS. Standards of **Doxo** and **TML-Doxo** were prepared in the four different buffers in the same manner, to provide for adequate calibration curves (0, 5 µM, 10 µM, 20 µM). Samples were measured by LC-MS/MS using an Agilent 1290 Infinity II UHPLC (Agilent) with an AB Sciex QTrap 6500 triple quadrupole mass spectrometer (AB Sciex Germany GmbH) in positive mode. Acetonitrile and water (Ultra Gradient HPLC Grade, J.T Baker) with 0.1% (V/V) formic acid (HiPerSolv CHROMANORMTM, VWR) were used as LC solvents. An acetonitrile-water gradient was run over Gemini® 3 µm NX-C18 110 Å 50 x 2 mm columns (Phenomenex) as follows: 0-1 min, 5% ACN, 1-5 min 5-95% ACN, 5-6 min 95 % ACN at flow rate of 0.8 ml/min, two columns were in continuous use, requilibrating one while running the sample on the other. Specific parent-daughter ion pairs (multiple reaction monitorings or MRMs) were used to monitor **Doxo** and **TML-Doxo**. The transitions for **Doxo** were adapted from literature [1] (Table S1). The data was analysed with the MacCoss Lab Software Skyline Targeted Mass Spec Environment by the University of Washington (2023).[2] **Doxo** release could be observed under all four conditions, providing a proof-of-principle that the antibiotic can be released from **TML-Doxo** under conditions similar to in vivo (Figure S3). The yield of the reaction was rather low – 1-3%, depending on the buffer – however this can be attributed to the low reactivity of the tetrazine under these conditions. The poor stability of Doxorubicin in these buffers at 37°C also contributed to the low yields. Stabilities measured over 65 h were: TBS: 83.0±6.3%; TBS-FBS: 45.8±0.4%; RPMI: 8.3±0.4%; RPMI-FBS: 3.6±0.2%. The comparison to reactions with **TML-Doxo** without the tetrazine shows that a slight deterioration of the compound with release of **Doxo** can be observed, however this is considerably less that for the specific release with tetrazine.

Table S1: MRM transitions for detection of Doxo and TML-Doxo

Analyte	Q1 mass (Da)	Q3 mass (Da)	Dwell time (ms)	Declustering potential (V)	Collision energy (V)	Entry potential (V)	Collision cell exit potential (V)
Doxorubicin	544.3	397.0	40	66	13	10	18
	544.3	361.0	40	66	33	10	28
TML-Doxo	774.4	360.3	40	1	19	10	20
	774.4	212.3	40	1	25	10	10

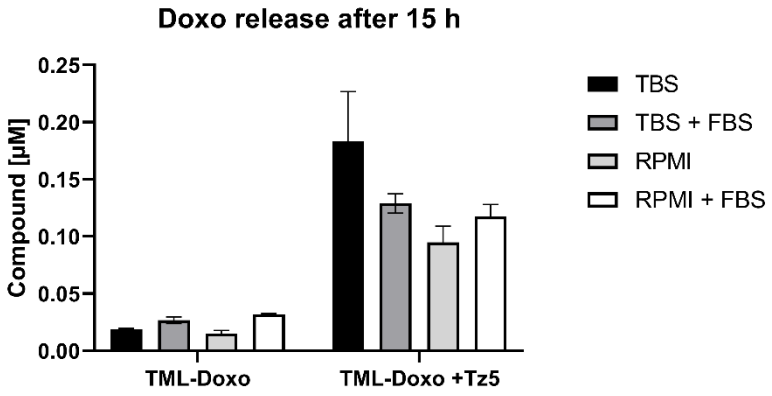


Figure S3: Concentration of **Doxo** measured in the samples after 15h in TBS (black), TBS+10%FBS (grey); RPMI (light grey) and RPMI+10% FBS (white) using specific transitions for **Doxo** (see Table S1). The error bars depict the standard deviation of the duplicates. The samples were quantified using calibration curves in each of the four buffers to attribute for matrix effects.

Methanol release in DMSO

13 (1.14 mg, 4.35 µmol, 1.0 eq.) was dissolved in 700 µL DMSO-d_6 in a 5 mm NMR tube. The homogeneity of the magnetic field was adjusted by gradient shimming on the z-axis, and a reference ^{1}H spectrum was acquired at 25 °C. Tetrazine **Tz6** (8.61 mg, 43.5 µmol, 10 eq.) was added to the solution and the tube was immediately placed back in the NMR spectrometer (BRUKER Avance III HD 700 with the probe head CPTCI ^{1}H-^{13}C/^{15}N/D Z-GRD). ^{1}H spectra were acquired after 2 min, 19 min, 48 min, 3.23 h, 3.95 h, 4.48 h, 11.4 h, 21.57 h, 31.75 h and 42.56 h. After 3.87 h another Tetrazine **Tz6** (5.39 mg, 27.2 µmol, 6.25 eq.) was added to the solution for addition of the same equivalents, due to stability of the tetrazine and the tube was immediately placed back in the NMR spectrometer. During the non-measuring times the sample was kept at 25 °C. ^{1}H spectra were acquired with a relaxation delay of 5 s and 16 scans per experiment. Data were processed and analyzed using TopSpin 3.6.4. Regions of interest were integrated and the values obtained were transferred to GraphPad Prism 9.1.2 (226) for further analysis. The proton, which was used for integration for the starting material is the proton at 6.59 ppm of **13.** For the product the integral at 6.72 ppm was used and for the

intermediate the integral at 6.30 ppm. A fast product formation of 29% in 3.23 hours could be observed, as well as intermediate formation and full disappearance of the intermediate after 11.4 hours.

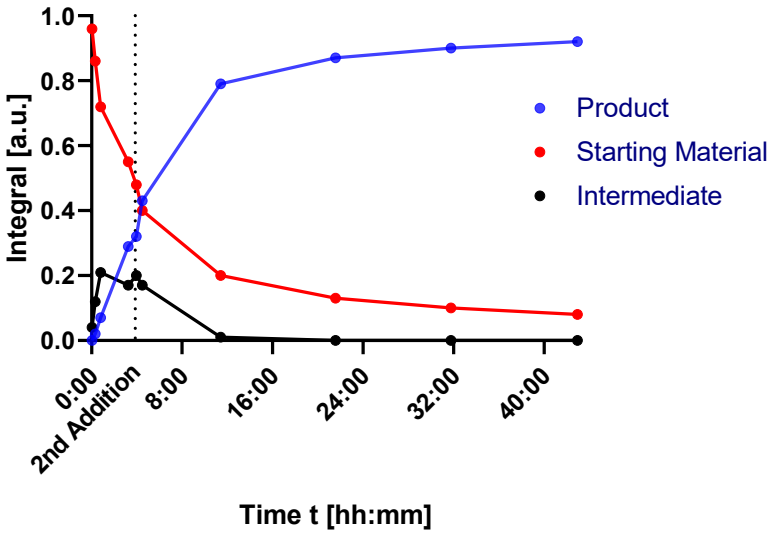


Figure S4: NMR-based assessment of methanol release upon treatment of **13** with **Tz6**. Integrals of the starting material, the intermediate and the product plotted over time as well as time point of second addition of tetrazine.

Synthetic procedures

4,4,5,7-tetramethylchroman-2-one (3)

3,5-Dimethylphenol (22.3 g, 182 mmol, 1.0 eq.) was dissolved in methanesulfonic acid (38 mL). Methyl-3-methylbut-2-enoate (28.6 mL, 24.9 g, 218 mmol, 1.2 eq.) was added and the resulting amber mixture was stirred at 70 °C for 4 h. The reaction mixture was allowed to cool to room temperature, poured into iced water and was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with water (3 × 50 mL) and dried over Na_2SO_4, filtered and evaporated *in vacuo* to give the crude product as colorless crystals, which were used in the next step without further purification. **TLC:** R_f = 0.90 (1% MeOH in DCM) [UV/$KMnO_4$].

2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenol (7)

$LiAlH_4$ (13.8 g, 364 mmol, 2.0 eq.) was added carefully to a solution of crude 4,4,5,7-tetramethylchroman-2-one (37.2 g, 182 mmol, 1.0 eq.) in THF (265 mL) at 0 °C. The mixture was stirred at room temperature over night. After the TLC showed full consumption of the starting material, a sat. NH_4Cl (100 mL) was slowly added to the solution and the mixture was filtered over sand. The cake was washed with PE (400 mL), dissolved in EtOAc (400 mL) and neutralized with HCl (2 N). The aqueous layer was extracted with EtOAc (4 × 25 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to give the crude product as off-white crystals. Additional product could be recovered from the filtrate. The filtrate was neutralized with HCl (2 N) and extracted with EtOAc (3 × 25 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo* to give the crude product as off-white crystals. The crude product was used in the next step without further purification. **TLC:** R_f = 0.18 (PE:EtOAc 3:1) [UV/$KMnO_4$] **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 6.48 (s, *J* = 9.0 Hz, 1H), 6.33 (s, 1H), 3.63 (t, *J* = 7.2 Hz, 2H), 2.47 (s, 3H), 2.27 – 2.23 (t, *J* = 7.2 Hz, 2H), 2.16 (s, 3H), 1.55 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 155.4, 137.8, 136.1, 128.5, 126.9, 116.2, 61.4, 44.9, 39.5, 31.9, 25.5, 20.2. **HRMS** (ESI) *m/z*: ($C_{13}H_{21}O_2^+$ [M+H]$^+$) calc.: 209.1536; found: 209.1539.

2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenol (8)

A 50% w/w solution of TBSCl in toluene (16.7 mL, 47.6 mmol, 1.2 eq.) was added slowly to a solution of **7** (8.26 g, 39.7 mmol, 1.0 eq.) in 1-methyl-imidazole (40 mL) at 0 °C. The mixture

was allowed to warm to room temperature and was stirred over night. The mixture was diluted with EtOAc (10 mL) and water (10 mL) and the pH was neutralized with HCl (2 N). The aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with water (10 mL) and brine (10 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The mixture was purified via flash column chromatography (silica, 1% → 5 % Et_2O in PE) to give 2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenol as colorless crystals (11.2 g, 34.8 mmol, 78% over three steps). **TLC**: R_f = 0.87 (PE:EtOAc 3:1) [UV/$KMnO_4$] **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 6.49 (s, 1H), 6.42 (s, 1H), 3.60 (t, *J* = 7.0 Hz, 2H), 2.46 (s, 3H), 2.19 (s, 3H), 2.12 (t, *J* = 7.0 Hz, 2H), 1.55 (s, 6H), 0.88 (s, 9H), 0.03 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 155.6, 137.8, 136.1, 129.3, 127.0, 116.9, 61.9, 45.0, 39.4, 32.2, 26.1, 25.6, 20.4, 18.4, -5.2. **HRMS** (ESI) *m/z*: ($C_{19}H_{35}O_2Si^+$ $[M+H]^+$) calc.: 323.2401; found: 323.2401.

(3-(2-(2-bromoethoxy)-4,6-dimethylphenyl)-3-methylbutoxy)(*tert*-butyl)dimethyl-silane (9)

Diisopropyl azodicarboxylate (7.30 mL, 7.51 mg, 37.2 mmol, 1.2 eq.) and 2-bromoethanol (2.64 mL, 4.65 mg, 37.2 mmol, 1.2 eq.) were added directly to a solution of **8** (10.0 g, 31.0 mmol, 1.0 eq.) and triphenylphosphine (9.76 g, 37.2 mmol, 1.2 eq.) in toluene (56 mL) at 0 °C. The solution was allowed to warm to room temperature and was stirred over the weekend. The cloudy, yellow mixture was filtered to give a clear, yellow solution which quickly turned cloudy again. MnO_2 (4.00 g, 46.5 mmol, 1.5 eq.) was added and the suspension stirred for 1 h. The mixture was filtrated, washed with water (2 × 20 mL) and brine (1 × 20 mL), dried over Na_2SO_4 and concentrated *in vacuo*. The yellow brown liquid was purified via column chromatography (silica, PE:Et_2O 1:0 → 1:1) to give (3-(2-(2-bromoethoxy)-4,6-dimethylphenyl)-3-methylbutoxy)(tert-butyl)dimethyl-silane as a pale-yellow oil (5.67 g, 13.0 mmol, 42%). **TLC**: R_f = 0.34 (PE:Et_2O 40:1) [UV/$KMnO_4$] **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 6.56 (s, 1H), 6.51 (s, 1H), 4.27 (t, *J* = 6.3 Hz, 2H), 3.70 (t, *J* = 6.3 Hz, 2H), 3.48 (t, *J* = 7.7 Hz, 2H), 2.49 (s, 3H), 2.25 (s, 3H), 2.16 (t, *J* = 7.7 Hz, 2H), 1.54 (s, 6H), 0.85 (s, 9H), -0.03 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 157.8, 138.0, 135.9, 131.2, 127.8, 111.7, 68.4, 61.2, 45.9, 40.0, 32.5, 29.4, 26.1, 26.0, 20.9, 18.4, -5.2. **HRMS** (ESI) *m/z*: ($C_{21}H_{38}O_2BrSi^+$ $[M+Na]^+$) calc.: 451.1638; found: 451.1638.

tert-butyl(3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutoxy)dimethylsilane (10)

To a solution of **9** (820 mg, 1.9 mmol) in DMSO (2.5 mL) was added the solution of potassium *tert*-butoxide (256 mg, 2.28 mmol) in DMSO (3 mL) at 15 ˚C. After the reaction mixture was stirred at room temperature for 3 h, 1 M HCl (30 mL) was added dropwise at 0 ˚C. The mixture

was extracted with diethyl ether (10 x 3 mL) and washed with sat. $NaHCO_3$ aq.. The collected organic layer was dried over Na_2SO_4. The solvent was evaporated and the residue was purified by chromatography (PE/ether 10:1) to give the product (548 mg, 82%). **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 6.67-6.66 (m, 1H), 6.61-6.59 (m, 1H), 6.43 (dd, *J* = 13.8, 6.1 Hz, 1H), 4.65 (dd, *J* = 13.8, 1.5 Hz, 1H), 4.33 (dd, *J* = 6.2, 1.5Hz, 1H), 3.51-3.47 (m, 2H), 2.50 (s, 3H), 2.23 (s, 3H), 2.12-2.08 (m, 2H), 1.51 (s, 6H), 0.84 (s, 9H), -0.03 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 156.9, 149.7, 138.2, 136.2, 133.0, 130.0, 118.3, 93.8, 61.4, 45.9, 39.8, 32.4, 26.1, 25.6, 20.5, 18.4, -5.1. **HRMS** (ESI) *m/z*: ($C_{21}H_{37}O_2Si^+$ $[M+H]^+$) calc.: 349.2557; found: 349.2560.

3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutan-1-ol (11)

Under N_2 atmosphere, TBAF (1.8 mL, 1.8 mmol, 1.2 eq.) was added to a THF solution (4 mL) of **10** (530 mg, 1.5 mmol) at room temperature. The mixture was stirred for 3 h (Monitored by TLC to achieve high conversion). Afterwards, the solvent was evaporated and the residue was purified by chromatography (PE/EA 5:1 v/v) to give the title product as an oil (300 mg, 71%). **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 6.69-6.67 (m, 1H), 6.63-6.62 (m, 1H), 6.46 (dd, *J* = 13.69, 6.1 Hz, 1H), 4.67 (dd, *J* = 13.8, 1.5 Hz, 1H), 4.36 (dd, *J* = 6.1, 1.5 Hz, 1H), 3.54 (t, *J* = 7.4 Hz. 2H), 2.51 (s, 3H), 2.23 (s, 3H), 2.17-2.13 (m, 2H), 1.52 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 156.9, 149.7, 138.17, 136.5, 132.7, 130.2, 118.5, 94.0, 61.2, 46.1, 39.7, 32.4, 25.7, 20.5. **HRMS** (ESI) *m/z*: ($C_{15}H_{23}O_2^+$ $[M+H]^+$) calc.: 235.1693; found: 235.1693.

3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutanal (12)

A solution of starting material **11** (300 mg, 1.28 mmol) in anhydrous DCM (10 mL) was slowly added to a suspension of pyridinium chlorochromate (594 mg, 2.68 mmol) in anhydrous DCM (20 mL) at room temperature and allowed to stir overnight at room temperature. The reaction mixture was concentrated and the residue dissolved in 30 mL of DCM followed by filtration through a short silica gel pad. The silica gel was flushed several times with ethyl ether. The filtrate was concentrated in vacuo to give the product as a viscous oil. The aldehyde was used in the next step without further purification (Also can be isolated by chromatography using PE/ether 10:1 v/v as eluent). The title compound is obtained as an oil (130 mg, 43%). **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 9.55 (t, J = 2.6 Hz, 1H), 6.44 (dd, J = 13.8, 6.1 Hz, 1H), 4.70 (dd, J = 13.8, 1.6 Hz, 1H), 4.41 (dd, J = 6.1, 1.6 Hz, 1H), 2.94 (d, J = 2.5 Hz, 2H), 2.53 (s, 3H), 2.24 (s, 3H), 1.59 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 203.8, 156.2, 149.1, 137.8, 137.1, 131.3, 130.3, 118.3, 95.0, 57.1, 38.8, 31.9, 25.6, 20.5. **HRMS** (ESI) *m/z*: ($C_{15}H_{21}O_2^+$ $[M+H]^+$) calc.: 233.1536; found: 233.1536.

Vinyl-TML

A solution of 80% $NaClO_2$ (668 mg, 5.37 mmol) in water (6.2 mL) was slowly added to a solution of aldehyde **12** (520 mg, 2.2 mmol) and sodium dihydrogen phosphate (290 mg, 2.2 mmol), and 2-methyl-2-butene (10 equiv.) in CH_3CN (4.6 mL) and water (1.8 mL) that had been cooled to 0 deg in an ice-salt water bath. The mixture was stirred at 0 deg for 1 h and then allowed to reach room temperature. Sodium sulfite (315 mg, 2.5 mmol) was added to the reaction to decompose HOCl and H_2O_2. The pH was adjusted to 2.0 with 1 N HCl, followed by extraction with ethyl acetate (500 mL). The organic layer was washed with brine (2×200mL) and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue was purified with silica gel column chromatography (PE/EA 2:1 v/v) to give the product (Quantitative yield). **^{1}H NMR** (500 MHz, $CDCl_3$): δ [ppm] = 10.31 (br, 1H), 6.67-6.66 (m, 1H), 6.61-6.59 (m, 1H), 6.42 (dd, *J* = 13.8, 6.1 Hz, 1H), 4.69 (dd, *J* = 13.8, 1.5 Hz, 1H), 4.37 (dd, *J* = 6.1, 1.5 Hz, 1H), 2.94 (s, 2H), 2.51 (s, 3H), 2.22 (s, 3H), 1.59 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$): δ [ppm] = 177.5, 156.5, 149.4, 138.0, 136.6, 131.7, 130.1, 118.0, 94.6, 47.5, 39.4, 31.8, 25.5, 20.5. **HRMS** (ESI) *m/z*: ($C_{15}H_{21}O_3^+$ $[M+H]^+$) calc.: 249.1485; found: 249.1485.

TML-Doxo

Doxorubicin (46.0 mg, 0.08 mmol) was suspendedin dry DCM (10 mL).**Vinyl-TML** (20.0 mg, 0.08 mmol) and HBTU (102 mg, 0.27 mmol) in dry DCM (20 mL) were added slowly to the reaction mixture. Next, DIPEA (88.0 mg, 0.68 mmol) was added to the reaction mixture at room temperature. The mixture was stirred for 3 h under nitrogen atmosphere. After completion of the reaction, water (50 mL) was added to the mixture and the crude product was extracted with DCM (3 × 40 mL). After removal of DCM under reduced pressure, the crude product was purified by column chromatography (silica, DCM/methanol, 15 %) to afford the product (55.0 mg, 71.0 µmol, 89%) as a red-orange solid. **^{1}H NMR** (500 MHz, DMSO): δ [ppm] = 14.04 (s, 1H), 13.27 (s, 1H), 7.92 (s, 1H), 7.91 (s, 1H), 7.66 (t, *J* = 4.8 Hz 1H), 6.97 (d, *J* = 8.4 Hz, 1H)6.58 – 6.51 (m, 3H), 5.76 (s, 1H), 5.44 (s, 1H), 5.17 (d, *J* = 3.4 Hz, 1H), 4.93 – 4.88 (m, 1H), 4.85 (t, *J* = 6.0 Hz, 1H), 4.70 (d, *J* = 6.0 Hz, 1H), 4.63 (dd, *J* = 13.7, 1.3 Hz, 1H), 4.55 (d, *J* = 6.0 Hz, 2H), 4.36 (dd, *J* = 6.1, 1.3 Hz, 1H), 4.10 (q, *J* = 6.2 Hz, 1H), 3.99 (s, 3H), 3.87 (ddd, *J* = 12.7, 7.8, 4.2 Hz, 1H), 3.27 (d, *J* = 4.0 Hz, 1H), 2.95 (q, *J* = 18.1 Hz, 2H), 2.58 (dd, *J* = 48.6, 14.1 Hz, 2H), 2.38 (s, 3H), 2.18 – 2.07 (m, 5H), 1.66 (td, *J* = 12.9, 3.8 Hz, 1H), 1.45 (s, 3H), 1.42 (s, 3H), 1.29 (dd, *J* = 12.5, 4.5 Hz, 2H), 1.09 (d, *J* = 6.5 Hz, 3H). **^{13}C NMR** (126 MHz, DMSO): δ [ppm] = 213.7, 186.6, 186.6, 169.9, 164.6, 160.8, 156.1, 156.1, 154.6, 149.8, 137.3, 136.3, 135.6, 135.5, 134.8, 134.1, 132.6, 129.6, 120.1, 119.8, 119.0, 117.6, 110.8, 110.7, 100.5, 94.2, 75.0, 70.1, 68.2, 66.7, 63.7, 56.6, 55.0, 47.8, 44.4, 39.5, 36.7, 32.1, 31.5, 31.4,

29.8, 25.1, 19.9, 17.0. **HRMS** (ESI) *m/z*: ($C_{42}H_{48}NO_{13}^+$ [M+H]$^+$) calc.: 774.3120; found: 774.3119.

Methyl 3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutanoate (13)

To a solution of **Vinyl-TML** (20.0 mg, 0.08 mmol), MeOH (32.0 μL, 0.80 mmol) and dry DCM (200 μL) 4-dimethylaminopyridine (0.98 mg, 8.05 μmol) and EDC·HCl (13.7 mg, 8.86 μmol) were added consecutively at 0 °C. The reaction was stirred for 5 min at 0 °C and after warming to room temperature, the reaction was stirred for 3 h. After reaction completion (monitored by TLC), the reaction was diluted with DCM (20 mL) and H_2O (15 mL) with consecutive separation of the two phases. The aqueous phase was extracted with DCM (2 × 10 mL) and the combined organic layers were washed with NH_4Cl (15 mL), $NaHCO_3$ (15 mL), H_2O (15 mL) and brine (15 mL). After drying over Na_2SO_4 and consecutive removing the solvent under reduced pressure the crude was purified by column chromatography (silica, EtOAc/cyclohexane, 2 %). The desired product was obtained as colorless oil (9.40 mg, 35.8 µmol, 45 %). **TLC**: R_f = 0.76 (EtOAc/cyclohexane 2%) [UV, $KMnO_4$]. **^{1}H NMR** (500 MHz, DMSO): fδ [ppm] = 6.67 (d, *J* = 1.2 Hz, 1H), 6.59 (d, *J* = 1.4 Hz, 1H), 6.54 (dd, *J* = 13.7, 6.1 Hz, 1H), 4.64 (dd, *J* = 13.7, 1.4 Hz, 1H), 4.40 (dd, *J* = 6.1, 1.4 Hz, 1H), 3.42 (s, 3H), 2.86 (s, 2H), 2.46 (s, 3H), 2.17 (s, 3H), 1.51 (s, 6H). **^{13}C NMR** (126 MHz, DMSO): δ [ppm] = 171.9, 155.9, 149.6, 137.5, 135.9, 131.4, 129.7, 117.6, 94.3, 50.8, 46.8, 31.3, 24.9, 20.0. **HRMS** (ESI) *m/z*: ($C_{16}H_{23}O_3^+$ [M+Na]$^+$) calc.: 285.1461; found: 285.1463.

3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (Tz4)

Hydrazine hydrate (3.40 mL) was added dropwise to picolinonitrile (354 mg, 3.40 mmol, 1.0 eq.). The reaction was heated to 80 °C and stirred for 35 min. After cooling down to room temperature sodium nitrate (2.89 g, 34.0 mmol, 10.0 eq.) in water (5 mL) was added to the reaction. Afterwards the product was precipitated by adding HCl (2 M) until the pH was ca. 3. The pink solid was separated by filtration. The filtrate was washed with HCl (0.5 M) and H_2O. The crude was purified by column chromatography giving 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (0.08 g, 0.34 mmol, 10%) as a pink solid. **TLC**: R_f = 0.48 (MeOH/DCM 10%) [UV]. **^{1}H NMR** (500 MHz, DMSO): δ [ppm] = 8.95 (ddd, *J* = 4.7, 1.6, 0.8 Hz, 1H), 8.62 (d, *J* = 7.9 Hz, 1H), 8.17 (td, *J* = 7.8, 1.8 Hz, 1H), 7.74 (ddd, *J* = 7.6, 4.7, 1.1 Hz, 1H). **^{13}C NMR** (126 MHz, DMSO): δ [ppm] = 163.3, 150.7, 150.1, 137.9, 126.7, 124.4. **HRMS** (ESI) *m/z*: ($C_{12}H_9N_6^+$ [M+H]$^+$) calc.: 237.0883; found: 237.0884.

4-(1,2,4,5-tetrazin-3-yl)benzoic acid (Tz3)

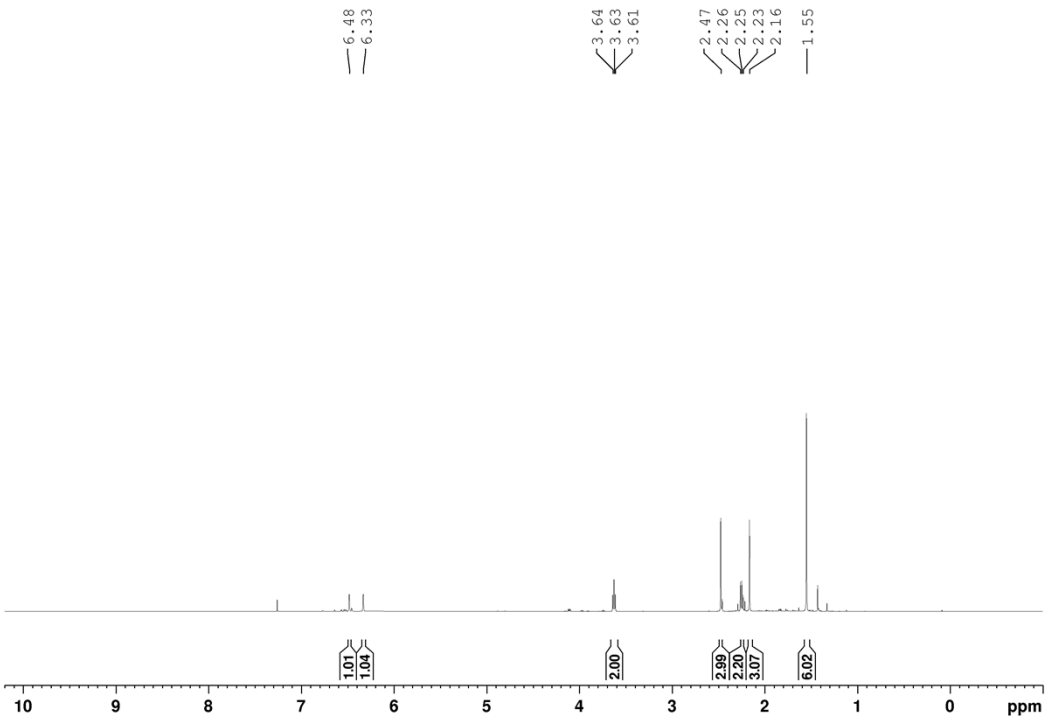
Hydrazine hydrate (34.0 mL) was added dropwise under Ar to Cyanobenzoic acid (5.00 g, 34.0 mmol, 1.0 eq.) and formamidine acetate (17.7 g, 170 mmol, 5.0 eq.). The reaction was heated to 80 °C and stirred for 35 min. After cooling down to RT sodium nitrate (11.7 g, 170 mmol, 5.0 eq.) in water (34 mL) was added to the reaction. Afterwards the product was precipitated by adding HCl (2 M) until the pH was ca. 3. The pink solid was separated by filtration. The filtrate was washed with HCl (0.5 M) and H_2O. The crude was purified by column chromatography and further purified by recrystallization ($CHCl_3$/^{i}PrOH). 4-(1,2,4,5-tetrazin-3-yl)benzoic acid (460 mg, 2.28 mmol, 7%) was obtained as a bright pink solid. **TLC**: R_f = 0.38 (10%MeOH/DCM) [UV]. **^{1}H NMR** (500 MHz, DMSO): δ [ppm] = 13.34 (br s, 1H), 10.66 (s, 1H), 8.62 (d, *J* = 8.6 Hz, 2H), 8.24 – 8.20 (d, *J* = 8.6 Hz, 2H). **^{13}C NMR** (126 MHz, DMSO): δ [ppm] = 166.8, 165.1, 158.3, 135.8, 134.4, 130.3, 128.0. **HRMS** (ESI) *m/z*: ($C_9H_7N_4O_2^+$ [M+H]$^+$) calc.: 203.0564; found: 203.0564.

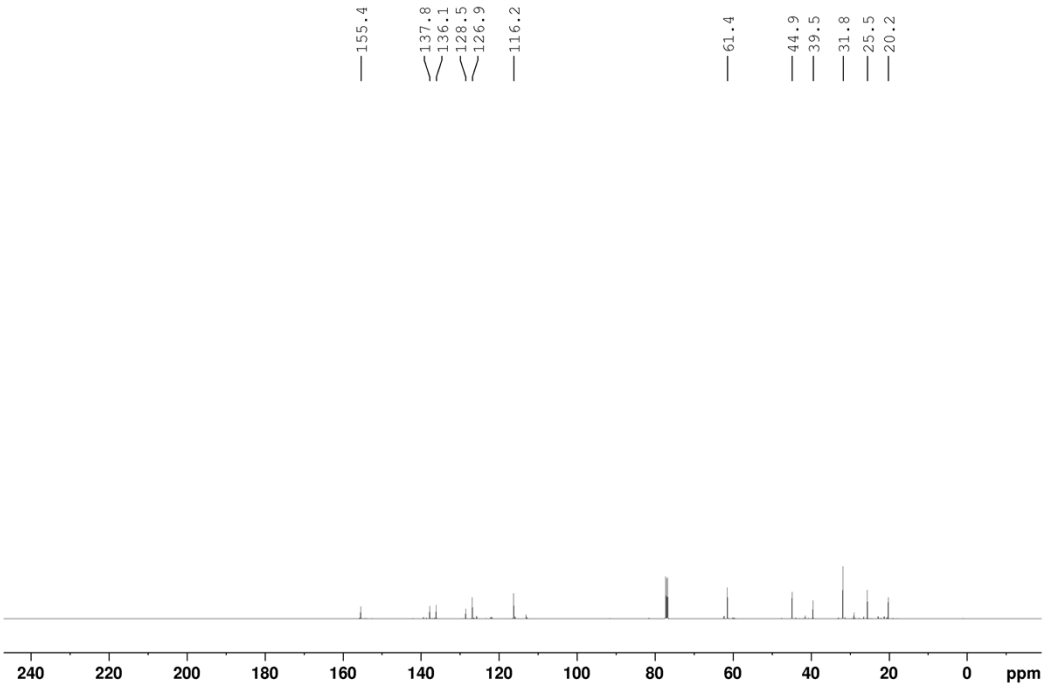
6-(1,2,4,5-tetrazin-3-yl)nicotinic acid (Tz5)

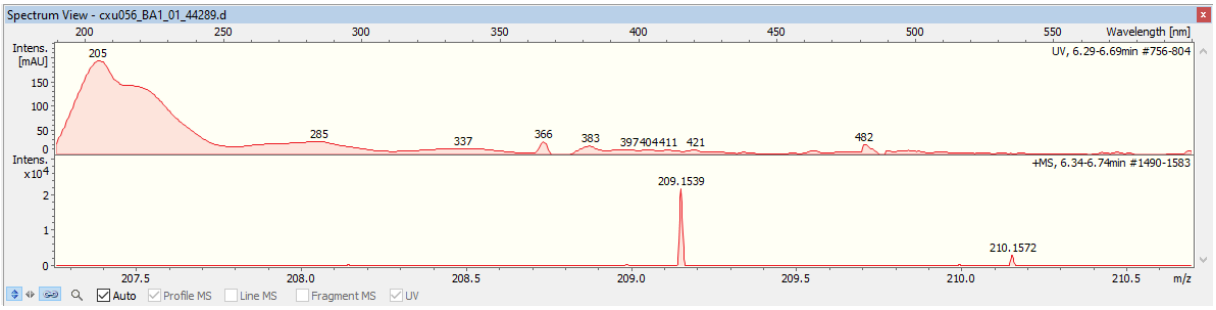
Hydrazine hydrate (1.68 mL) was added to a mixture of 6-cyanopyridine-3-carboxylic acid (250 mg, 1.68 mmol, 1.0 eq.) and formamidine acetate (875 mg, 8.40 mmol, 5.0 eq.). The resulting clear and yellow solution was stirred at 80 °C for 30 min. After cooling to RT, the solution was acidified to pH = 3 by addition of 1 M HCl and 2 M HCl while cooling with an ice bath. The orange precipitate was filtered and washed with water (3 × 10 mL), and dried to give 185 mg of solid. The solid was suspended in MeOH (25 mL) via sonification. To this suspension was added solid tetrachloro-1.4-benzoquinone (206 mg, 0.84 mmol, 0.5 eq.) and the suspension was stirred for 15 min. The solution was filtered and washed with MeOH (2 × 5 mL) to give 6-(l,2,4,5-tetrazin-3-yl)nicotinic acid (40.0 mg, 197 µmol, 12%) as a pink solid. **TLC**: R_f = 0.11 (MeOH/DCM 10%) [UV]. **^{1}H NMR** (500 MHz, DMSO): δ [ppm] = 13.83 (br s, 1H), 10.76 (s, 1H), 9.36 (dd, *J* = 2.1, 0.8 Hz, 1H), 8.68 (dd, *J* = 8.2, 0.8 Hz, 1H), 8.59 (dd, *J* = 8.2, 2.1 Hz, 1H). **^{13}C NMR** (126 MHz, DMSO): δ [ppm] = 165.8, 165.0, 158.6, 153.4, 151.0, 138.8, 128.7, 124.1. **HRMS** (ESI) *m/z*: ($C_8H_6N_5O_2^+$ [M+H]$^+$) calc.: 204.0516; found: 204.0516.

NMR Spectra, HPLC Chromatograms and HRMS Spectra

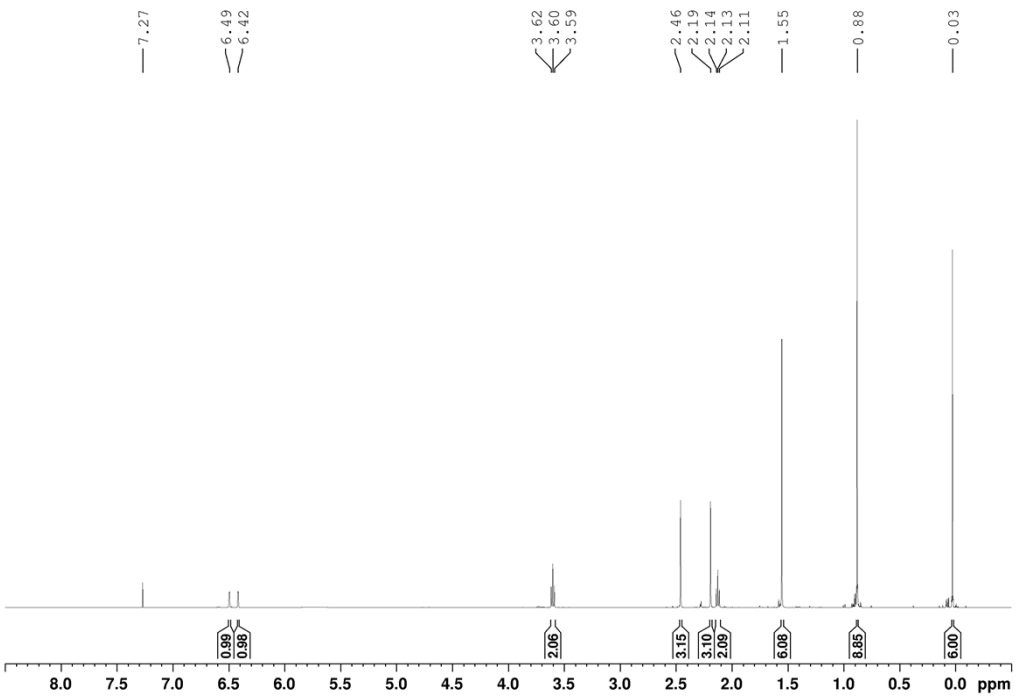
2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenol (7)

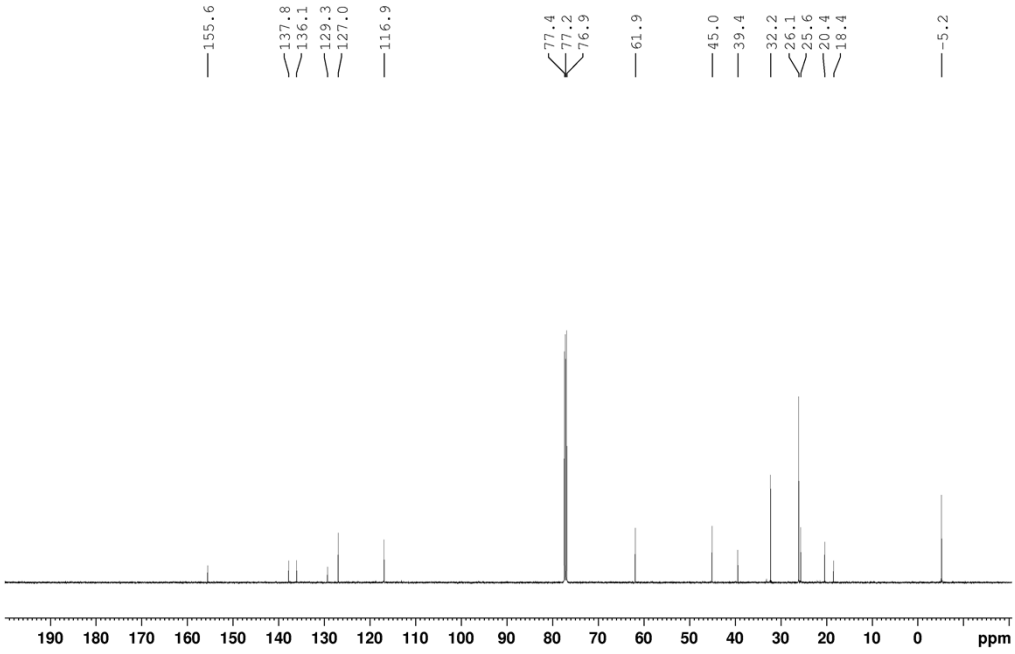






2-(4-((*tert*-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenol (8)

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45.0
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26.1
25.6
20.4
18.4
-5.2
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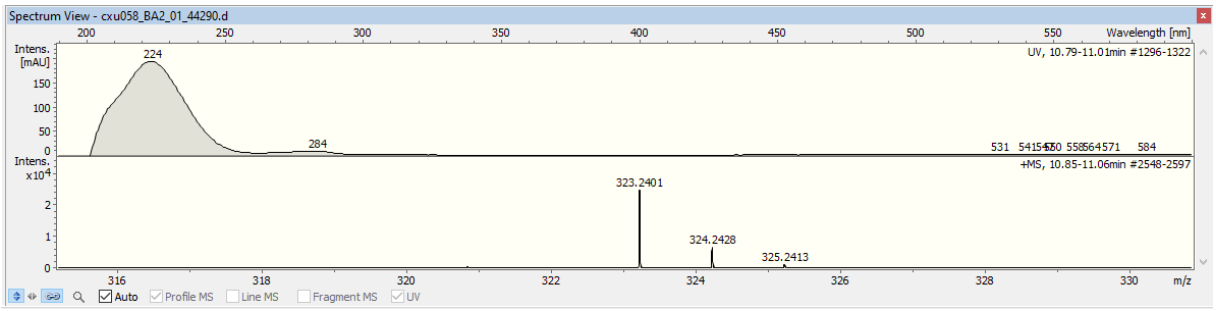
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325.2413
m/z
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Line MS
Fragment MS
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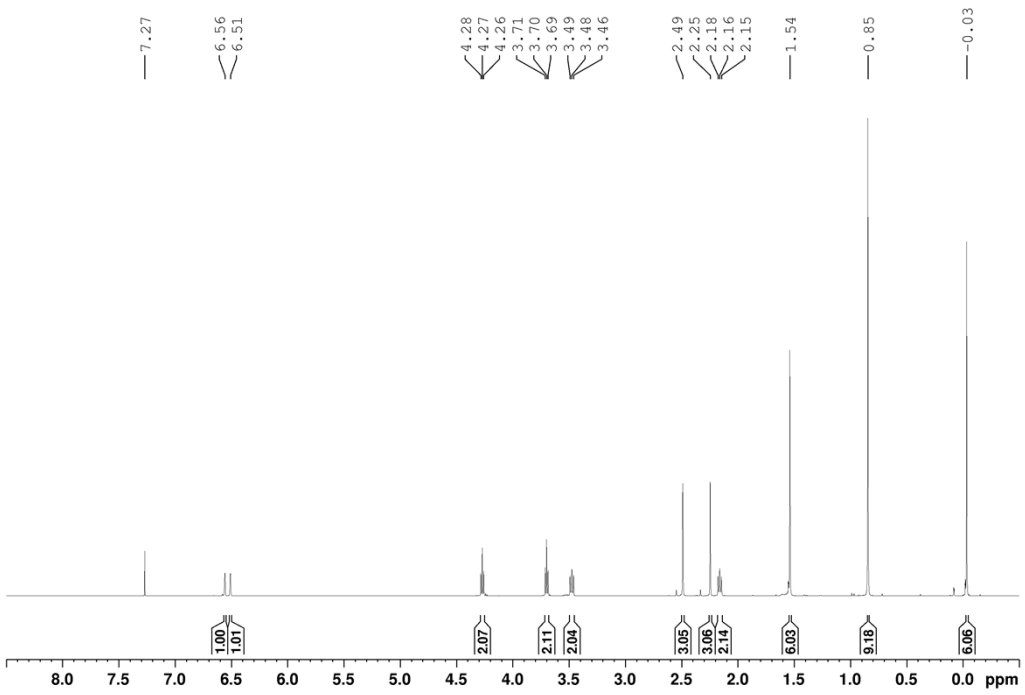
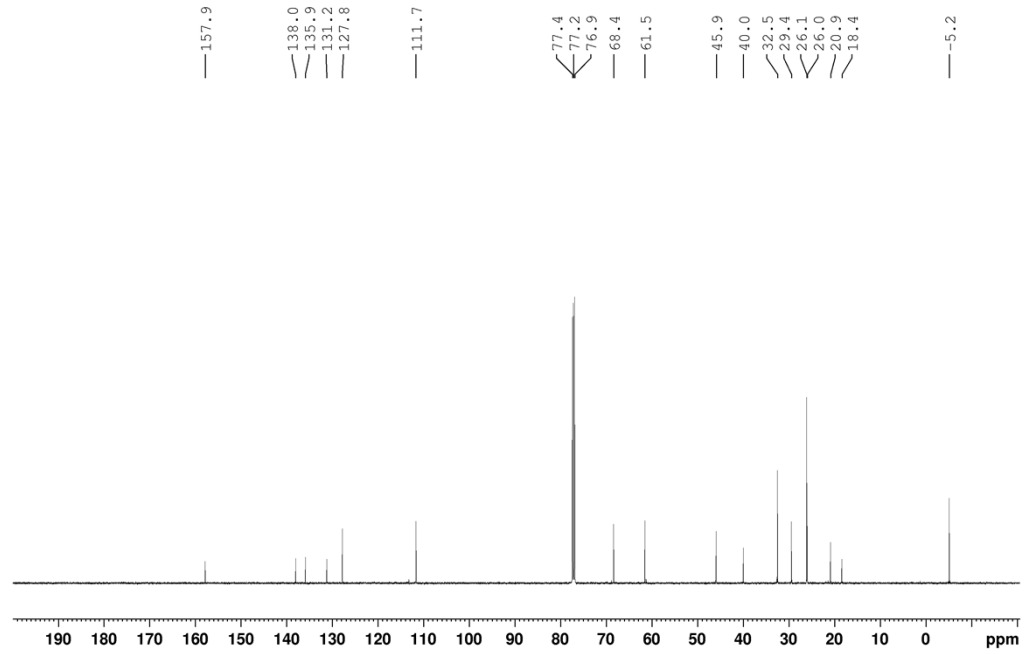
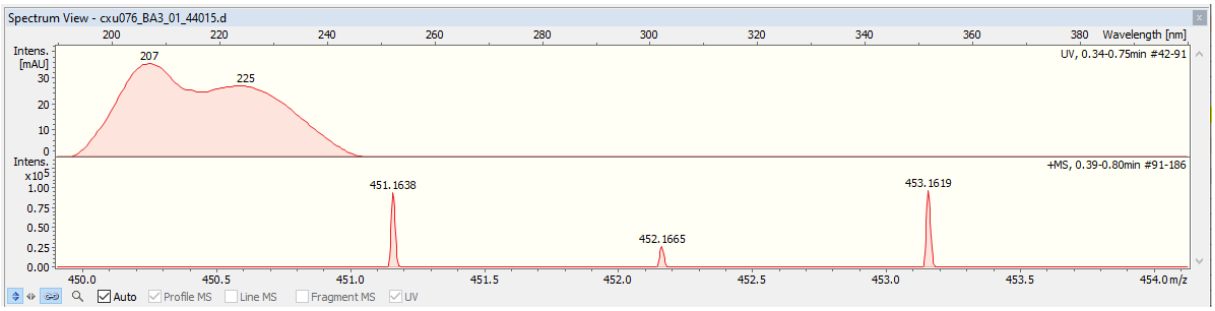
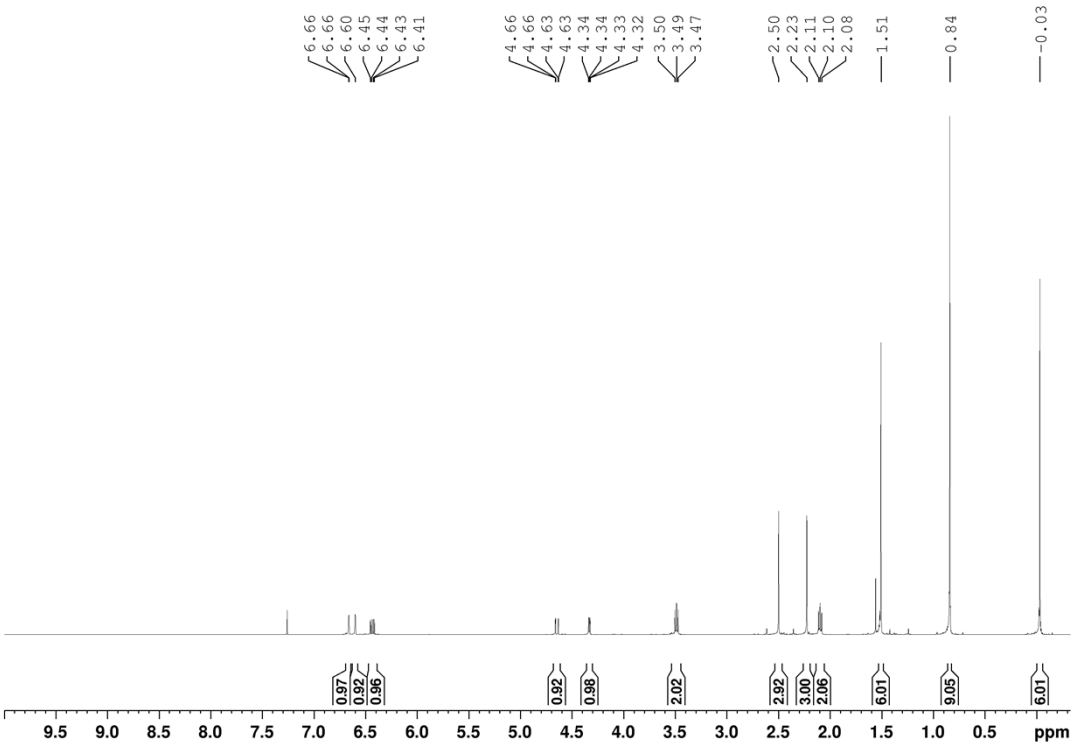
(3-(2-(2-bromoethoxy)-4,6-dimethylphenyl)-3-methylbutoxy)(*tert*-butyl)dimethyl-silane (9)

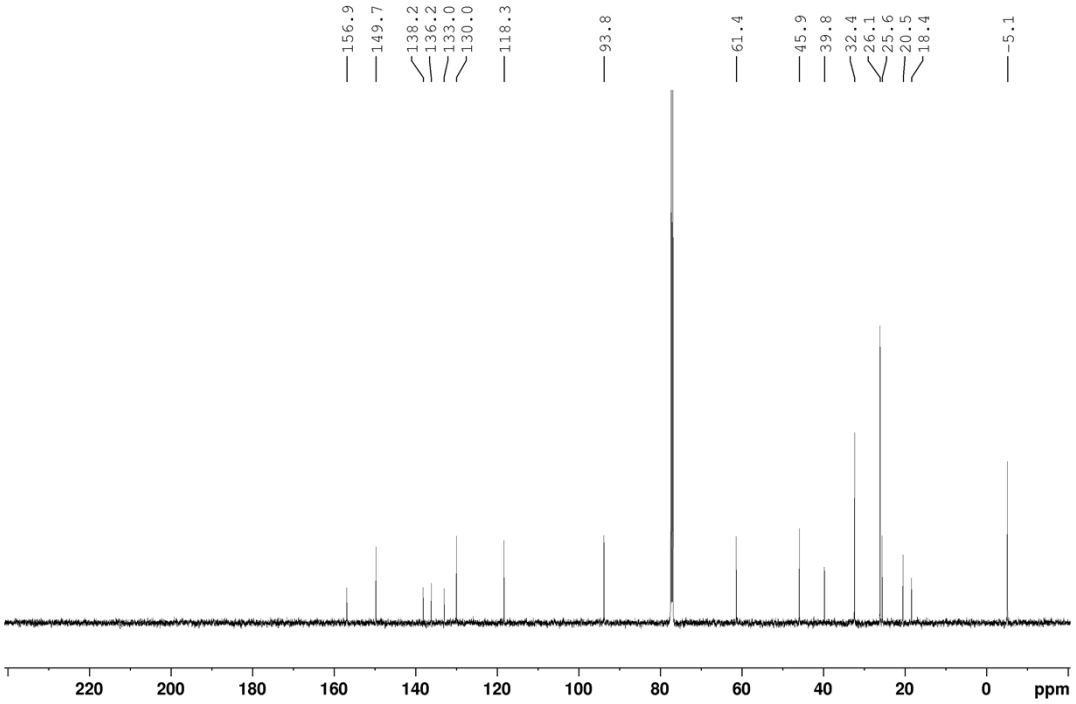






tert-butyl(3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutoxy)dimethylsilane (10)

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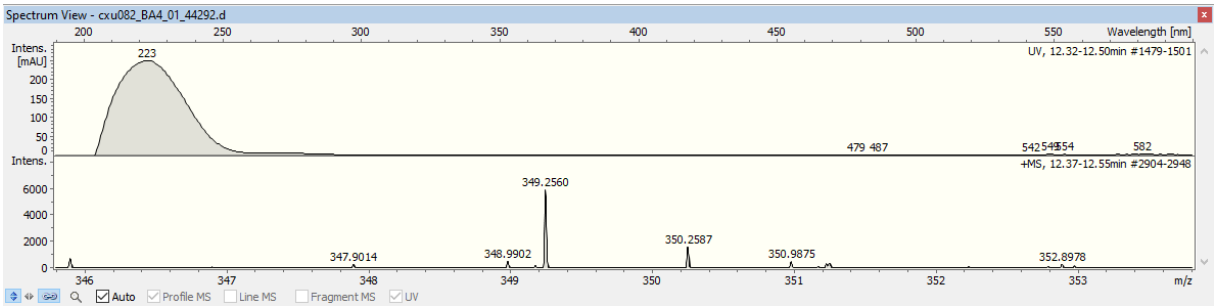
ppm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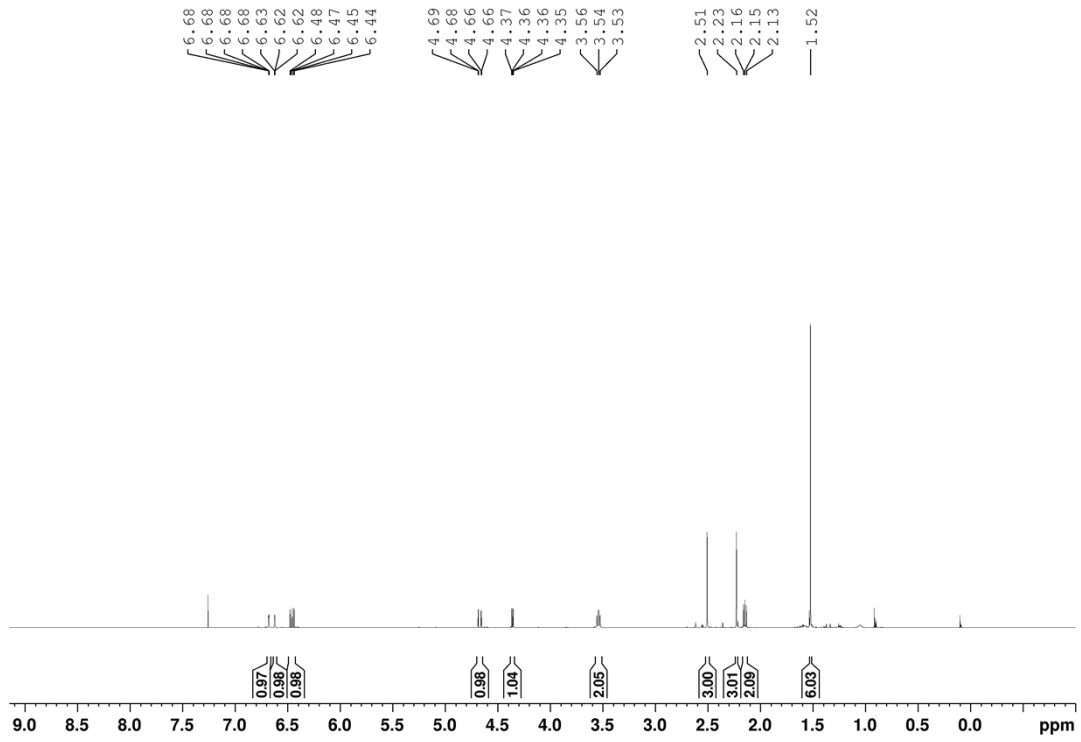
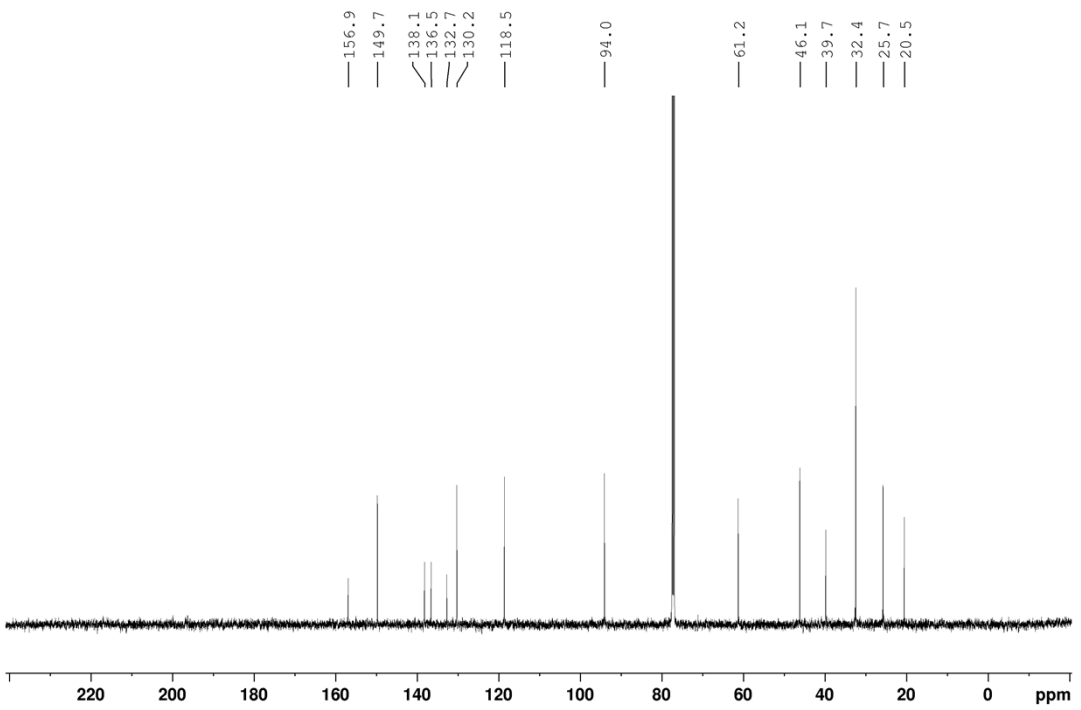
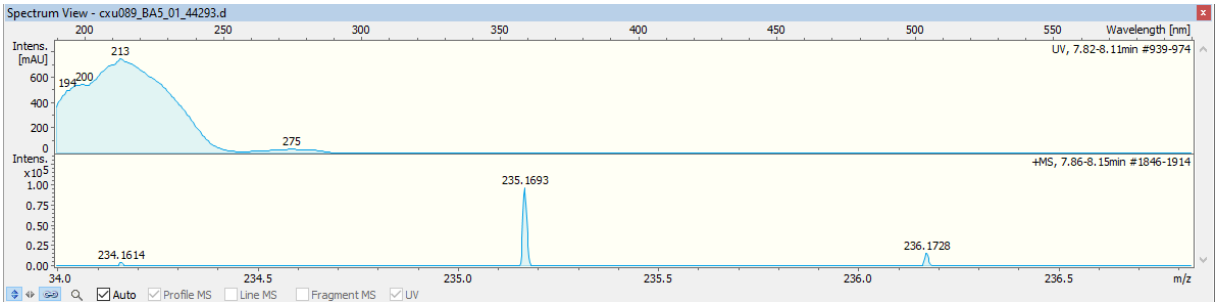
Fragment MS
UV

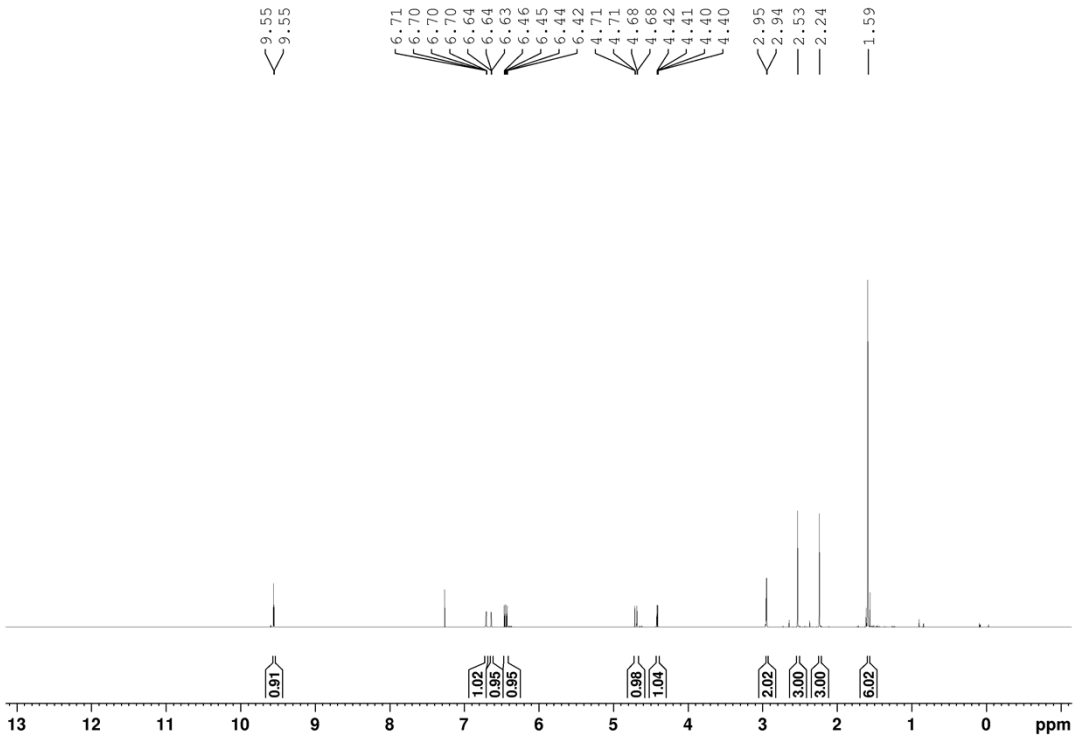
3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutan-1-ol (11)

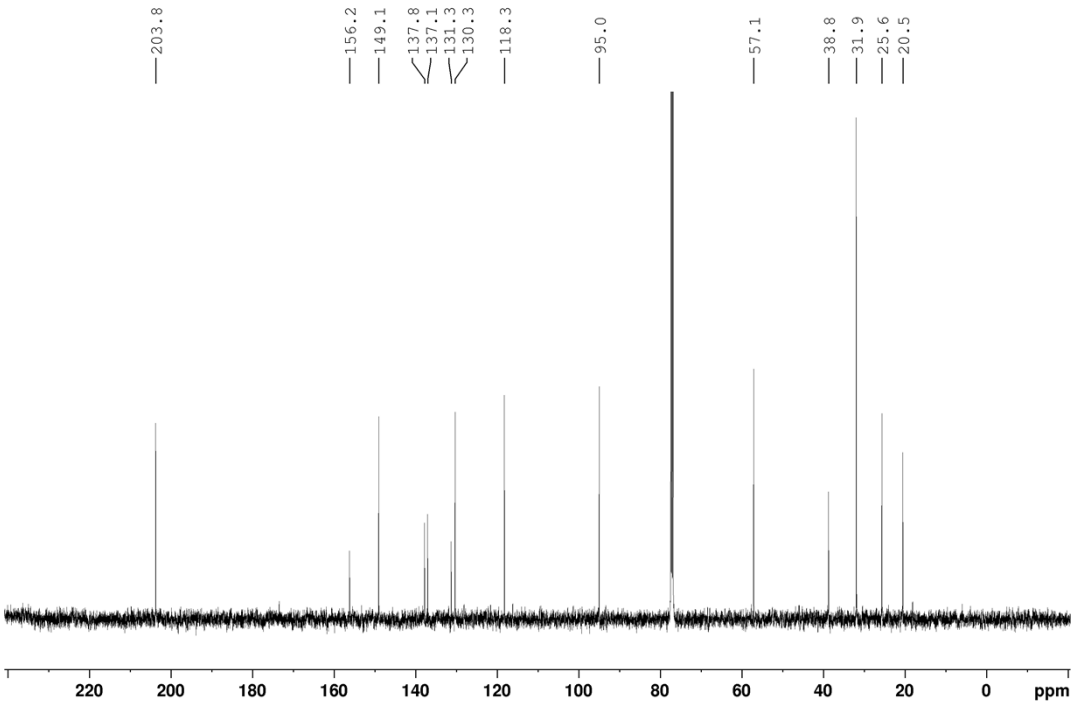






3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutanal (12)

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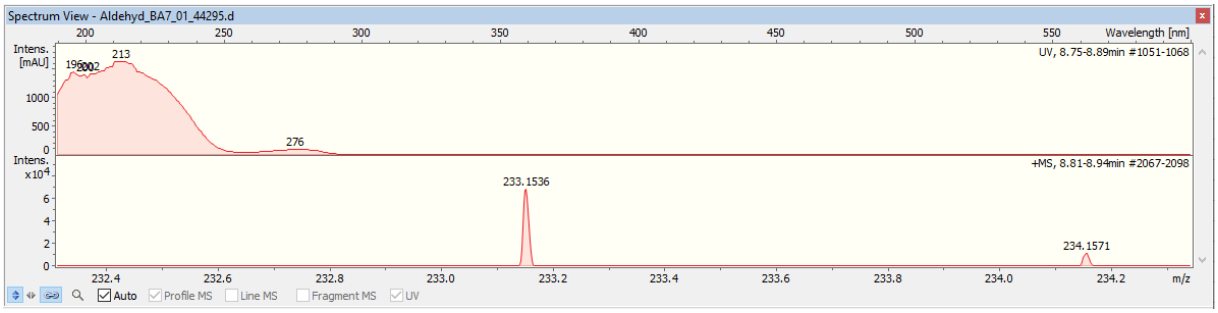
ppm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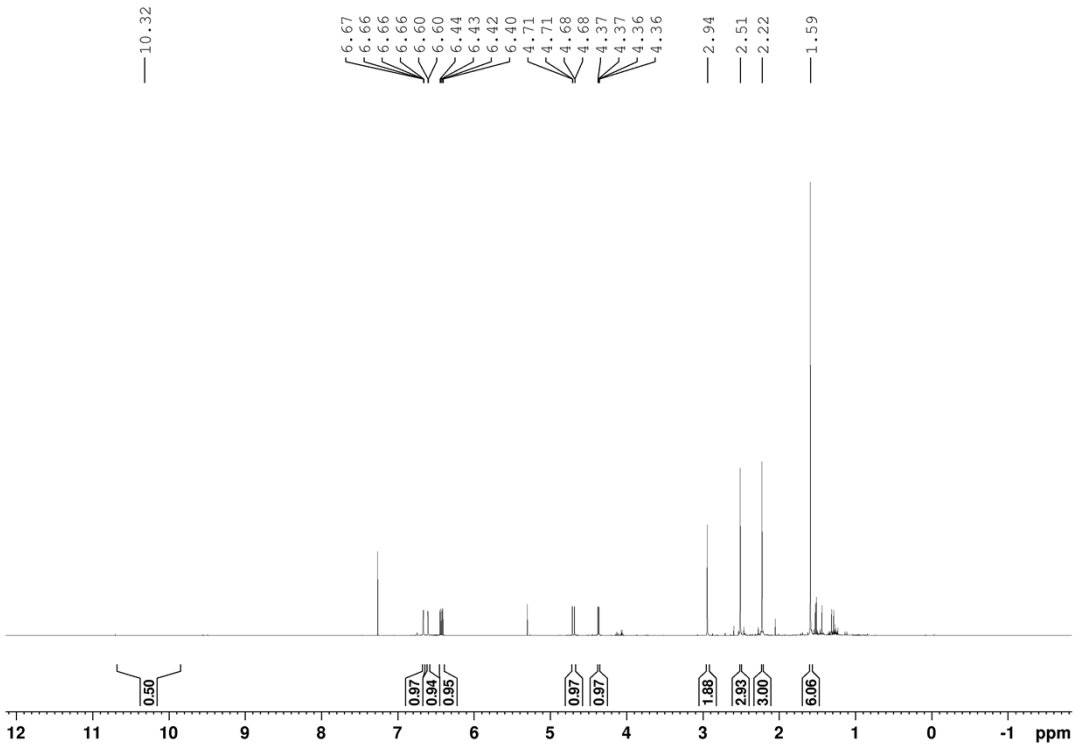
Fragment MS
UV

Vinyl-TML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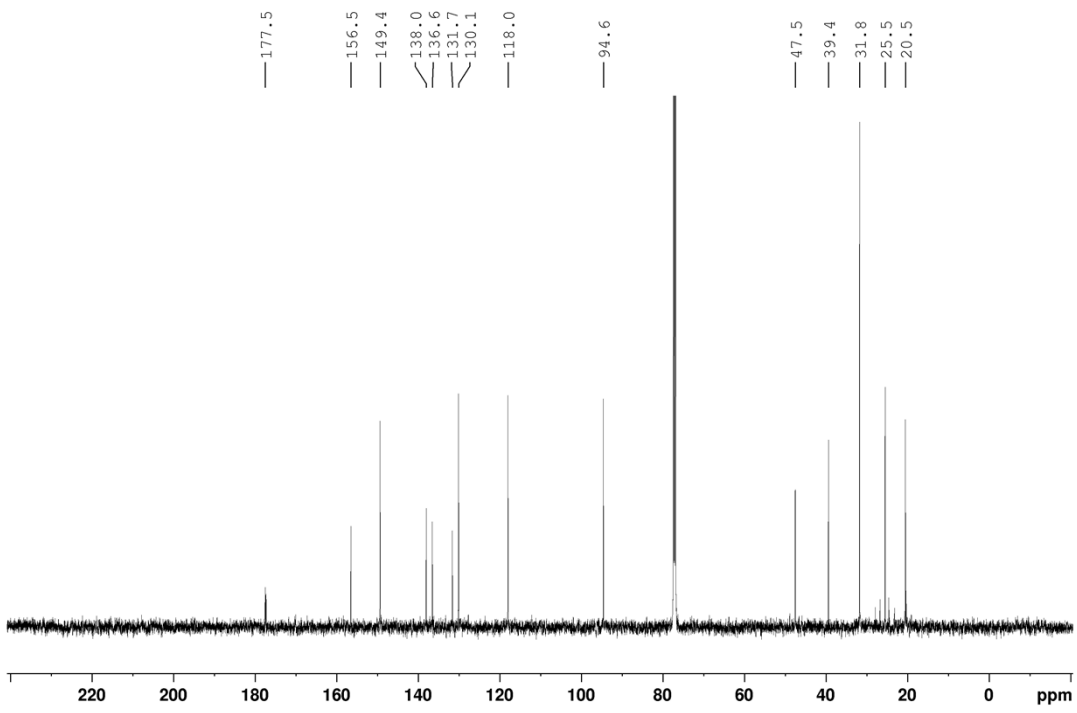
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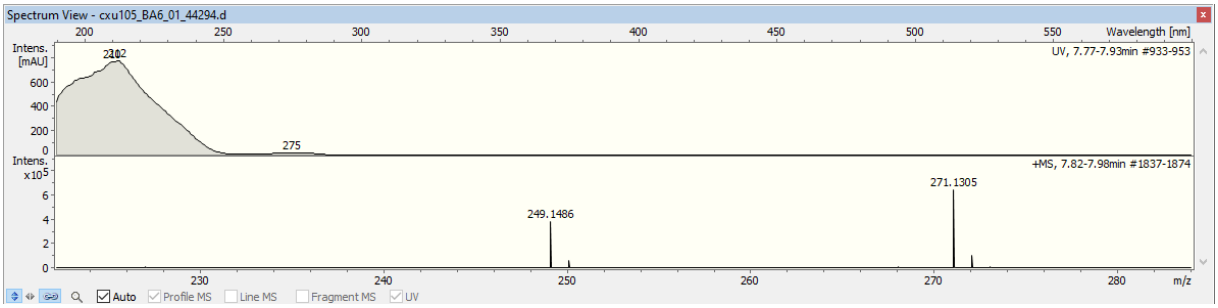
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m/z
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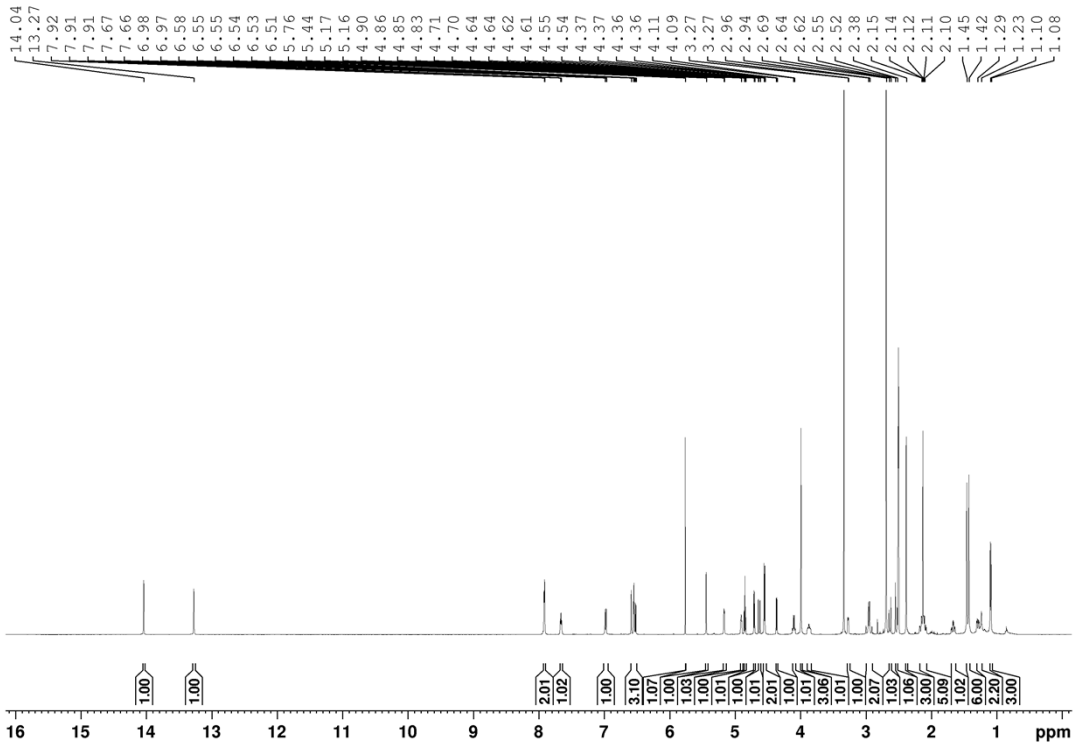
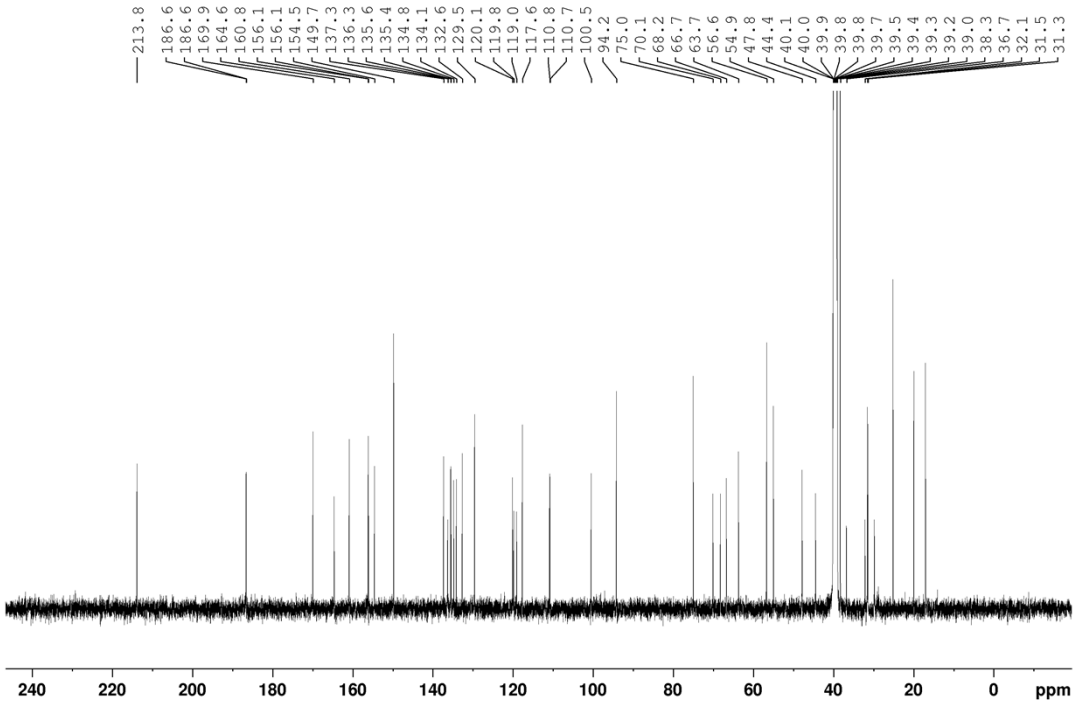
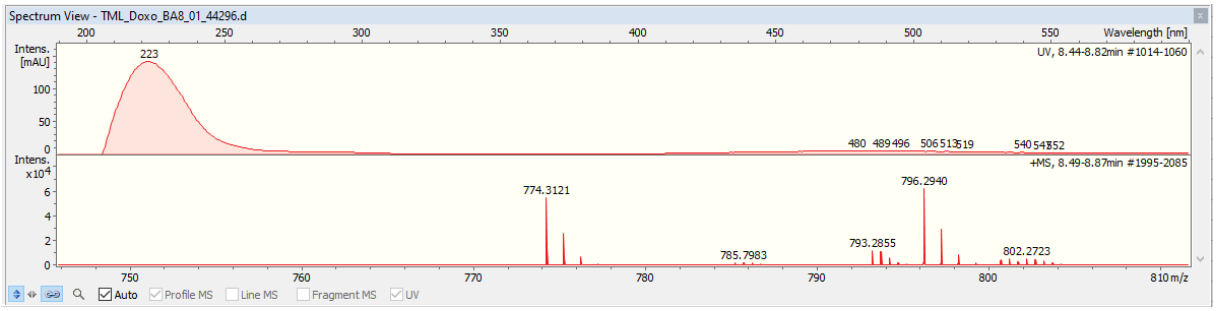
Line MS
Fragment MS
UV

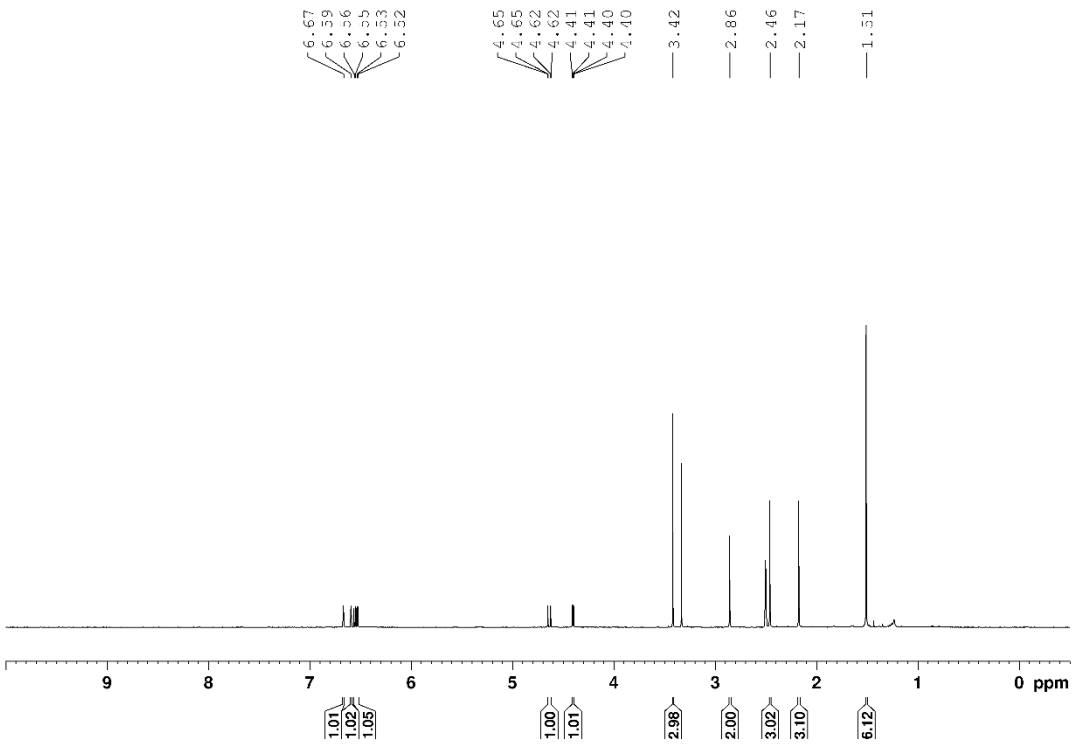
TML-Doxo

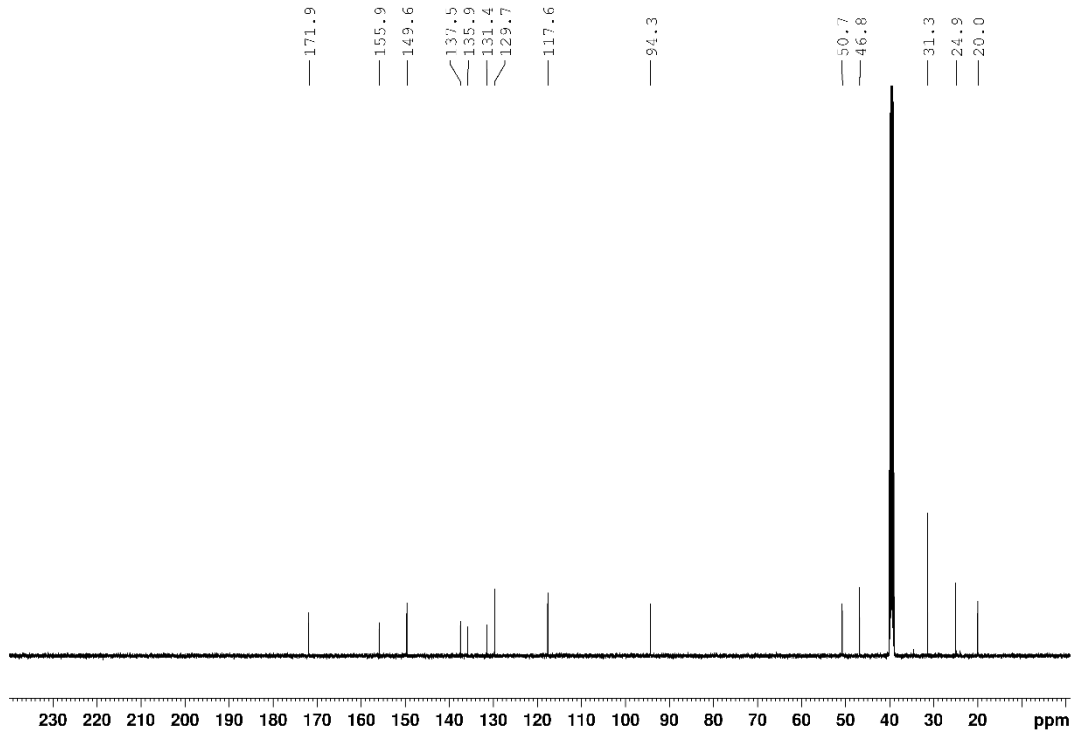

14.04
13.27
7.92
7.91
7.91
7.67
7.66
6.98
6.97
6.58
6.55
6.55
6.54
6.53
6.51
5.76
5.44
5.17
5.16
4.90
4.86
4.85
4.83
4.71
4.70
4.64
4.64
4.62
4.61
4.55
4.54
4.37
4.37
4.36
4.36
4.11
4.09
3.27
3.27
2.96
2.94
2.69
2.64
2.62
2.55
2.52
2.38
2.15
2.14
2.12
2.11
2.10
1.45
1.42
1.29
1.23
1.10
1.08
ppm

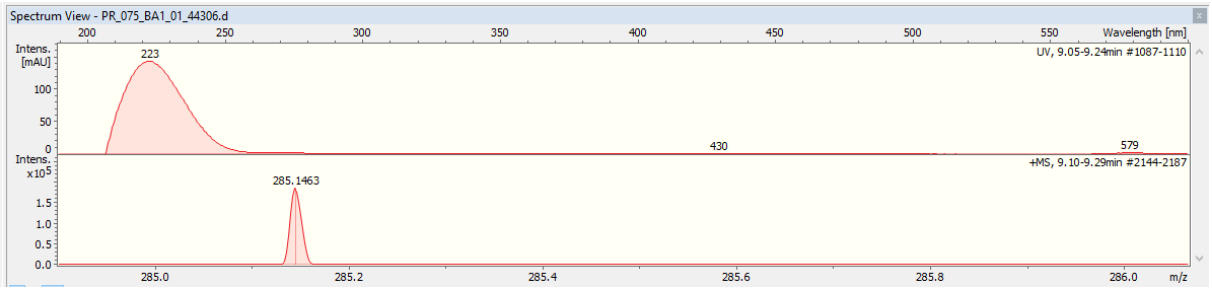

213.8
186.6
186.6
169.9
164.6
160.8
156.1
156.1
154.5
149.7
137.3
136.3
135.6
135.4
134.8
134.1
132.6
129.5
120.1
119.8
119.0
117.6
110.8
110.7
100.5
94.2
75.0
70.1
68.2
66.7
63.7
56.6
54.9
47.8
44.4
40.1
40.0
39.9
39.8
39.8
39.7
39.5
39.4
39.3
39.2
39.0
38.3
36.7
32.1
31.5
31.3
240
220
200
180
160
140
120
100
80
60
40
20
0
ppm


Spectrum View - TML_Doxo_BA8_01_44296.d
Wavelength [nm]
Intens. [mAU]
UV, 8.44-8.82min #1014-1060
223
480
489
496
506
513
519
540
543
552
Intens. x10^4
+MS, 8.49-8.87min #1995-2085
774.3121
796.2940
785.7983
793.2855
802.2723
m/z
Auto
Profile MS
Line MS
Fragment MS
UV

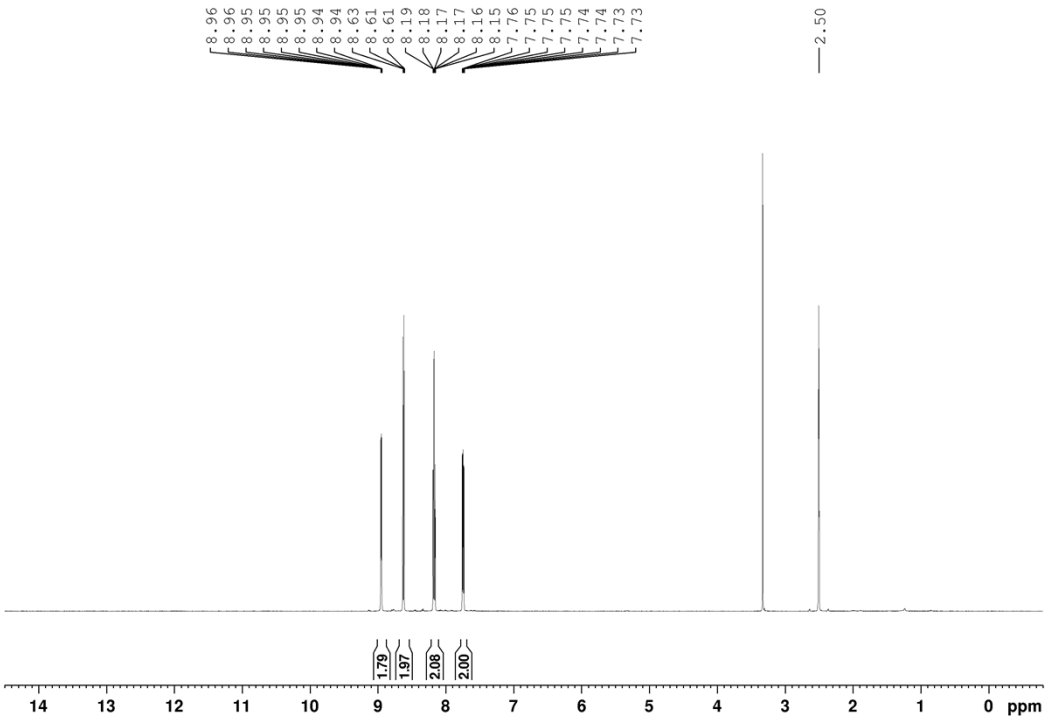
Methyl 3-(2,4-dimethyl-6-(vinyloxy)phenyl)-3-methylbutanoate (13)

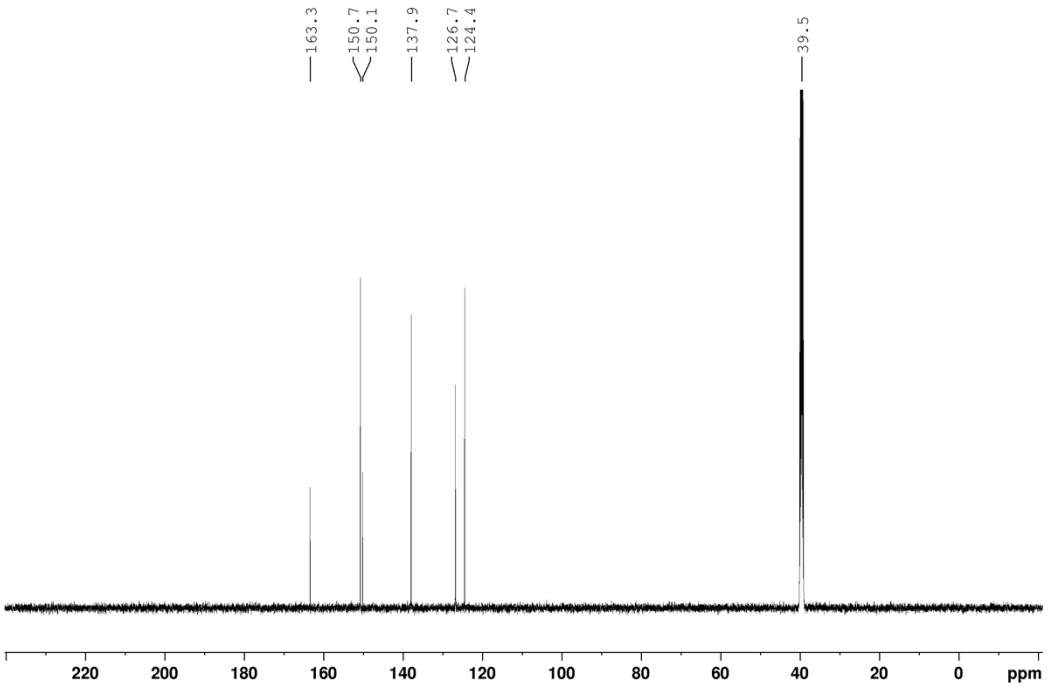


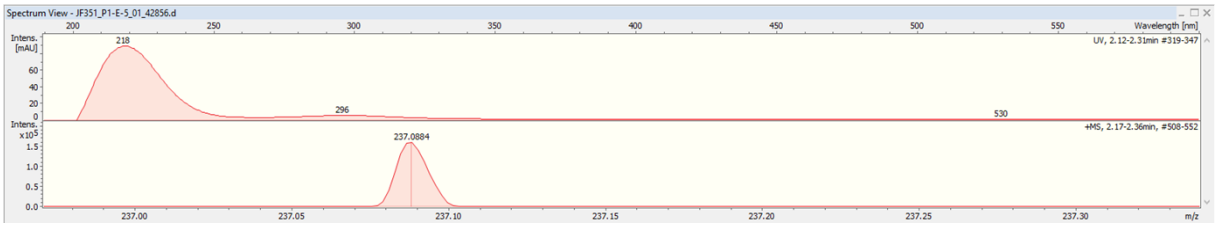




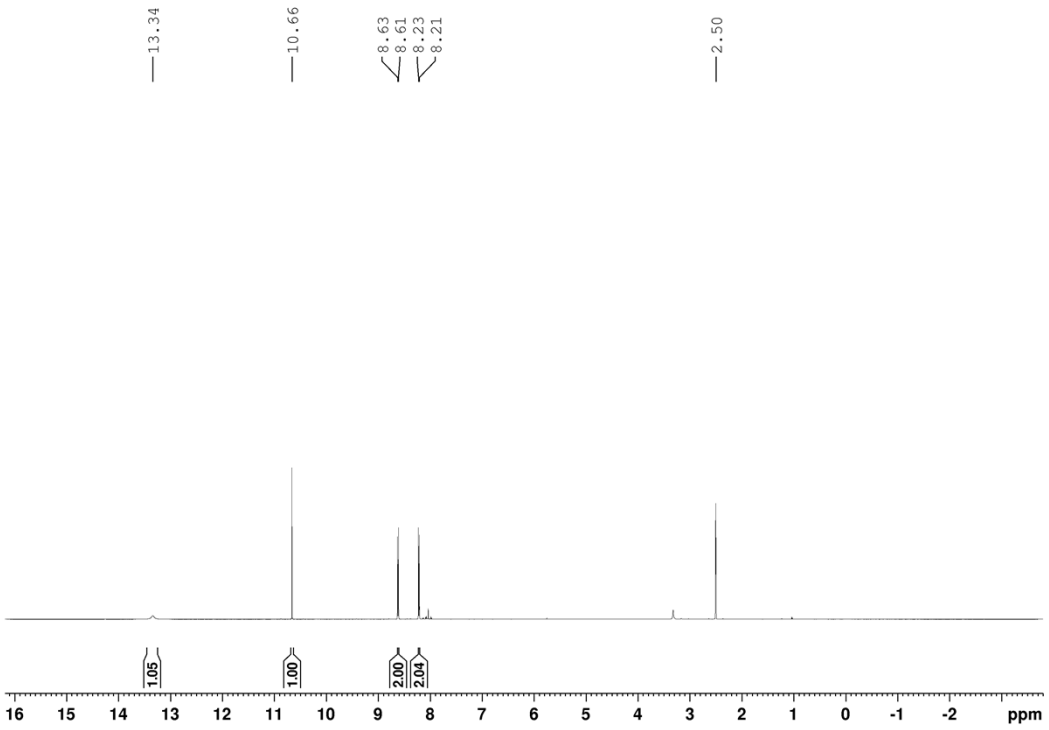
3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (Tz4)

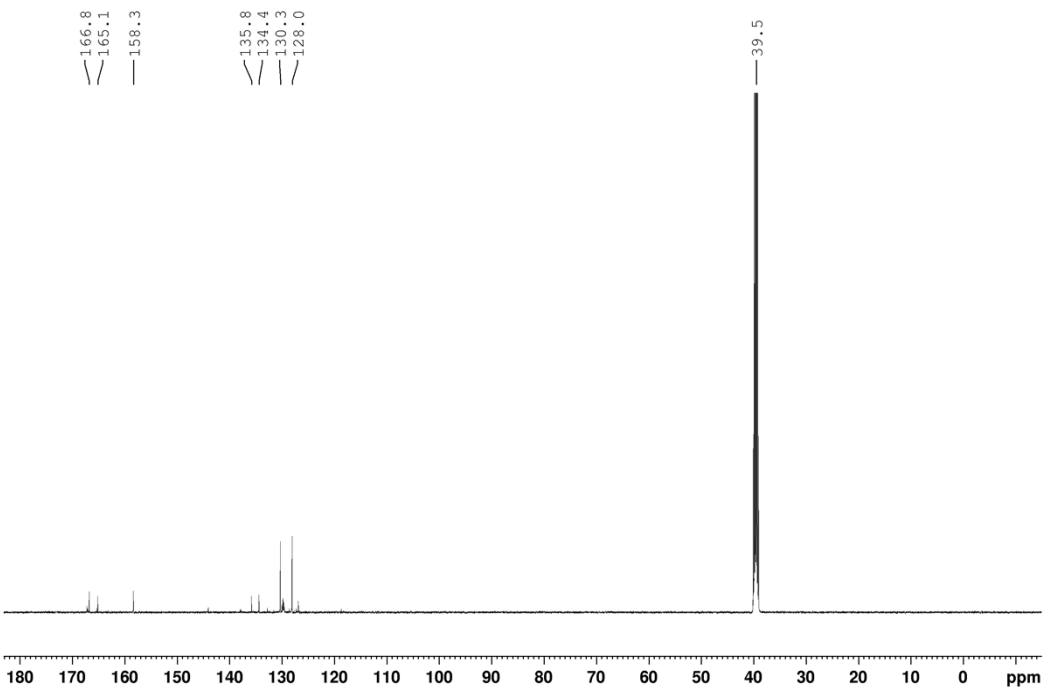


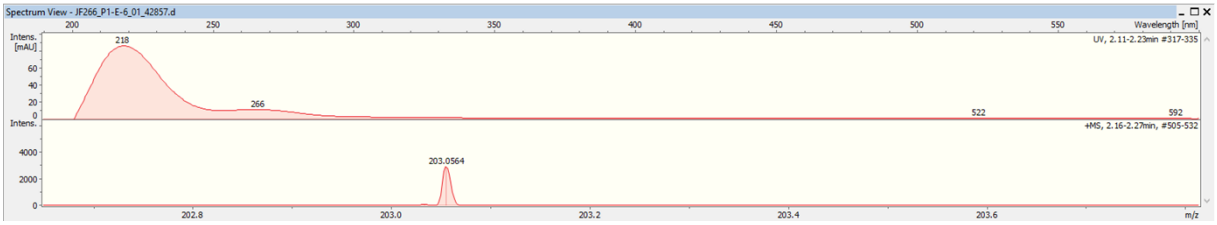

163.3
150.7
150.1
137.9
126.7
124.4
39.5
220
200
180
160
140
120
100
80
60
40
20
0
ppm


Spectrum View - JF351_P1-E-5_01_42856.d
Wavelength [nm]
Intens. [mAU]
218
296
530
UV, 2.12-2.31min #319-347
Intens. x10^5
237.0884
+MS, 2.17-2.36min, #508-552
m/z

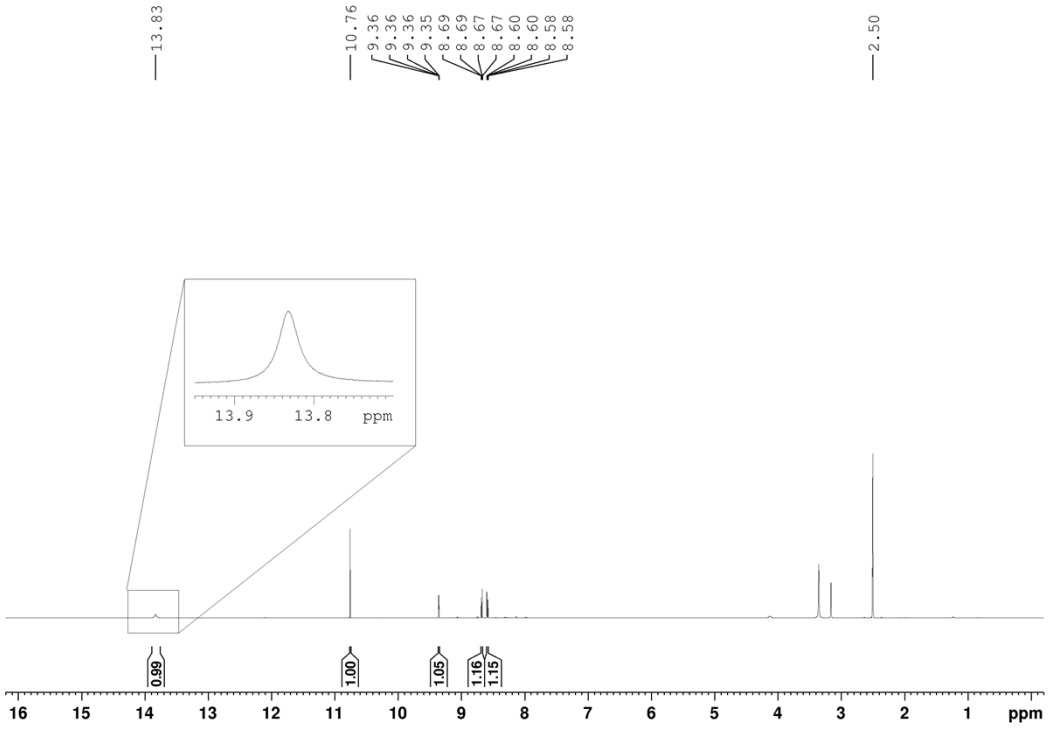
4-(1,2,4,5-tetrazin-3-yl)benzoic acid (Tz3)

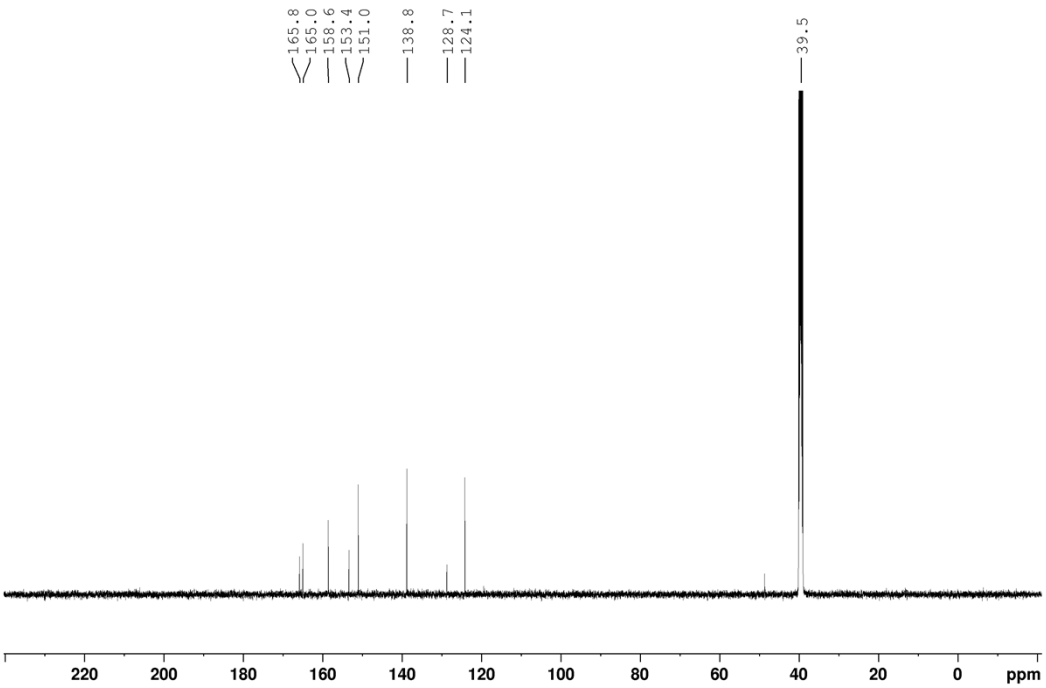


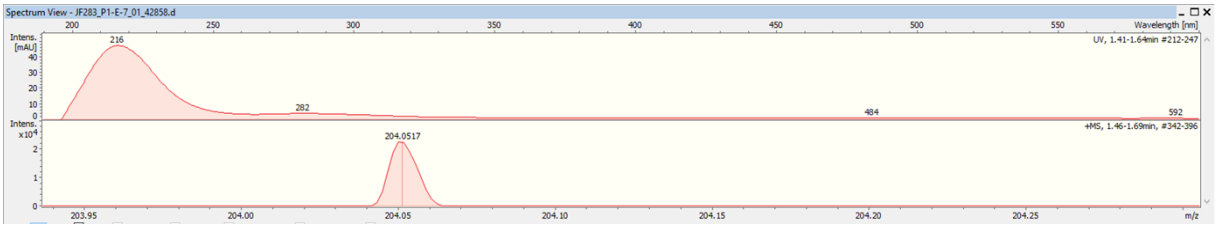




6-(1,2,4,5-tetrazin-3-yl)nicotinic acid (Tz5)

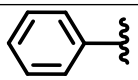
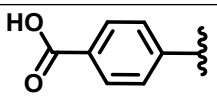
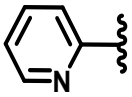
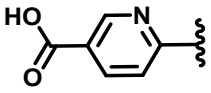
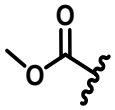



165.8
165.0
158.6
153.4
151.0
138.8
128.7
124.1
39.5
220
200
180
160
140
120
100
80
60
40
20
0
ppm


Spectrum View - JF283_P1-E-7_01_42858.d
Wavelength [nm]
UV, 1.41-1.64min #212-247
216
282
484
592
+MS, 1.46-1.69min, #342-396
204.0517
m/z

Hammett constants

Table S2: Hammett constant σ*p* (experimental[3] and predicted[4])

	Hammett constant *σp*
	0.02
	0.11
	0.16
	0.17
	0.29
	0.45

Acknowledgements

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Author Contributions

J.F. performed chemical synthesis, conducted the kinetic evaluation and wrote the manuscript. C.X. conducted chemical synthesis. M.B. conceptualized the study, coordinated the research and wrote the manuscript. P.R. performed chemical synthesis, analyzed release kinetics, and contributed to writing the manuscript H.L.S.F. designed and performed LC-MS/MS experiments and contributed to writing the manuscript. All authors analyzed the results, participated in the final revision of the manuscript, and gave approval for publication.

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1. A. Villa, K. Tremolet, B. Martinez, O. D. S. Cacao, B. Atgé, D. Ducint, K. Titier-Debeaupuis, C. Verdun-Esquer, M. Molimard and M. Canal-Raffin, *Journal of Chromatography B*, 2020, **1156**, 122305.

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3. C. Hansch, A. Leo and R. W. Taft, *Chemical Reviews*, 1991, **91**, 165-195.
4. https://bitly.com/getsigmas, https://bitly.com/getsigmas, (accessed last access 27.02.2023).