Biosynthesis of Brevinic Acid from Lawsone

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SUPPORTING INFORMATION

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Experimental Procedures

Bacterial Strains

E. coli BAP1 were cultivated at 37 °C in LB medium supplemented with a suitable selection antibiotic while shaking at 180 rpm, or on LB-Agar supplemented with selection antibiotic at 37 °C unless otherwise specified. DNA was kept in MilliQ water for short-term storage.

Analytical and preparative HPLC

Analytical high-performance liquid chromatography (HPLC) was performed on an Azura HPLC device manufactured by Knauer, consisting of the following components: AS 6.1L sampler, P 6.1L pump, DAD 2.1L detector. Components were separated on a Phenomenex Luna 3u C-18 column (150 × 4.6 mm) at a flowrate of 1 mL/min with the eluents water (A) and acetonitrile (B), both supplemented with 0.05% trifluoracetic acid. The elution method consisted of equilibration at 5% B for 2 min, followed by a gradient of 5–100% B over 28 min. Column washing was performed at 100% B for 5 min and the column was re-equilibrated at 5% B for 2 min before the next measurement.

Preparative HPLC was performed on a Jasco HPLC system consisting of an UV-1575 Intelligent UV/Vis detector, two PU-2068 Intelligent preparation pumps, a Mika 1000 dynamic mixing chamber (1,000 µl; Portmann Instruments AG Biel-Benken) and a LC-NetII/ADC anda Rheodyne injection valve. The system was controlled by Galaxie software. Chromatographic separation was performed on a Eurospher II 100-5 C18 A (250 × 16 mm) column with precolumn (30 × 16 mm) provided by Knauer at a flow-rate of 10 ml/min and the eluents were water (A) and acetonitrile (B). The gradient was adjusted depending on the polarity of the compounds. Collected product fractions were combined, the organic solvent was evaporated under reduced pressure at 40 °C and water was removed by lyophilization.

HR LC-MS measurement

For liquid chromatography (LC) coupled to high resolution mass spectrometry (HR-MS), a Bruker Elute UHPLC-system with a Intensity Solo 2 C18-column (100 × 2.1 mm) coupled to a Bruker Impact II ultra-high resolution Q-TOF mass spectrometer with electron-spray ionization (ESI) were used. For LC, water (A) and acetonitrile (B) were used as eluents, both supplemented with 0.1% formic acid, at a flowrate of 0.3 mL/min. The elution method consisted of equilibration at 5% B for 2 min, a gradient of 5–95% B over 23 min, washing at 95% B for 3 min and re-equilibration at 5% B for 2 min.

NMR-measurement

^{1}H and ^{13}C Nuclear Magnetic Resonance spectra (NMR) were recorded on Bruker AVANCE 300 and AVANCE 600 spectrometers at room temperature. The chemical shifts are given in δ-values (ppm) downfield from TMS and are referenced on the residual peak of the deuterated solvent ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.2 ppm; D_2O: δ_H = 4.80 ppm). The coupling constants *J* are given in Hertz [Hz].

Production and Isolation of Brevinic Acid (1)

E. coli BAP1 were chemically transformed with an expression vector as described previously[1] or plated from a cryostock in the case of plasmid-free *E. coli* BAP1. A single positive clone was selected on agar-plates and was used to inoculate a preculture. An expression culture of 1 L terrific broth in a 2 L Erlenmeyer flask was inoculated with 1% preculture (vol/vol) and was incubated while shaking at 180 r.p.m. at 37 °C until an OD_{600} of 0.8 was reached. The culture was cooled to 4 °C for 60 min and expression was induced by adding 0.5 µg/mL tetracycline. The cultures were incubated at 20 °C while shaking at 180 r.p.m in darkness.

After incubation, cultures were centrifuged (6,000 g for 15 min) to separate *E. coli* biomass from growth medium. The culture supernatants were adjusted to a pH of 3–4 by addition of conc. HCl and extracted with ethyl acetate (2 × 700 mL per 1 L of growth medium). The combined extracts were washed with saturated brine, dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure at 40 °C. The crude extract was purified on a silica column (dichloromethane/methanol = 30:1), which yielded a crude violet product. Final purification ensued on a preparative HPLC-system with a gradient of 25–45% B over 15 min, where **1** eluted between 8.5 and 9 min. Brevinic acid (**1**) was isolated as purple crystals (4.8 mg).

^{1}H NMR (600 MHz, CD_3OD): δ = 8.00 (*br*d, *J* = 7.6 Hz, 1H), 7.96 (*br*d, *J* = 7.6 Hz, 1H), 7.72 (tt, *J* = 7.5, 1.3 Hz, 1H), 7.67 (tt, *J* = 7.5, 1.3 Hz, 1H), 5.41 (dd, *J* = 11.5, 5.2 Hz, 1H), 3.69 (ddd, *J* = 14.8, 11.4, 5.8 Hz, 1H), 3.12 (ddd, *J* = 14.8, 6.8, 1.7 Hz, 1H), 2.49 (dddd, *J* = 13.3, 11.7, 6.8, 5.1 Hz, 1H), 2.24 (dddd, *J* = 13.3, 11.3, 5.7, 1.9 Hz, 1H) ppm; **^{13}C NMR** (151 MHz, CD_3OD): δ = 182.9, 180.0, 174.6, 147.5, 135.5, 134.2, 133.9, 131.7, 127.4, 126.9, 116.7, 55.9, 32.2, 31.5 ppm; **HRMS** (m/z): $[M]^+$ calcd. for $C_{14}H_{12}NO_4S^+$, 290.0482; found, 290.0483. The spectroscopic data was in agreement with those stated in literature.[2]

Synthesis of (3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)homocysteine (7)

Lawsone (**5**, 40.0 mg, 0.23 mmol, 1.0 eq.) and homocysteine (**2**, 31.1 mg, 0.23 mmol, 1 eq.) are dissolved in 8 mL water and stirred at room temperature. Pyridine (74.2 µL, 0.92 mmol, 4 eq.) is slowly added and the reaction is stirred for 3 h. The reaction mixture is lyophilized, and the product is purified on a preparative HPLC with a gradient of 20–45% B over 20 min, where **7** elutes between 8 and 9 min. Compound **7** is obtained as orange crystals (27.6 mg, 0.090 mmol, 39%).

^{1}H NMR (300 MHz, D_2O): δ = 7.82 (t, *J* = 7.9 Hz, 2H), 7.70 (t, *J* = 7.3 Hz, 1H), 7.65 (t, *J* = 7.1 Hz, 1H), 4.11 (t, *J* = 6.3 Hz, 1H), 3.01 (bs, 2H), 2.22–2.04 (m, 2H) ppm; **^{13}C NMR** (151 MHz, D_2O): δ = 184.0, 183.9, 172.3, 162.3, 135.1, 133.7, 132.3, 129.7, 126.6, 126.5, 126.5, 52.3, 30.1, 28.4 ppm; **HRMS** (m/z): $[M]^+$ calcd. for $C_{14}H_{14}NO_5S^+$, 308.0587; found, 308.0588.

Synthesis of 2-diethylphosphate-1,4-naphthoquinone (8)

In a round-bottom flask, **5** (40.0 mg, 0.23 mmol, 1.0 eq.) is dissolved in 6 mL dichloromethane and triethylamine (95.5 mL, 0.69 mmol, 3 eq.) is added under stirring. Diethylchlorophosphate (134 µL, 0.92 mmol, 4 eq.) is dissolved in 3 mL dichloromethane and slowly added to the reaction. The mixture

is stirred over night at room temperature and finally heated to 40 °C for 30 min, before the solvent is removed under reduced pressure. The product is purified on a preparative HPLC with a gradient of 35–55% B over 25 min, where **8** elutes at 14 min. Compound **8** is obtained as a red oil (15.7 mg, 0.050 mmol, 22%).

^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.14–8.07 (m, 2H), 7.79–7.74 (m, 2H), 6.89 (d, *J* = 1.5 Hz, 1H), 4.33 (dq, *J* = 7.1, 8.2 Hz, 4H), 1.42 (td, *J* = 1.0, 7.1 Hz, 6H) ppm; **^{13}C NMR** (151 MHz, $CDCl_3$): δ = 184.7, 179.0, 152.7, 134.5, 133.9, 131.8, 130.9, 126.8, 126.4, 122.1, 65.7, 16.0 ppm; **^{31}P NMR** (242 MHz, $CDCl_3$): δ = –7.66 ppm; **HRMS** (m/z): $[M]^+$ calcd. for $C_{14}H_{16}O_6P^+$, 311.0679; found, 311.0675.

Synthesis of 2-acetoxy-1,4-naphthoquinone (9)

In a round-bottom flask, **5** (40.0 mg, 0.23 mmol, 1.0 eq.) is dissolved in 5 mL acetic acid anhydride. After addition of 5 drops of concentrated sulfuric acid, the reaction is stirred at room temperature for 5 h. The reaction is stopped by addition of 100 mL aqueous $NaHCO_3$-solution (5%), followed by extraction with dichloromethane (3 × 40 mL) and washing with brine. The solvent is removed under reduced pressure and the crude product is purified on a silica column (cyclohexane:ethyl acetate 10:1 → 7:1). Compound **9** is obtained as yellow crystals (18.9 mg, 0.087 mmol, 38%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.14–8.07 (m, 2H), 7.80–7.75 (m, 2H), 6.76 (s, 1H), 2.39 (s, 3H) ppm; **HRMS** (m/z): $[M]^+$ calcd. for $C_{12}H_9O_4^+$, 217.1995; found, 217.1998. The spectroscopic data was in agreement with those stated in literature.[3]

Brevinic Acid (1) Synthesis Assays

Four substrates are tested for their performance in enzyme-free formation of **1** under physiological conditions. In four different reaction flasks, L-homocysteine (5 mg, 0.037 mmol, 1 eq.) is dissolved in 10 mL PBS (8.0 g NaCl, 0.20 g KCl, 1.42 g Na_2HPO_4, 0.27 g KH_2PO_4, MilliQ-Water to 1 L, pH adjusted to 7.4 with HCl). Compounds **3** (5 mg, 0.037 mmol, 1 eq.), **5** (negative control, 6 mg, 0.037 mmol, 1 eq.), **8** (10.8 mg, 0.037 mmol, 1 eq.) and **9** (7.5 mg, 0.037 mmol, 1 eq.) are separately dissolved in 200 µL DMSO each and individually introduced into the four flasks containing L-homocysteine. The assays are incubated at 37 °C at 200 r.p.m. and samples for HPLC-measurement are taken at 0, 30, 60, 120 and 600 min timepoints. To prevent continued reaction, the samples were quenched in 0.01 M HCl and stored at –80 °C prior to measurement.

NMR-Data

Brevinic Acid (1)

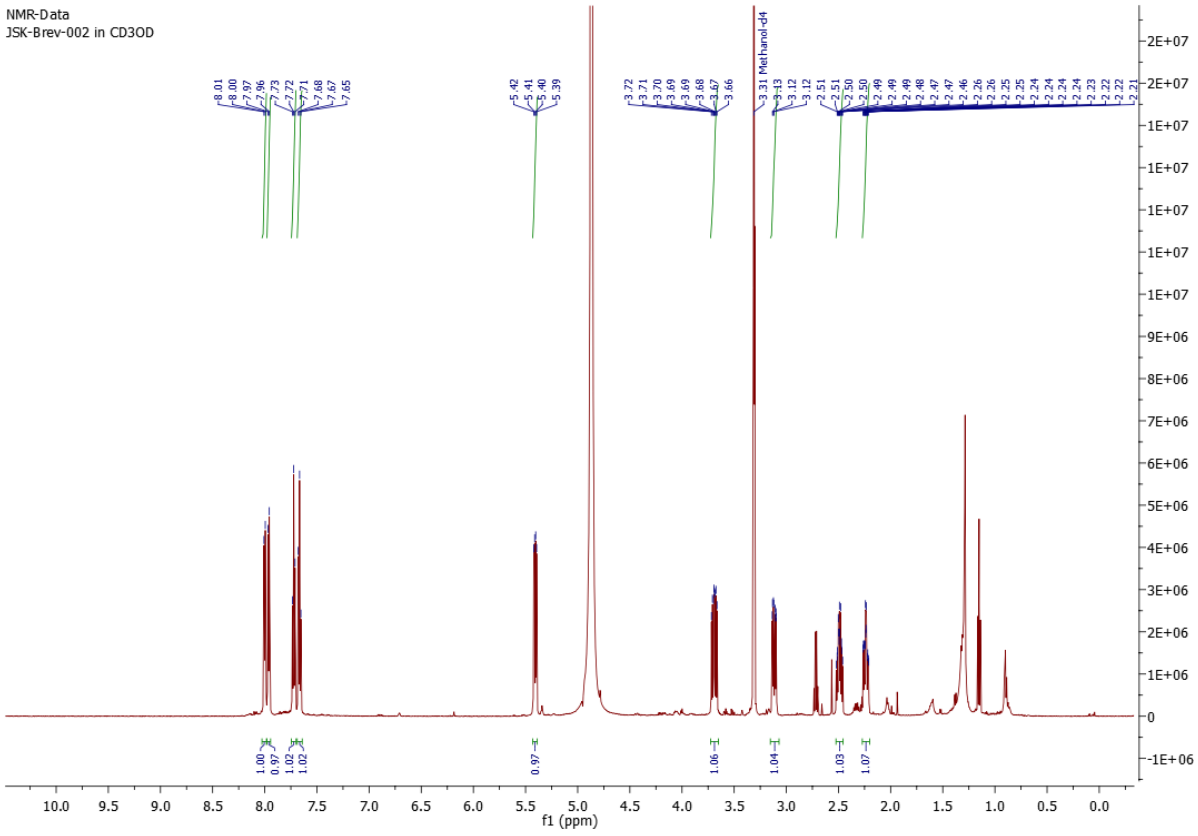

Figure S1. ^{1}H-NMR spectrum of **1** in CD_3OD.

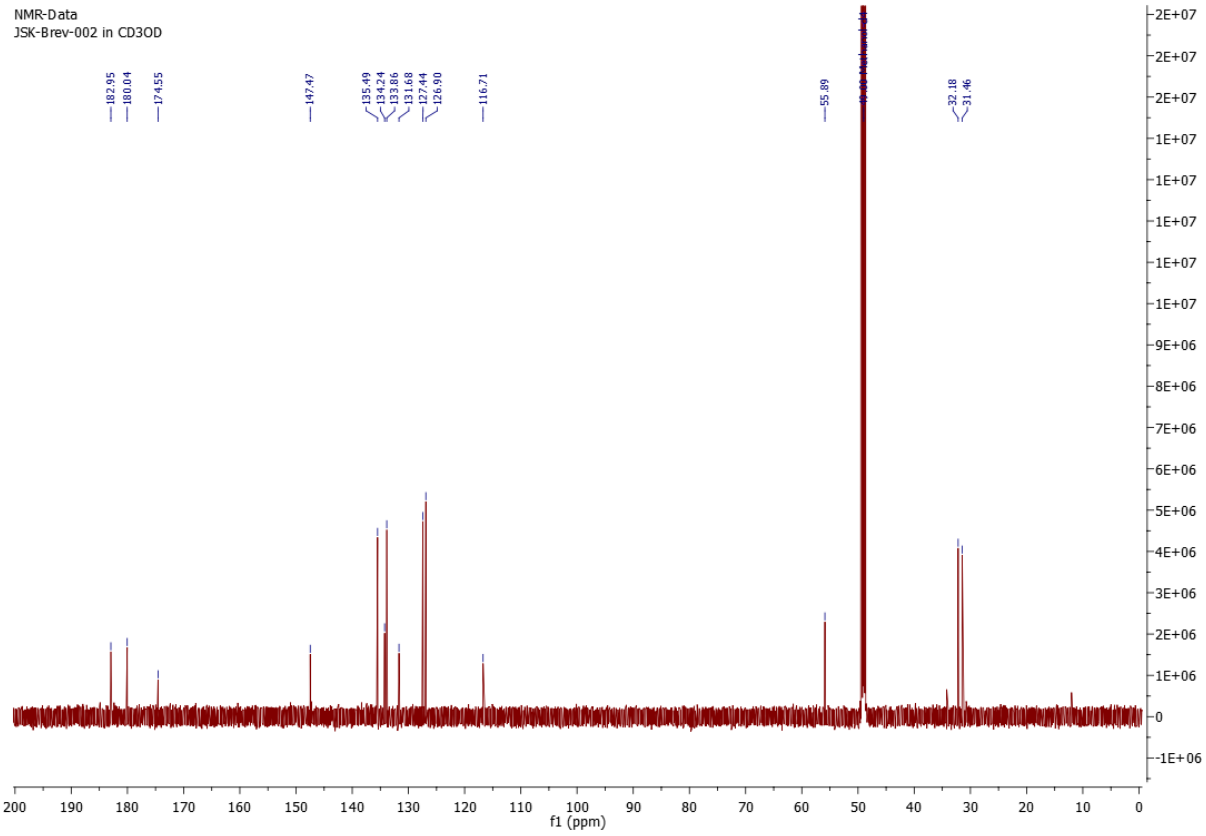

Figure S2. ^{13}C-NMR spectrum of **1** in CD_3OD.

(3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)homocysteine (7)

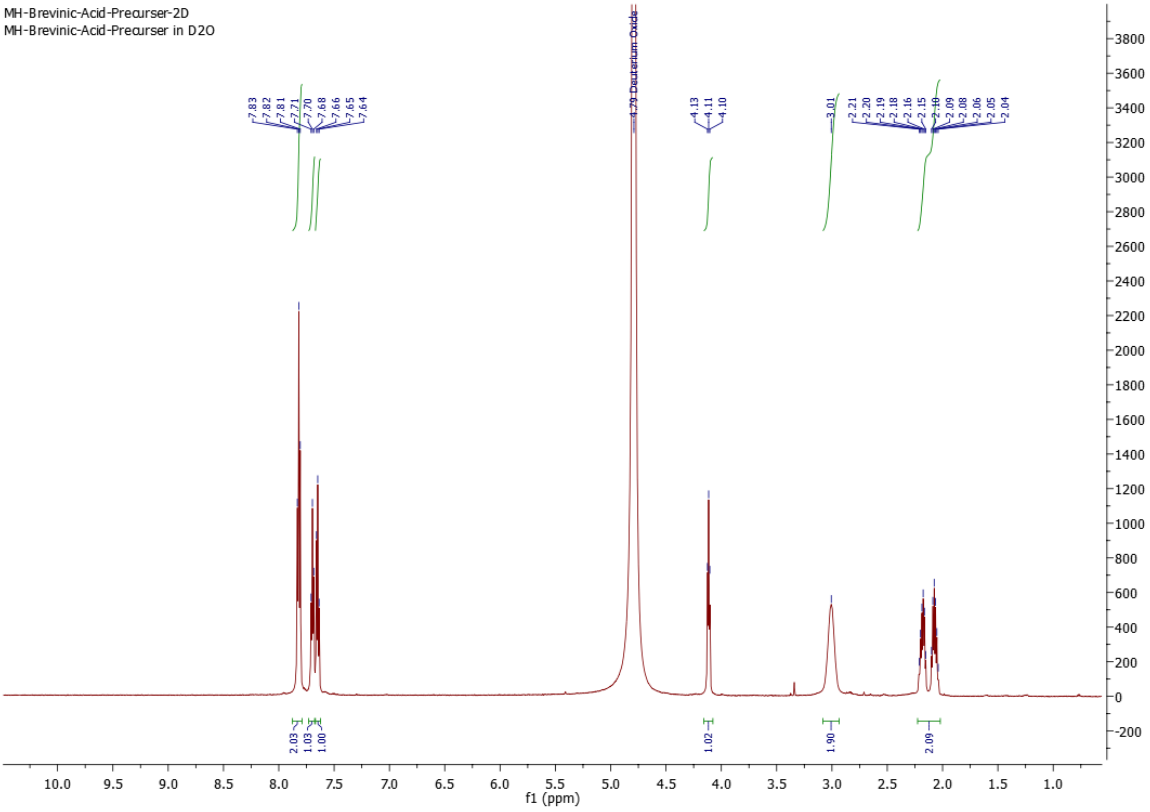

Figure S3. ^{1}H-NMR spectrum of **7** in D_2O.

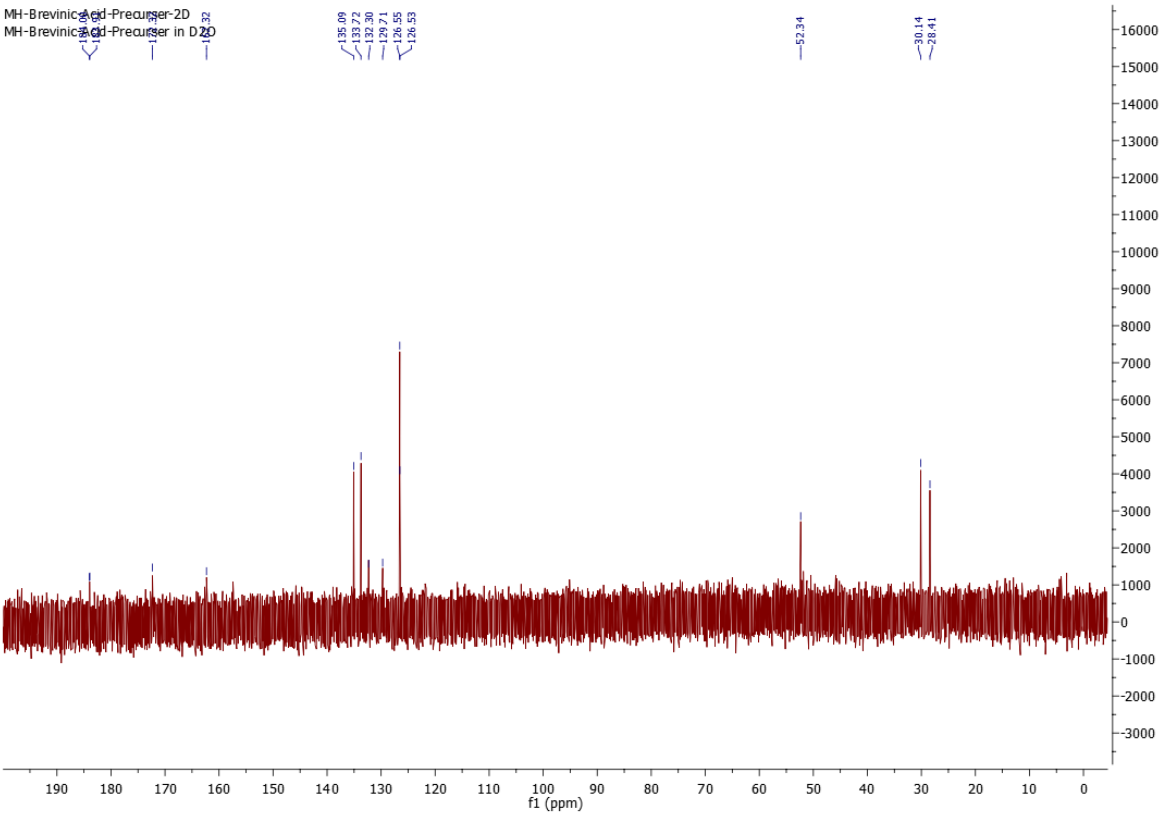

Figure S4. ^{13}C-NMR spectrum of **7** in D_2O.

2-diethylphosphate-1,4-naphthoquinone (8)

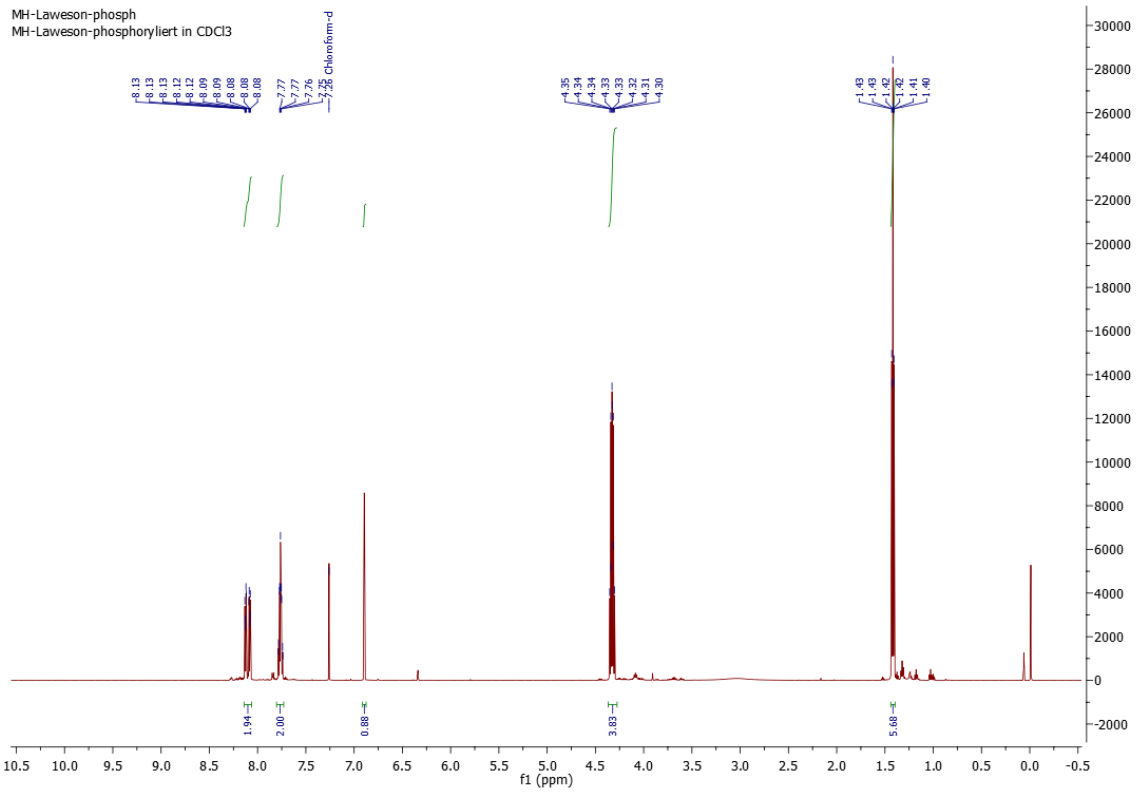

Figure S5. ^{1}H-NMR spectrum of **8** in $CDCl_3$.

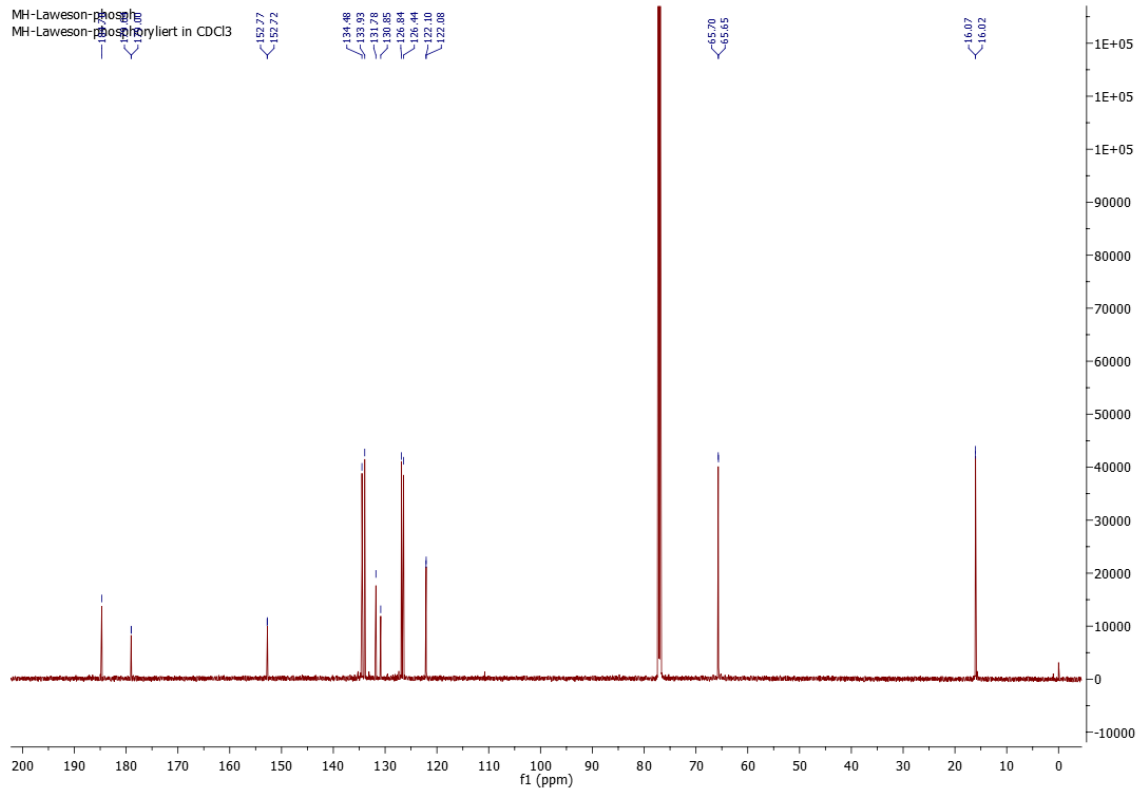

Figure S6. ^{13}C-NMR spectrum of **8** in $CDCl_3$.

2-acetoxy-1,4-naphthoquinone (9)

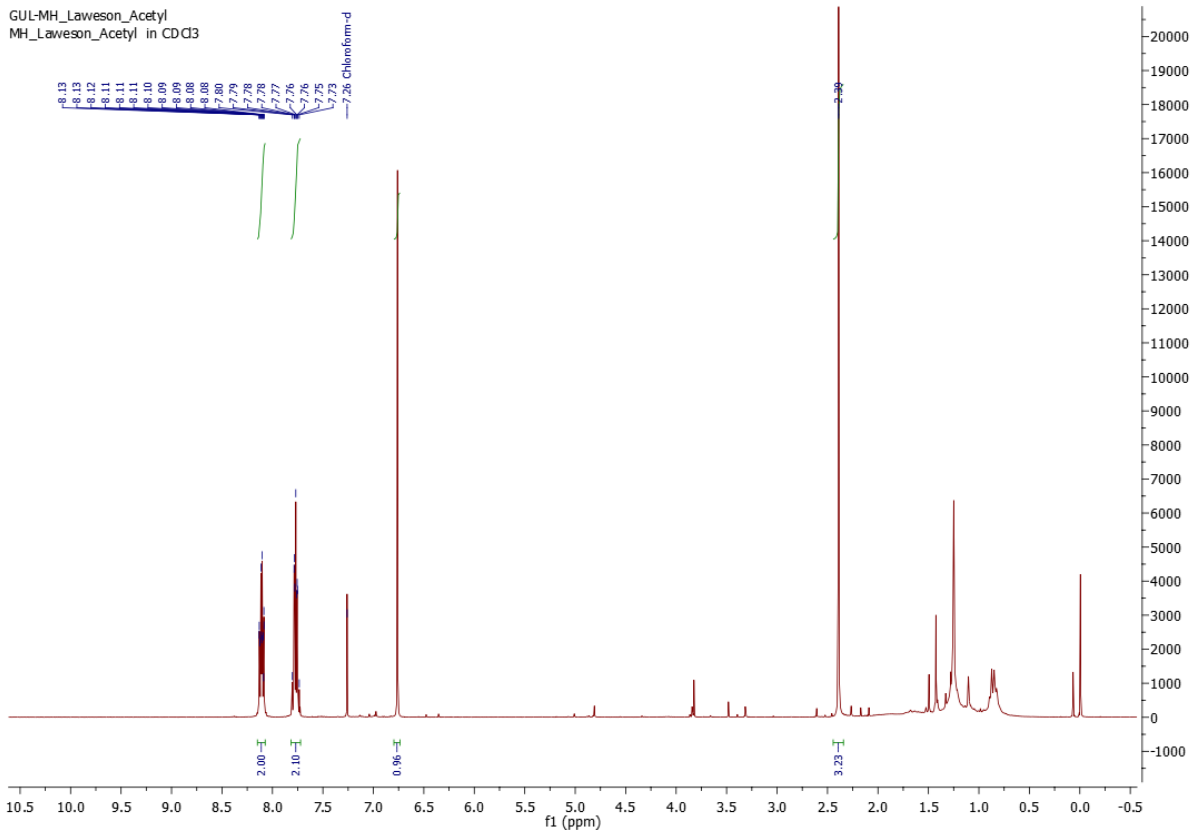

Figure S7. ^{1}H-NMR spectrum of **9** in $CDCl_3$.

Supplementary References

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3 R. B. Smith, C. Canton, N. S. Lawrence, C. Livingstone and J. Davis, Molecular anchors—mimicking metabolic processes in thiol analysis, *New J. Chem.*, 2006, **30**, 1718–1724.