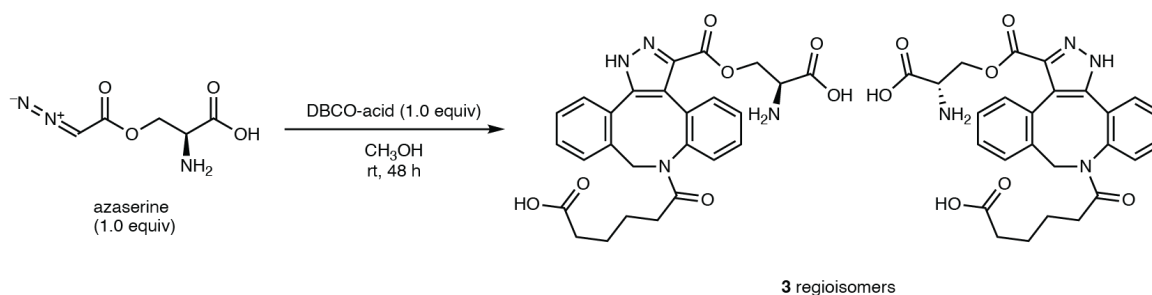

Supplementary information

**Chemical capture of diazo metabolites
reveals biosynthetic hydrazone oxidation**

In the format provided by the
authors and unedited

Supporting Information

Synthesis of (S)-6-(3-((2-amino-2-carboxyethoxy)carbonyl)-1,9-dihydro-8H-dibenzo[*b,f*]pyrazolo[4,3-*d*]azocin-8-yl)-6-oxohexanoic acid & (S)-6-(3-((2-amino-2-carboxyethoxy)carbonyl)-1,8-dihydro-9H-dibenzo[*b,f*]pyrazolo[3,4-*d*]azocin-9-yl)-6-oxohexanoic acid (3 regioisomers)



A suspension of azaserine (5.85 mg, 33.8 μ mol, 1.0 equiv.) and DBCO-acid (11.3 mg, 33.8 μ mol, 1.0 equiv.) in MeOH (1.35 mL) was stirred for 48 h, upon which the suspension dissolved completely to yield a colorless solution. The reaction mixture was directly purified by preparatory-HPLC (5 – 50% ACN in water, with 0.1% formic acid additive throughout, over 30 min) to yield an inseparable mixture of regioisomers of **1** (5.0 mg, 9.87 μ mol, 29%) as a fluffy white powder after lyophilization.

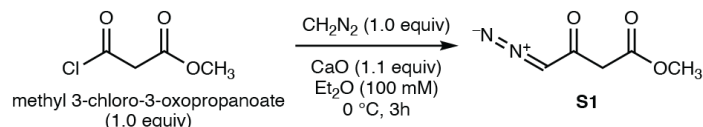
¹H NMR (Supplementary Figure 26): (400 MHz, CD₃OD) δ 7.73 – 6.95 (m, 8H), 5.98 (d, *J* = 16.7 Hz, 0.6H), 5.66 (dd, *J* = 15.4, 9.9 Hz, 0.4H), 4.79 – 4.63 (m, 1.6H), 4.58 – 4.38 (m, 1.4H), 4.08 – 3.89 (m, 1H), 2.23 – 2.04 (m, 2H), 1.95 – 1.72 (m, 1.6H), 1.65 (dd, *J* = 15.8, 7.4 Hz, 0.4H), 1.45 – 1.25 (m, 4H).

¹³C NMR (Supplementary Figure 27): (101 MHz, CD₃OD) δ 177.84, 177.55, 175.12, 174.63, 170.81, 170.53, 141.71, 141.23, 141.19, 136.15, 134.81, 134.51, 134.41, 133.24, 133.22, 132.95, 132.77, 132.05, 130.91, 130.88, 130.66, 130.56, 130.52, 130.38, 129.84, 129.62, 129.58, 129.06,

128.51, 128.45, 128.41, 128.24, 128.16, 65.03, 64.78, 55.30, 55.19, 53.40, 52.87, 34.87, 34.85,
34.74, 34.70, 34.66, 25.59, 25.54, 25.51, 25.47, 25.40.

HRMS (ESI+, m/z): $[M+H]^+$ calculated for $C_{26}H_{27}N_4O_7^+$, 507.1874; found 507.1876

Synthesis of methyl 4-diazo-3-oxobutanoate (**S1**)



A freshly prepared and titrated solution of diazomethane in diethyl ether (0.25 M, 4.0 mL, 1.0 mmol, 1.0 equiv)⁷¹ was added to a suspension of calcium oxide (61.7 mg, 1.0 mmol, 1.1 equiv) in anhydrous diethyl ether (5.0 mL) at 0 °C.⁷² The suspension was stirred at 0 °C for 5 min, and methyl 3-chloro-3-oxopropanoate (0.107 mL, 1.0 mmol, 1.0 equiv) in anhydrous diethyl ether (0.5 mL) was added dropwise. The reaction mixture was stirred at 0 °C for 3 h, allowed to warm to room temperature, filtered, and concentrated under reduced pressure (ambient water bath) to yield a crude light-yellow oil. Purification by flash-column chromatography (30 – 50% ethyl acetate in hexanes) afforded methyl 4-diazo-3-oxobutanoate (85.0 mg, 0.598 mmol, 60% yield) as a light-yellow oil.

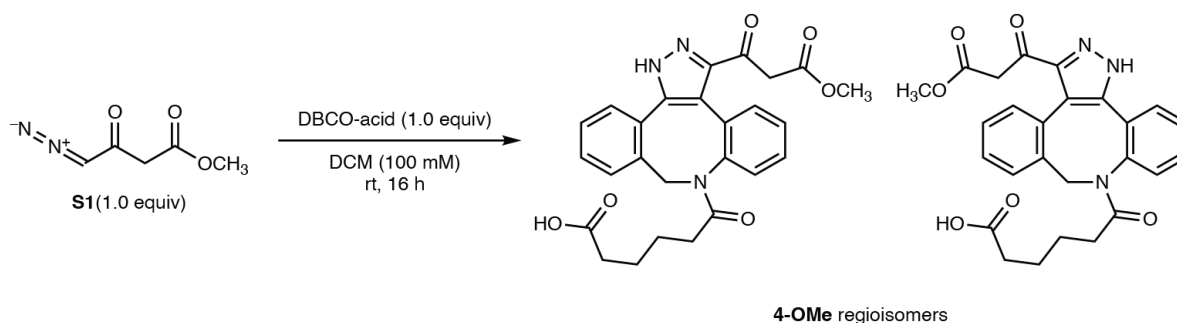
¹H NMR (Supplementary Figure 28): (400 MHz, $CDCl_3$) δ 5.53 (s, 1H, $N^-=N^+-CH-CO$), 3.74 (s, 3H, $-OCH_3$), 3.36 (s, 2H, $CO-CH_2-CO$).

¹³C NMR (Supplementary Figure 29): (101 MHz, $CDCl_3$) δ 186.07 ($N^-=N^+-CH-CO$), 167.85 ($-COOCH_3$), 56.12 ($N^-=N^+-CH-CO$), 52.68 ($-OCH_3$), 46.87 ($CO-CH_2-CO$).

FTIR (neat), cm^{-1} : 3102 (m), 2956 (w), 2107 (s), 1739 (s), 1636 (s).

HRMS (ESI+, m/z): $[M+H-N_2]^+$ calculated for $C_5H_7O_3^+$, 115.0390; found 115.0387.

Synthesis of 6-(3-(3-methoxy-3-oxopropanoyl)-1,9-dihydro-8*H*-dibenzo[*b,f*]pyrazolo[4,3-*d*]azocin-8-yl)-6-oxohexanoic acid & 6-(3-(3-methoxy-3-oxopropanoyl)-1,8-dihydro-9*H*-dibenzo[*b,f*]pyrazolo[3,4-*d*]azocin-9-yl)-6-oxohexanoic acid (4-OMe regioisomers)



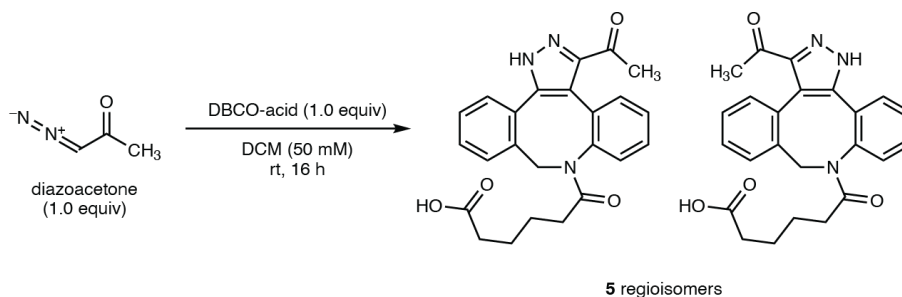
DBCO-C₆-Acid (14.00 mg, 42.0 μ mol, 1.0 equiv) was dissolved in DCM (420 μ L) and **S1** (5.97 mg, 42.0 μ mol, 1.0 equiv) dissolved in DCM (420 μ L) was added. The colorless solution was stirred for 16 h, concentrated under reduced pressure, and purified by preparatory-HPLC (5 – 50% ACN in water, with 0.1% formic acid additive throughout, over 30 min) to yield an inseparable mixture of regioisomeric **4-OMe** in approximately a 2:1 ratio (16.7 mg, 35.1 μ mol, 84%) as a fluffy white powder after lyophilization.

¹H NMR (Supplementary Figure 30): (400 MHz, CDCl₃) δ 7.53 – 7.47 (m, 1H), 7.47 – 7.32 (m, 4H), 7.30 – 7.15 (m, 5H), 7.13 – 7.08 (m, 1H), 6.94 – 6.88 (m, 1H), 5.82 (d, *J* = 17.0 Hz, 1H), 5.62 (d, *J* = 15.3 Hz, 0.5H), 4.44 (d, *J* = 15.4 Hz, 0.5H), 4.39 (d, *J* = 16.3 Hz, 1H), 4.19 (d, *J* = 17.1 Hz, 1H), 4.00 (d, *J* = 16.2 Hz, 1H), 3.84 (d, *J* = 9.5 Hz, 0.5H), 3.77 (d, *J* = 8.4 Hz, 0.5H), 3.74 (s, 3H), 3.70 (s, 1.5H), 2.31 – 2.11 (m, 3H), 1.93 – 1.79 (m, 3H), 1.76 – 1.69 1.72 (m, 1H), 1.58 – 1.20 (m, 7H).

¹³C NMR (Supplementary Figure 31): (101 MHz, CDCl₃) δ 189.50, 185.77, 178.19, 177.24, 174.49, 173.03, 169.21, 167.78, 147.31, 146.04, 142.42, 141.18, 140.57, 139.65, 134.56, 134.33, 132.86, 132.20, 132.16, 132.08, 131.45, 131.10, 130.82, 130.65, 129.83, 129.62, 129.16, 129.11, 128.92, 128.63, 127.71, 126.91, 126.55, 126.13, 123.44, 117.25, 52.92, 52.64, 52.46, 52.18, 46.55, 45.97, 33.88, 33.49, 33.43, 24.36, 24.27, 24.23, 23.90.

HRMS (ESI⁺, *m/z*): [M + H]⁺ calculated for C₂₆H₂₆N₃O₆⁺, 476.1816; found 476.1824.

Synthesis of 6-(3-acetyl-1,9-dihydro-8*H*-dibenzo[*b,f*]pyrazolo[4,3-*d*]azocin-8-yl)-6-oxohexanoic acid (5 regioisomers)



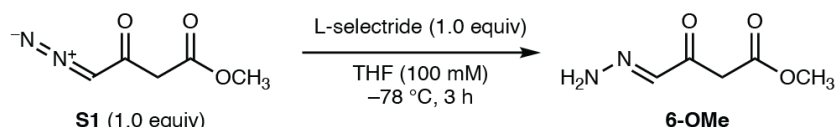
39.7 mg of DBCO-acid (0.12 mmol, 1.0 equiv) were added to 100 mg of diazoacetone (0.12 mmol, 1 equiv) (purchased from Enamine) in 2.4 mL of dichloromethane (DCM) in a capped vial. The reaction mixture was stirred overnight, and 0.5 mL of DCM were added the next morning to replace evaporated solvent. The mixture was concentrated under vacuum and purified using reversed phase chromatography on a Biotage Selekt using a 6 g C18 column with a 2% to 95% gradient of ACN in H₂O. Fractions were concentrated under reduced pressure and then lyophilized. The product was re-purified using a Dionex UltiMate HPG-3200Bx Semi-Preparative HPLC using a HypersilGold column with a 5% to 95% ACN (+ 0.1% formic acid) in H₂O (+ 0.1% formic acid) gradient. The fractions were concentrated under reduced pressure and lyophilized. NMR spectra were obtained using a Bruker AVANCE NEO 400B spectrometer (400 MHz, 100 MHz) and correspond to a 1:1 mixture of both pyrazole regioisomers. HRMS data were collected using a Thermo Orbitrap IQ-X Tribrid mass spectrometer coupled to a Horizon Vanquish UHPLC as described above.

¹H NMR (Supplementary Figure 32): (400 MHz, CDCl₃) δ 7.50 – 7.32 (m, 7H), 7.30 – 7.27 (m, 2H), 7.25 – 7.17 (m, 11H), 7.07 – 6.99 (m, 2H), 5.92 (d, *J* = 16.9 Hz, 1H), 5.77 (d, *J* = 15.5 Hz, 1H), 4.42 (d, *J* = 15.5 Hz, 1H), 4.26 (d, *J* = 17.0 Hz, 1H), 2.64 (s, 3H), 2.31 (s, 3H), 2.26 – 2.12 (m, 3H), 1.84 (m, 3H), 1.71 (m 1H), 1.53 – 1.22 (m, 4H).

¹³C NMR (Supplementary Figure 33): (101 MHz, CDCl₃) δ 194.50, 190.58, 178.09, 177.44, 174.14, 173.27, 148.89, 145.76, 143.20, 140.45, 140.07, 139.78, 134.78, 134.49, 133.34, 132.52, 132.11, 131.99, 131.06, 130.76, 130.49, 129.84, 129.57, 129.27, 129.20, 128.63, 128.57, 128.46, 127.59, 127.11, 126.78, 126.72, 123.35, 117.42, 52.70, 52.26, 33.88, 33.66, 33.52, 28.63, 27.76, 24.27, 24.20, 23.93.

HRMS (ESI⁺, *m/z*): [M + H]⁺ calculated for C₂₄H₂₄N₃O₄⁺, 418.1761; found 418.1760.

Synthesis of methyl (*E*)-4-hydrazono-3-oxobutanoate (**6-OMe**)



S1 (15.0 mg, 106 μmol, 1.0 equiv) was dissolved in tetrahydrofuran (THF) (1.06 mL) and cooled to -78 °C. L-selectride in THF (1.0 M, 106 μL, 1.0 equiv) was added dropwise and the resultant canary-yellow solution was stirred at the same temperature for 3 h.⁷³ The reaction mixture was quenched at -78 °C with MeOH (42.7 μL, 10 equiv), warmed to room temperature, and concentrated under reduced pressure. Purification by flash-column chromatography (0 – 5% MeOH in DCM) afforded methyl (*E*)-4-hydrazineylidene-3-oxobutanoate (**6-OMe**) (5.0 mg, 34.7 μmol, 33% yield) as a light-yellow film.

¹H NMR (Supplementary Figure 34): (400 MHz, CD₂Cl₂) δ 7.09 (s, 1H, H₂N-N=CH-CO), 6.49 (s, 2H, H₂N-N=), 3.72 (s, 2H, CO-CH₂-CO), 3.68 (s, 3H, -OCH₃).

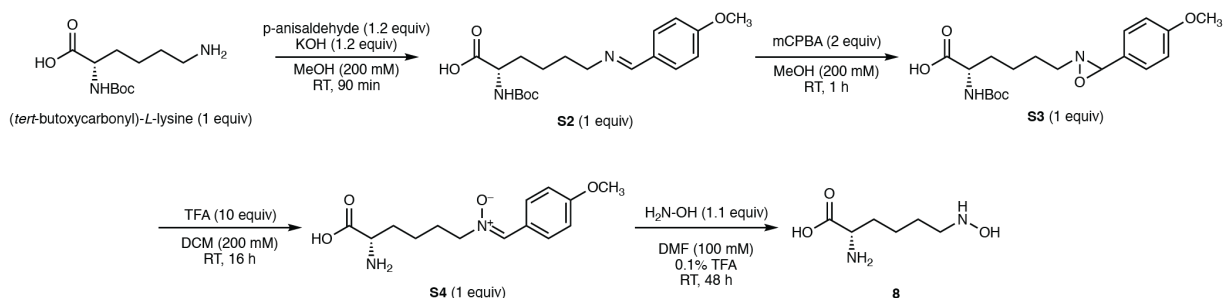
¹³C NMR (Supplementary Figure 35): (101 MHz, CD₂Cl₂) δ 192.14 (H₂N-N=CH-CO), 169.02 (-COOCH₃), 137.67 (H₂N-N=CH-CO), 52.57 (-OCH₃), 43.99 (CO-CH₂-CO).

FTIR (neat), cm⁻¹: 3430 (m), 3308 (m), 3225 (m), 2954 (m), 1739 (s), 1664 (s), 1596 (s).

HRMS (ESI⁺, *m/z*): [M+H]⁺ calculated for C₅H₉N₂O₃⁺, 145.0608; found 145.0609.

NB: When dissolved in CDCl₃, the (*E*)-hydrazone was observed to isomerize to the (*Z*)-hydrazone.

Synthesis of *N*-6-hydroxylysine (**8**)

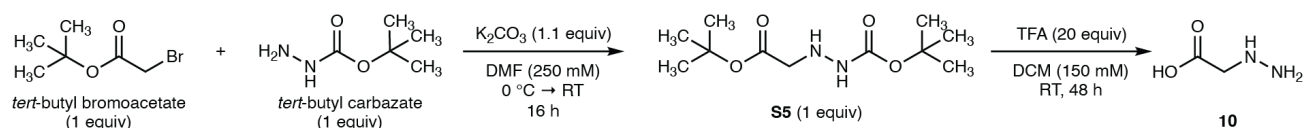


8 was synthesized according to a previously reported procedure.²⁰ NMR and HRMS data matched the previously reported values.

¹H NMR (Supplementary Figure 36): (400 MHz, D₂O) δ 3.73 (t, J = 6.1 Hz, 1H), 3.33 – 3.24 (m, 2H), 1.93-1.82 (m, 2H), 1.79-1.72 (m, 2H), 1.58 – 1.36 (m, 2H).

¹³C NMR (Supplementary Figure 37): (101 MHz, D₂O) δ 172.03, 50.06.

Synthesis of hydrazinoacetic acid (**10**)

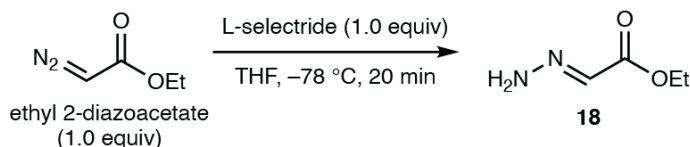


10 was synthesized according to a previously reported procedure.²⁰ NMR and HRMS data matched previously reported values.

¹H NMR (Supplementary Figure 38): (400 MHz, D₂O) δ 3.82 (s, 2H).

¹³C NMR (Supplementary Figure 39): (101 MHz, D₂O) δ 172.03, 50.06.

Synthesis of Ethyl (*E*)-2-hydrazineylideneacetate (**18**)



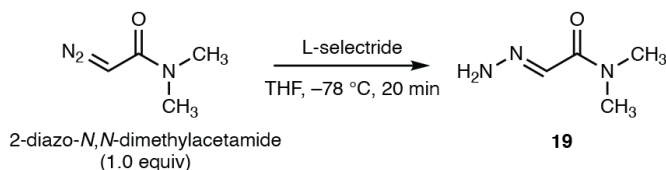
To a yellow solution of ethyl 2-diazoacetate (87 Wt% in DCM, 100 μ L, 827 μ mol, 1.0 equiv.) dissolved in THF (4.14 mL) at -78 $^{\circ}$ C was added 1.0 M L-selectride in THF (827 μ L, 827 μ mol, 1.0 equiv.) dropwise. The reaction was stirred at the same temperature for 20 min and quenched at -78 $^{\circ}$ C by the addition of a minimal amount of ethanol. The solution was allowed to warm up to room temperature and concentrated to yield a crude blood-orange oil. The crude oil was purified by flash column chromatography (50% ethyl acetate in hexanes) to yield **18** (48.0 mg, 413 μ mol, 50%) as a colorless film.

1 H NMR (Supplementary Figure 40): (400 MHz, CD_2Cl_2) δ 7.02 (s, 1H, $\text{H}_2\text{N}-\text{N}=\text{CH}-\text{CO}$), 6.38 (s, 2H, $\text{H}_2\text{N}-\text{N}=\text{CH}-\text{CO}$), 4.21 (q, J = 7.1 Hz, 2H, $-\text{OCH}_2\text{CH}_3$), 1.29 (t, J = 7.2 Hz, 3H, $-\text{OCH}_2\text{CH}_3$).

^{13}C NMR (Supplementary Figure 41): (101 MHz, CD_2Cl_2) δ 164.46 ($\text{H}_2\text{N}-\text{N}=\text{CH}-\text{CO}$), 130.02 ($\text{H}_2\text{N}-\text{N}=\text{CH}-\text{CO}$), 61.12 ($-\text{OCH}_2\text{CH}_3$), 14.60 ($-\text{OCH}_2\text{CH}_3$).

HRMS (ESI+, m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_4\text{H}_9\text{N}_2\text{O}_2^+$, 117.0659; found 117.0657.

139 **Synthesis of (*E*)-2-hydrazineylidene-*N,N*-dimethylacetamide (**19**)**



141 To a yellow solution of 2-diazo-*N,N*-dimethylacetamide⁷⁴ (68 mg, 601 μmol, 1 equiv.) dissolved in
 142 THF (3.0 mL) at –78 °C was added 1.0 M L-selectride in THF (601 μL, 601 μmol, 1.0 equiv.)
 143 dropwise. The reaction was stirred at the same temperature for 20 min and quenched at –78 °C
 144 by the addition of a minimal amount of ethanol. The solution was allowed to warm up to room
 145 temperature and concentrated to yield a crude orange oil. The crude oil was purified by flash
 146 column chromatography (0 – 5% MeOH in DCM) to yield **19** (38.2 mg, 332 μmol, 55%) as a white
 147 film.

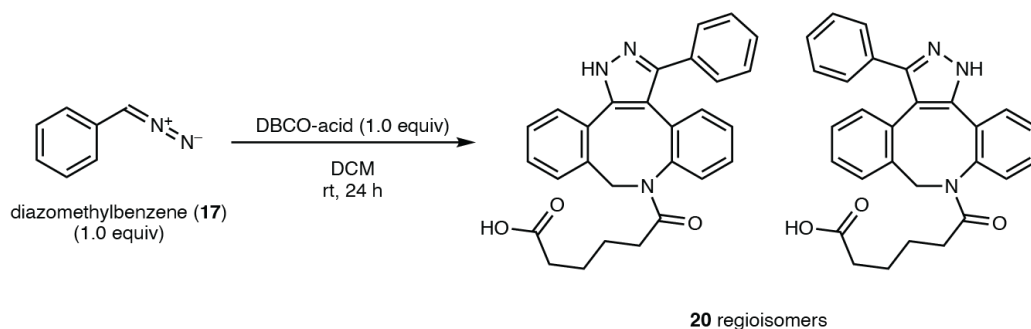
148 **¹H NMR (Supplementary Figure 42):** (400 MHz, CD₂Cl₂) δ 7.33 (s, 1H, H₂N–N=CH–CO), 5.94
 149 (s, 2H, H₂N–N=), 3.14 (s, 3H, –N(CH₃)₂), 2.95 (s, 3H, –N(CH₃)₂).

150 **¹³C NMR (Supplementary Figure 43):** (101 MHz, CD₂Cl₂) δ 164.56 (H₂N–N=CH–CO), 164.37*
 151 (H₂N–N=CH–CO), 134.46 (H₂N–N=CH–CO), 120.74* (H₂N–N=CH–CO), 38.11 (–N(CH₃)₂),
 152 37.54* (–N(CH₃)₂), 36.20 (–N(CH₃)₂), 35.08* (–N(CH₃)₂).

153 NB: The (*E*)-hydrazone was observed to isomerize (~50% conversion) to the (*Z*)-hydrazone over
 154 the timespan of the ¹³C NMR experiment in CD₂Cl₂. Therefore, eight ¹³C peaks, rather than the
 155 expected four, were observed. Peaks corresponding to the (*Z*)-hydrazone are labeled with an
 156 asterisk.

157 **HRMS (ESI+, *m/z*):** [M+H]⁺ calculated for C₄H₁₀N₃O⁺, 116.0818; found 116.0818.

Synthesis of 6-oxo-6-(3-phenyl-1,9-dihydro-8*H*-dibenzo[*b,f*]pyrazolo[4,3-*d*]azocin-8-yl)hexanoic acid & 6-oxo-6-(3-phenyl-1,8-dihydro-9*H*-dibenzo[*b,f*]pyrazolo[3,4-*d*]azocin-9-yl)hexanoic acid (20 regioisomers)



A peach-colored solution of diazomethylbenzene (19.7 mg, 167 μmol , 1.33 equiv.) in DCM-diethyl ether-pentane⁷⁵ (1:9:10 ratio, 3.34 mL) was added to DBCO-acid (41.6 mg, 125 μmol , 1.0 equiv.) in DCM (333 μL) and stirred for 24 h. The reaction mixture was concentrated and purified by flash column chromatography (0 – 5% MeOH in DCM) to yield an inseparable mixture of regioisomeric **20** (12.0 mg, 26.6 μmol , 16%) as a fluffy white powder after lyophilization.

¹H NMR (Supplementary Figure 44): (400 MHz, CDCl_3) δ 7.58 – 7.27 (m, 8.6H), 7.25 – 7.07 (m, 3.4H), 7.00 (dtd, J = 15.1, 7.5, 1.4 Hz, 0.6H), 6.79 (dd, J = 7.7, 1.4 Hz, 0.4H), 6.00 (d, J = 16.3 Hz, 0.6H), 5.93 (d, J = 15.6 Hz, 0.4H), 4.51 – 4.35 (m, 1H), 2.17 – 1.96 (m, 2.6H), 1.91 – 1.78 (m, 1H), 1.54 – 1.17 (m, 4.4H).

¹³C NMR (Supplementary Figure 45): (101 MHz, CDCl_3) δ 177.69, 177.18, 173.12, 172.80, 147.85, 147.06, 143.18, 142.73, 140.94, 140.71, 134.75, 134.65, 133.51, 133.14, 132.86, 131.95, 131.90, 131.50, 130.44, 130.25, 130.12, 129.64, 129.42, 129.38, 129.33, 129.19, 128.94, 128.89, 128.81, 128.76, 128.72, 128.50, 128.40, 127.90, 127.50, 127.47, 127.11, 118.21, 115.16, 53.03, 52.88, 34.14, 33.94, 33.92, 33.75, 24.51, 24.40, 24.26.

HRMS (ESI+, m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{28}\text{H}_{26}\text{N}_3\text{O}_3^+$, 452.1969; found 452.1974.

Supplementary Table 1: Primers used in this study.

Primers	Sequence (5'-3')	Description
DobA_ninae_NcoI_HindIII_F	ctttaagaaggagatatacATGACCGCGGT CCTGC	HindIII
DobA_ninae_NcoI_HindIII_R	ggtgctcgagtgcggccgcaCGTTGCTCCT GGGACAGC	NcoI
DobB_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGGGATGGG CTGAGCTGG	NdeI
DobB_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCATTCGACGG CGCCC	HindIII
DobC_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGGATTCGG TGATCAACC	NdeI
DobC_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCAGGCATGTGT TCTTCC	HindIII
DobE_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGATCATCAA TCGTTTCGACAC	NdeI
DobE_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCAAGCGCTCC CCAC	HindIII
DobF_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGACGAACA	NdeI

	ACACCGTCGC	
DobF_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCACGCGGGAC TCATCTC	HindIII
DobG_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGAGCCGGA GCTCGC	NdeI
DobG_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCATCGCTCTAC GCTCCGG	HindIII
DobM_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGAGCTTCG ATGCGGGC	NdeI
DobM_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCATTCGCCCCG CCCC	HindIII
DobQ_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGCCTGATCT GGAAAAGGCC	NdeI
DobQ_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaTCAGACCGCAC TTTCGGAG	HindIII
Dob2_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGAGTGCGT TCCTGTCTG	NdeI
Dob2_ninae_NdeI_HindIII_R	tgctcgagtgcggccgcaCTATCCCTCCAT TTCTCTCCGG	HindIII
Dob3_ninae_NdeI_HindIII_F	ggtgccgcgcggcagccatATGACATTGCC	NdeI

	GCAATATCC	
Dob3_ninae_NdeI_HindIII_R	tgctcgagtgcgccgcaTCACTGTCCCTC GATCGTC	HindIII
Dob3_E137A_F	gggtgtgtactg cg ctcgagcatcgc	ctc → cgc
Dob3_E137A_R	gcgatgctcgac gcgc cagtaccacacc	gag → gcg
Dob3_E101A_F	tcaccacgtgctg cg cggtgtccatgatg	ctc → cgc
Dob3_E101A_R	catcatggacacc gcgc cagcacgtggtga	gag → gcg
Dob3_H140A_F	gagatgcatgaggggt ggc gtactgctcgcgagc	gtg → ggc
Dob3_H140A_R	gctcgacgagcagtac gcc accctcatgcatctc	cac → gcc
Dob3_H225A_F	ctcgtcgcggtt ggc catggtcgcggtg	gtg → ggc
Dob3_H225A_R	caccgcgaccatg gcca aaccgcgacgag	cac → gcc
Dob3_E198A_F	gttgatcgagat cg ggcgaccgtggc	ctc → cgc
Dob3_E198A_R	gccacggtcgcc gcgc atctcgatcaac	gag → gcg
Dob3_E229A_F	ggagtggcagtac cg ctcgcggttgtg	ctc → cgc
Dob3_E229A_R	cacaaccgcgac gcgc tactgccactcc	gag → gcg
Dob3_H232A_F	cggcgatcgaggag ggc gcagtactcgtcgc	gtg → ggc
Dob3_H232A_R	gcgacgagtactgc gct cctcgcgacgccg	cac → gcc

181

Supplementary Table 2: Vectors used in this study

Vector	Description	Source
pET-28a(+)	Protein expression vector	Invitrogen
pDualP	Dual inducible BAC for whole-cluster expression	Terra Bioforge

182

183

Supplementary Table 3: Strains used in this study.

Strain	Description	Source
<i>Glycomyces harbinensis</i> ATCC 43155	Azaserine producer	ATCC
<i>Streptomyces coelicolor</i> M1152	Heterologous host for <i>dob</i> gene cluster expression	Lab stock
<i>Escherichia coli</i> Top10	Maintenance of protein expression vectors	Invitrogen
<i>Escherichia coli</i> BL21(DE3)	Protein expression	Invitrogen
<i>Escherichia coli</i> BAP1	Expression of phosphopantetheinylated proteins	Lab stock
<i>Escherichia coli</i> BacOpt2.0	Dh10B derivative – maintenance of <i>dob</i> -pDualP and <i>dob</i> -pDualP Δ Dob3	Terra Bioforge
<i>Escherichia coli</i> ET12567/pUZ8002	Methylation deficient donor for <i>dob</i> -pDualP and <i>dob</i> -pDualP Δ Dob3 conjugation	Lab Stock
<i>Nocardia ninae</i> NBRC 108245	Production of 1 and 2	DSMZ
<i>Nocardia tenerifensis</i> DSM 44704	Production of 1 and 2	DSMZ
<i>Streptomyces coelicolor dob</i> -pDualP	Cluster confirmation	This study
<i>Streptomyces coelicolor dob</i> -pDualP Δ <i>dob3</i>	In vivo investigation	This study

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Supplementary Table 4: Protein sequences used in genome mining

Protein	Pfam	Accession ID (NCBI)
AzaA	03417	WP_218125324.1
AzaB	13302	WP_091038445.1
AzaC	00501	WP_218125325.1
AzaE	07883, 09334	WP_091038156.1
AzaF	01266	WP_091038158.1
AzaG	13434	WP_091038161.1
AzaM	02770	WP_091038175.1

Supplementary Table 5: Annotations of proteins in the *dob* biosynthetic gene cluster in *Nocardia ninae*.

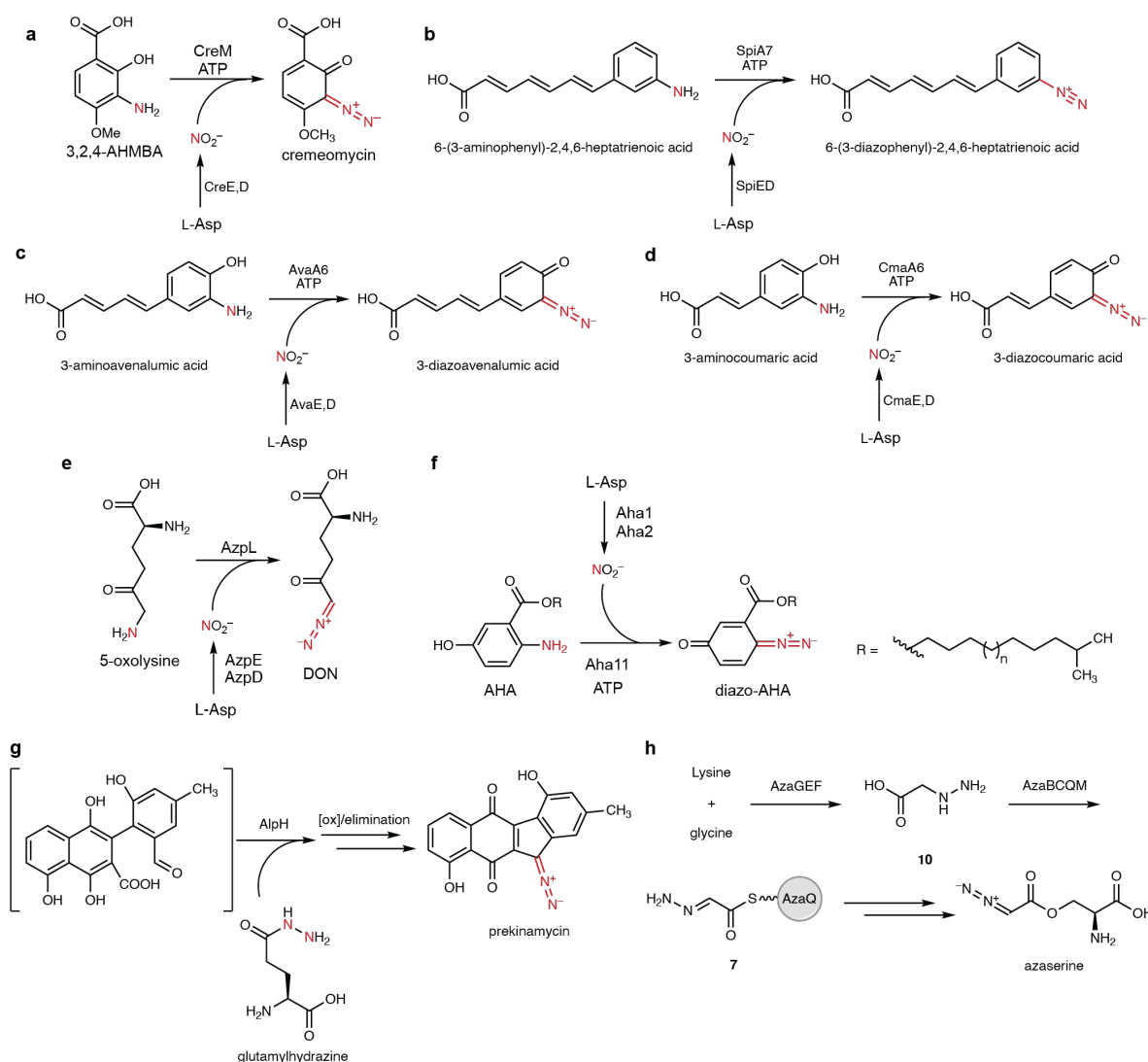
Name	Size (aa)	Annotation	Homolog origin	Proposed function	Accession ID (NCBI)	ID (% aa)	Query coverage (%)
DobA	350	C45 peptidase	<i>Nocardia</i> sp. CS682	hydrazone-formation	WP_135232797.1	96.0	100
DobB	204	GCN5 <i>N</i> -acetyl transferase	<i>Nocardia</i> sp. XZ_19_369	hydrazone-formation	WP_194834407.1	99.0	100
DobC	495	AMP-dependent synthetase	<i>Nocardia</i> sp. CS682	hydrazone-formation	WP_135232795.1	98.0	100
DobE	671	Methionine tRNA ligase/cupin domain	<i>Nocardia</i> sp. CS682	hydrazone-formation	WP_206055239.1	97.8	100
DobF	358	FAD-binding oxidoreductase	<i>Nocardia</i> sp. XZ_19_369	hydrazone-formation	WP_194834410.1	96.1	100
DobG	439	L-lysine 6-monooxygenase	<i>Nocardia</i> sp. CS682	hydrazone-formation	WP_135232793.1	98.4	100
DobM	391	Acyl-CoA dehydrogenase	<i>Nocardia</i> sp. CS682	hydrazone-formation	WP_135232798.1	97.4	100
DobQ	86	Acyl carrier protein	<i>Nocardia suis</i> <i>massiliensis</i>	hydrazone-formation	WP_107658748.1	98.8	100
Dob1	342	ParB transcriptional regulator	<i>Nocardia</i> sp. CS682	regulation	WP_135232792.1	98.3	100
Dob2	1239	Polyketide synthase	<i>Nocardia</i> sp. XZ_19_369	PKS	WP_194834404.1	95.8	100
Dob3	322	Ferritin-like diiron oxidase or oxygenase	<i>Nocardia</i> sp. CS682	Diazo formation	WP_135232800.1	98.8	100
Dob4	405	Major facilitator superfamily 1	<i>Nocardia suis</i> <i>massiliensis</i>	Export/resistance	WP_107658754.1	97.8	100

Supplementary Table 6: Organisms containing *dob* biosynthetic gene clusters.

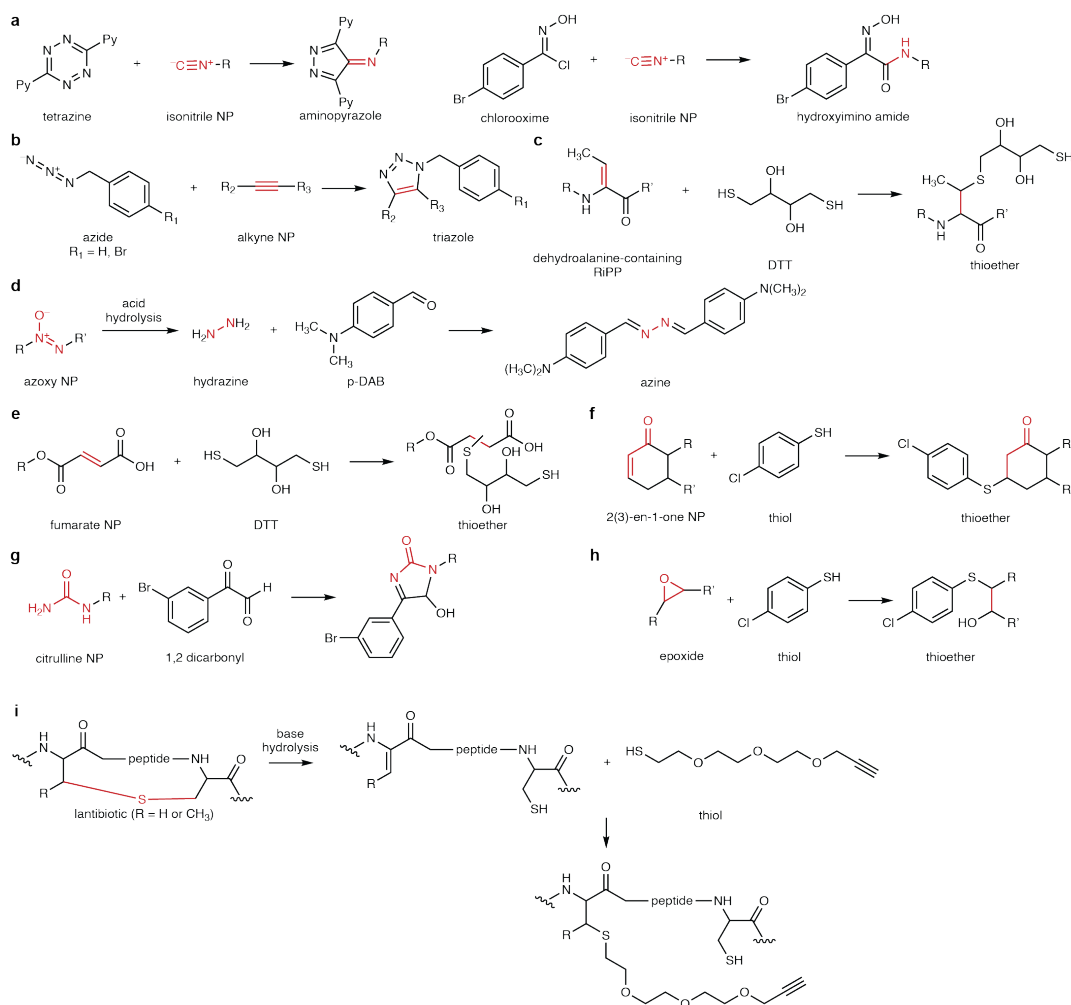
Organism	Description	Accession	Source database
<i>Actinobacteria</i> sp. 051321	Soil microbe	IMG 2931719839	IMG JGI
<i>Actinomadura</i> sp. J1-007	Industrial maduramicin producer	IMG 3003019599	IMG JGI
<i>Nocardia ninae</i> NBRC 108245	Human pathogen	NZ_BJXA000000000.1	NCBI
<i>Nocardia tenerifensis</i> DSM 44704	Animal pathogen	NZ_QJKF01000002.1	NCBI
<i>Nocardia pseudobrasiliensis</i> DSM 44290	Human pathogen	NZ_QQBC01000001.1	NCBI
<i>Nocardia colli</i> CICC11023	Human pathogen	NZ_VXLC01000004.1	NCBI
<i>Nocardia suismassiliense</i> S-137	Boar gut microbe	NZ_LT985361.1	NCBI
<i>Nocardia</i> sp. CS682	Soil microbe	CP029710.1	NCBI
<i>Nocardia</i> sp. NPDC 6044	Soil microbe	NZ_JBIAID010000001.1	NCBI
<i>Nocardia</i> sp. NPDC 50175	Soil microbe	NZ_JBITJI010000016.1	NCBI
<i>Nocardia</i> sp. NPDC 51321	Soil microbe	NZ_JBITEM010000001.1	NCBI
<i>Nocardia</i> sp. NPDC 51756	Soil microbe	NZ_JBFAYV010000001.1	NCBI
<i>Nocardia</i> sp. NPDC 57030	Soil microbe	NZ_JBHUSN010000373.1	NCBI
<i>Nocardia</i> sp. NPDC 60255	Soil microbe	NZ_JBHXAU010000066.1	NCBI
<i>Nocardia</i> sp. NPDC 52316	Soil microbe	NZ_JBIUBB010000001.1	NCBI
<i>Nocardia</i> sp. NPDC 46473	Soil microbe	NZ_JBEYZJ010000003.1	NCBI
<i>Halostreptopolyspora alba</i> YIM 96095	Soil microbe	IMG 2861794813	IMG JGI

Supplementary Table 7: Protein sequences for queries used in previous genome mining efforts

Reference	Protein	NCBI Accession Number
55	Lom29	AHZ61863.1
	Lom30	AHZ61864.1
	Lom32	AHZ61866.1
	Lom33	AHZ61867.1
	Lom34	AHZ61868.1
	Lom35	AHZ61869.1
12	CreD	ALA99201.1
	CreE	ALA99202.1
	CreM	ALA99210.1
14	AzpL	BCN13448.1
20	AzaD	WP_091038154.1
	AzaH	WP_091038164.1
	AzaI	WP_091038167.1
	AzaL	WP_143014975.1
	AzaN	WP_091038177.1
	AzaP	WP_091038183.1



Supplementary Figure 1: Characterized and putative diazo biosynthetic enzymes. The first diazo-containing intermediate or product in each pathway is shown. **a)** CreM catalyzes formation of the diazo functionality in cremeomycin biosynthesis. 3,2,4-AHMB = 3-amino-2-hydroxy-4-methoxybenzoic acid.^{12,6} **b)** SpiA7 catalyzes diazo formation in spinamycin biosynthesis.¹⁷ **c)** AvaA6 catalyzes 3-diazoavenalamic acid formation in avenalamic acid biosynthesis.¹⁶ **d)** CmaA6 catalyzes 3-diazocoumaric acid formation in *p*-coumaric acid biosynthesis.¹⁸ **e)** AzpL catalyzes DON formation in alazopeptin biosynthesis.¹⁴ **f)** Aha11 catalyzes diazo formation in tasikamide biosynthesis. AHA = alkyl 5-hydroxyanthranilate.¹⁵ **g)** AlpH catalyzes condensation of an acyl hydrazide with a carbonyl in kinamycin biosynthesis.¹⁹ **h)** AzaGEF catalyzes conversion of L-lysine and glycine to **10** and subsequent transformations by AzaBCQM yields **7-AzaQ**.^{20–22} The enzyme responsible for oxidation of the hydrazone to the diazo has not yet been elucidated.



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211 **Supplementary Figure 2:** Chemical trapping facilitates identification of natural products. **a)**212 Tetrazine²⁸ and chlorooxime²⁷ probes identify isonitrile-containing natural products. **b)** Azide213 probes undergo 1,3-dipolar cycloadditions to identify alkyne-containing natural products.^{24,25} **c)**

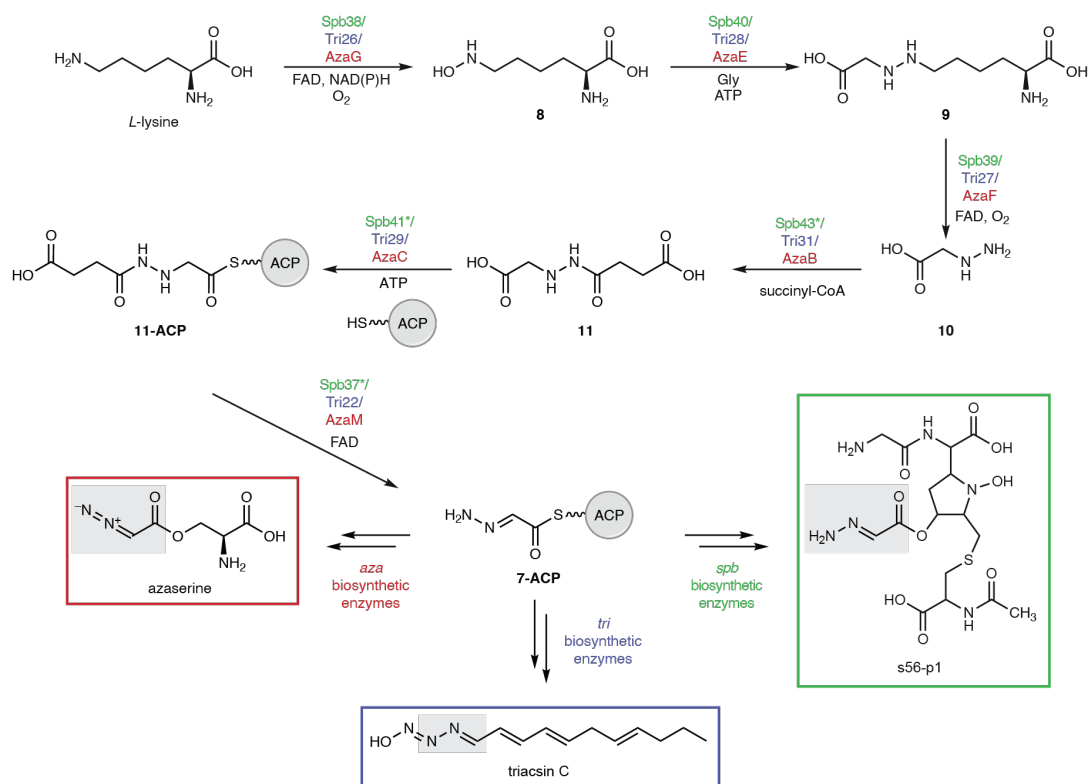
214 Dithiothreitol (DTT) undergoes 1,4-nucleophilic additions to identify dehydroalanine-containing

215 RiPPs.^{2,23} **d)** Acid hydrolysis of azoxy natural products yields hydrazine which can be derivatized216 by para-dimethylamino benzaldehyde (p-DAB) to identify azoxy natural products.⁷⁶ **e)** DTT217 undergoes 1,4-nucleophilic additions to identify fumarate-containing natural products.⁷⁷ **f)** Thiol218 probes undergo 1,4-nucleophilic additions to identify 2(3)-en-1-one-containing natural products.⁷⁸219 **g)** 1,2-dicarbonyl probes identify citrulline-containing natural products.⁷⁹ **h)** Thiol probes identify220 epoxide-containing natural products.⁸⁰ **i)** Base hydrolysis of lantibiotics yields dehydrated amino

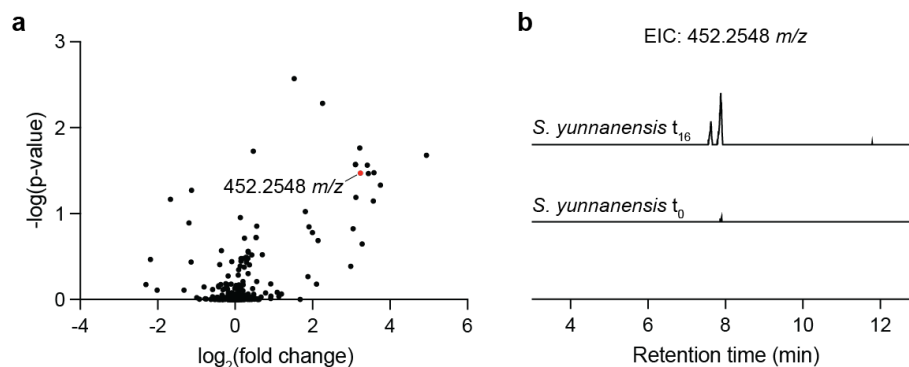
221 acids which undergo 1,4-nucleophilic additions with thiol probes to introduce an alkyne

222 functionality. A subsequent click reaction with an azide enables enrichment of the derivatized

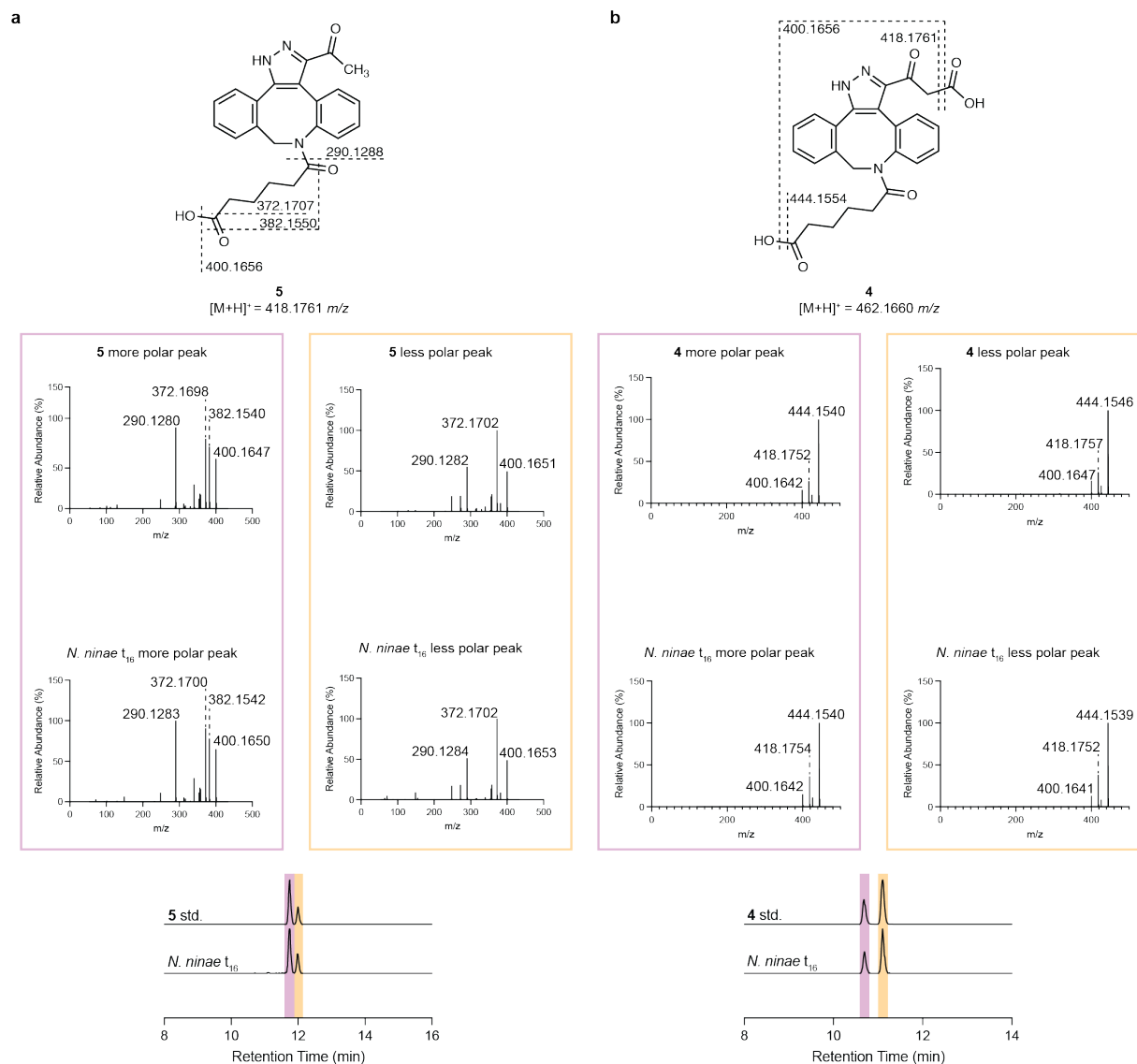
223 natural product.⁸¹



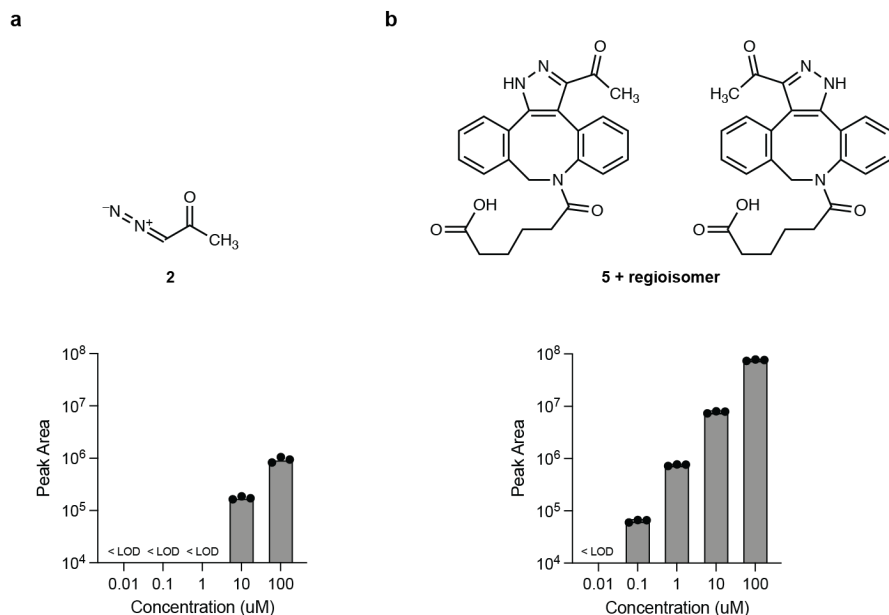
224
 225 **Supplementary Figure 3:** Previously reported hydrazone biosynthetic pathways. Atoms
 226 highlighted in gray boxes originate from **10**. * = enzymes were identified bioinformatically and
 227 have not been biochemically characterized.



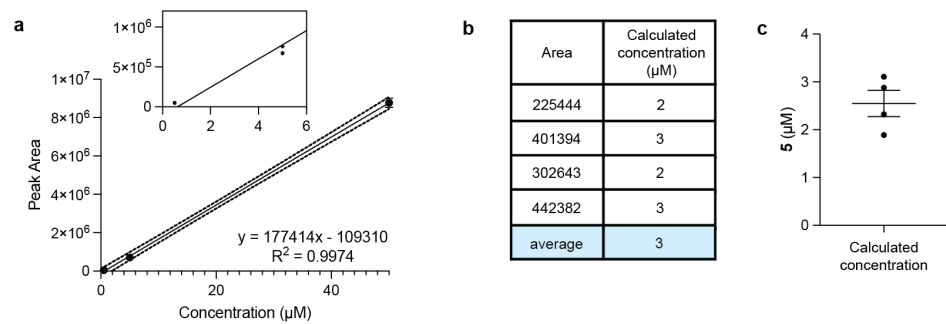
Supplementary Figure 4: Reactivity-guided comparative metabolomics identifies a putative diazo-containing natural product from *S. yunnanensis*. **a)** Volcano plot for comparative metabolomics of *S. yunnanensis* spent medium treated with DBCO-acid at t_{16} vs t_0 . P-values are calculated by a one-way ANOVA model with Tukey as post-hoc test. **b)** EICs of $m/z = 452.2548 \pm 5$ ppm from DBCO-acid treated spent medium at $t = 16$ h and $t = 0$ h. Low abundance of this metabolite meant the more polar (lower retention time) regioisomer fell below the threshold of detection for the volcano plot, resulting in only a single detected mass feature. Manual inspection of the EICs from highly differentially produced mass features in the volcano plot ($\log_2(\text{fold change}) > 2$) led to the observation of two peaks for $m/z = 452.2548 \pm 5$ ppm, consistent with a DBCO-acid derivatized metabolite. This hit was de-prioritized due to its low abundance compared to that of the hit from *N. ninae*.



Supplementary Figure 5: MS/MS spectra of both regioisomers of synthetic **5** and **4**, and comparison with derivatized spent media from *N. ninae*. **a)** Fragmentation pattern of DBCO-acid derivatized *N. ninae* spent media matches that of synthetic **5**. Extracted ion chromatogram (EIC) ($m/z = 418.1761 \pm 5$ ppm) of the DBCO-acid derivatized *N. ninae* spent media compared to a synthetic standard. **b)** Fragmentation pattern of DBCO-acid derivatized *N. ninae* spent media matches that of synthetic **4**. **4** was prepared through incubation of **4-OMe** with PLE. EIC ($m/z = 462.1660 \pm 5$ ppm) of the DBCO-acid derivatized *N. ninae* spent media compared to a synthetic standard.



Supplementary Figure 6: DBCO-acid derivatization improves LC–MS sensitivity for detection of **2**. **a)** LC-MS peak areas of different starting concentrations of **2** in 10% ACN, 10% MeOH, and 80% H₂O. **b)** LC-MS peak areas of different starting concentrations of **5** in 10% ACN, 10% MeOH, and 80% H₂O. Peak areas were calculated as the sum of the regioisomer peaks. Error bars indicate mean ± standard deviation. Experiments were run in biological triplicates.



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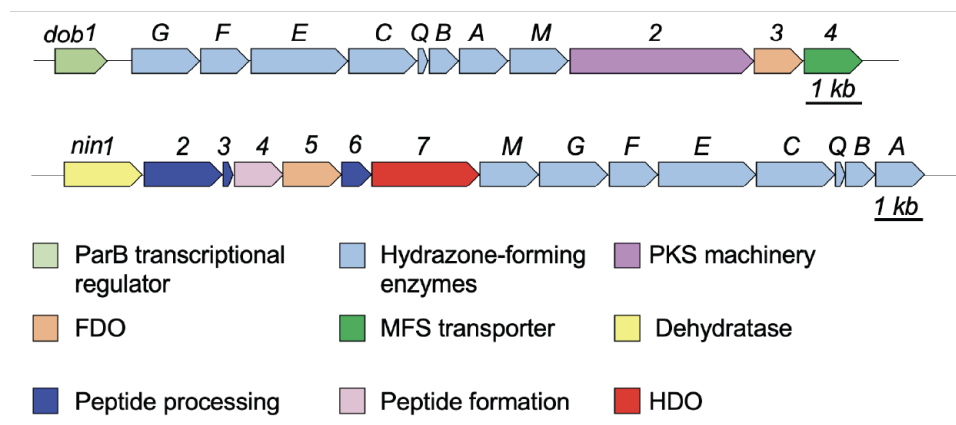
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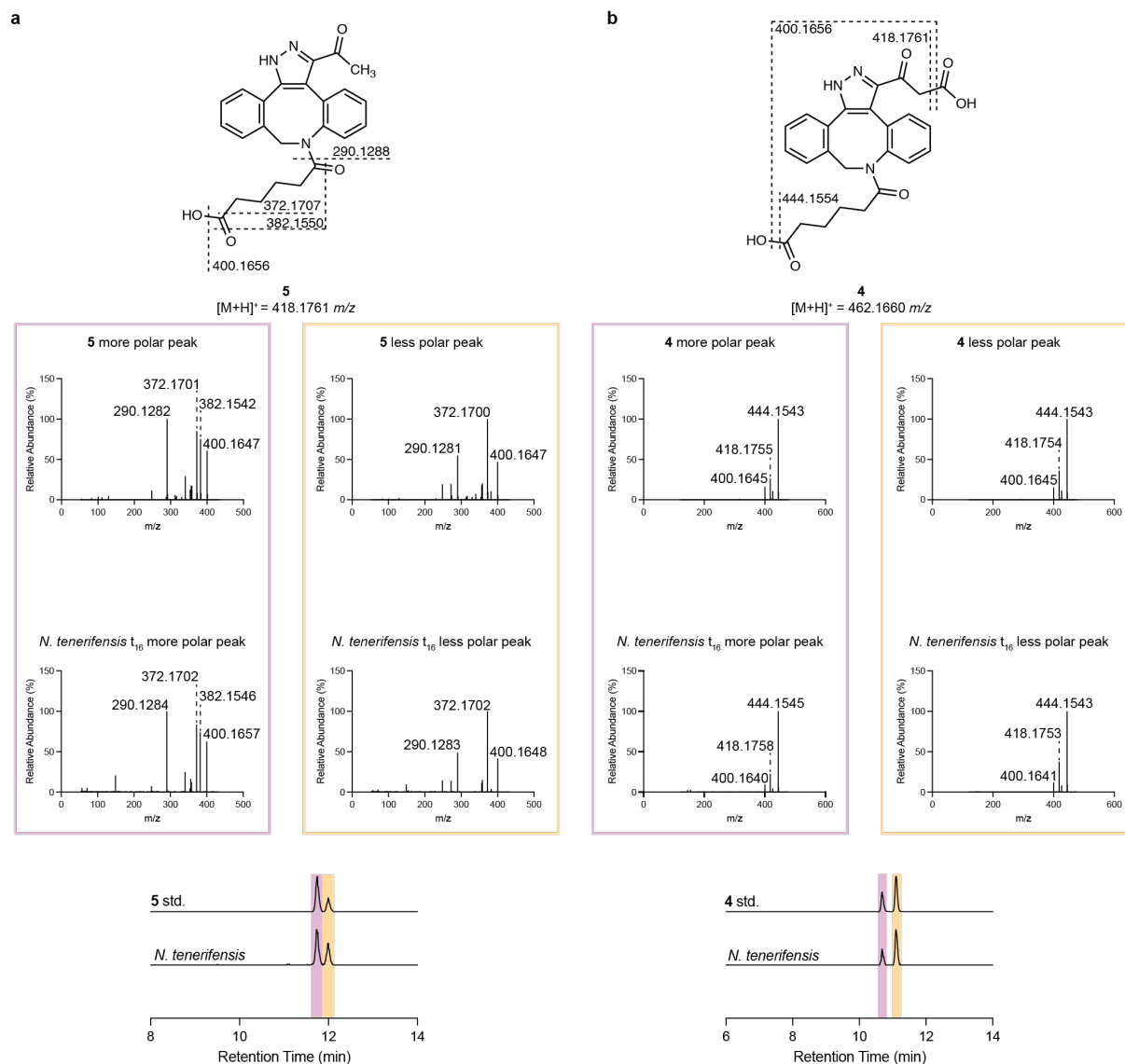
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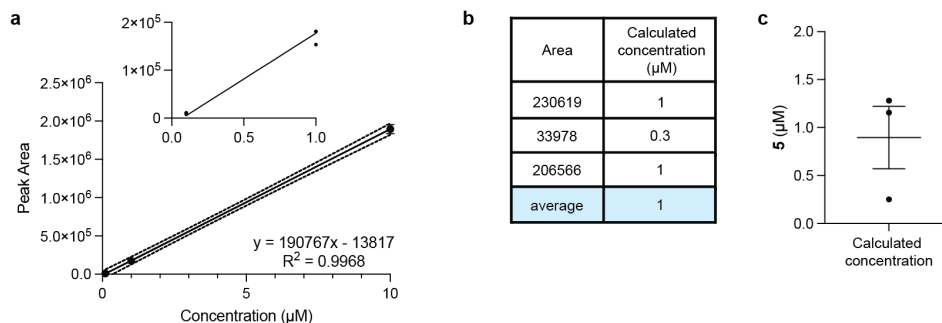
Supplementary Figure 7: Approximation of **5** in *N. ninae* spent medium. **a)** Standard curve of **5** at 500 nM, 5 μM, 50 μM in GYM medium. Peak areas from DBCO-derivatized *N. ninae* spent medium are highlighted in blue. Standards were run in biological triplicates. The dashed line indicates the 95% CI. Error bars indicate the SEM. **b)** Concentrations calculated from raw peak areas of *N. ninae* spent medium. *N. ninae* samples were run in biological quadruplicates. **c)** Concentration of **5** in *N. ninae* supernatants. The mean and SEM are shown.



Supplementary Figure 8: Genome-mining for hydrazone-forming enzymes identified the *dob* and *nin* biosynthetic gene clusters in *N. ninae*. The PKS gene encoded in the *dob* gene cluster suggests *dob* is responsible for production of **1** and **2**.

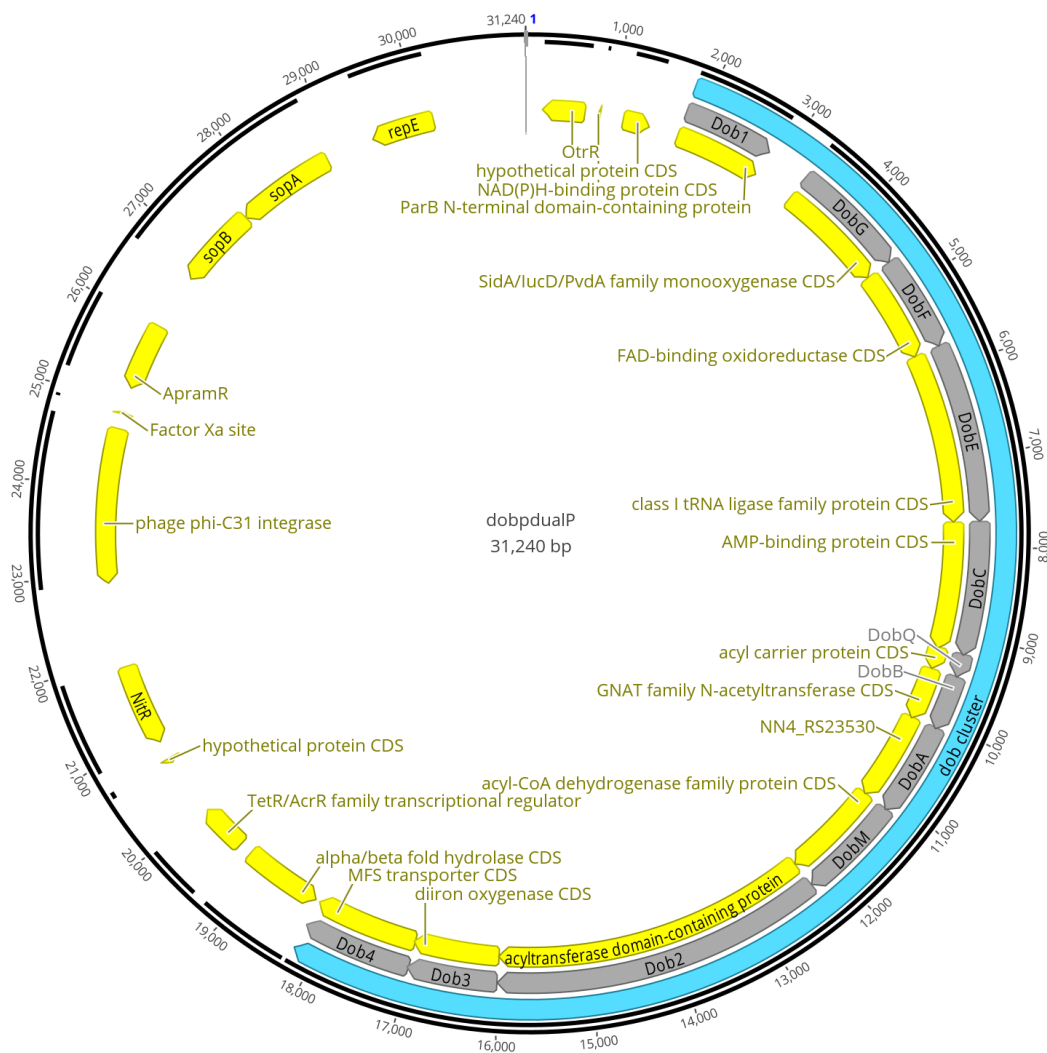


Supplementary Figure 9: MS/MS spectra of both regioisomers of synthetic **5** and **4**, and comparison with derivatized spent media from *N. tenerifensis*. **a)** Fragmentation pattern of DBCO-acid derivatized *N. tenerifensis* spent media matches that of synthetic **5**. EIC ($m/z = 418.1761 \pm 5$ ppm) of the DBCO-acid derivatized *N. tenerifensis* spent media compared to a synthetic standard. **b)** Fragmentation pattern of DBCO-acid derivatized *N. tenerifensis* spent media matches that of synthetic **4**. **4** was prepared through incubation of **4-OMe** with PLE. EIC ($m/z = 462.1660 \pm 5$ ppm) of the DBCO-acid derivatized *N. tenerifensis* spent media compared to a synthetic standard.



Supplementary Figure 10: Approximation of **5** in *N. tenerifensis* spent medium. **a)** Standard curve of **5** at 100 nM, 1 μM , 10 μM in molasses medium. Peak areas from DBCO-derivatized *N. tenerifensis* spent medium are highlighted in blue. Standards were run in biological triplicates. The dashed line indicates the 95% CI. Error bars indicate the SEM. **b)** Concentrations calculated from raw peak areas of *N. tenerifensis* spent medium. *N. tenerifensis* samples were run in biological triplicates. **c)** Concentration of **5** in *N. tenerifensis* supernatants. The mean and SEM are shown.

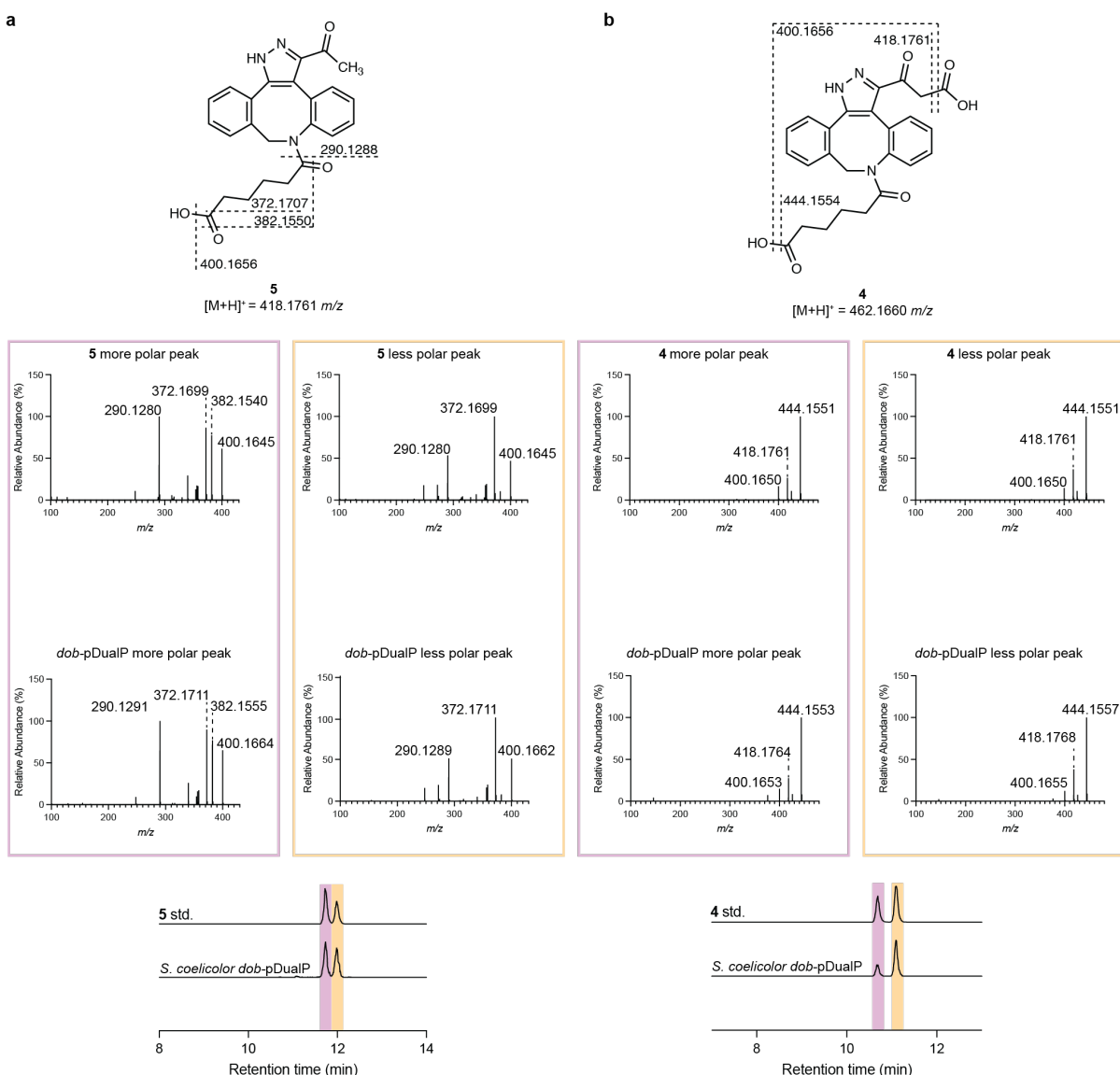
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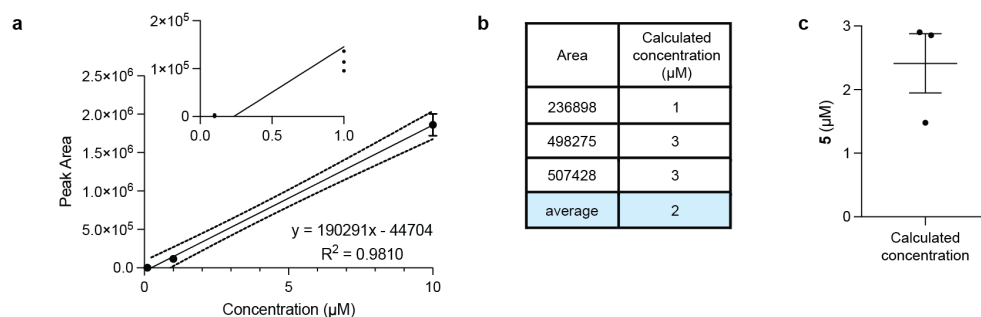
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294 **Supplementary Figure 11: Vector map of *dob*-pDualP.**

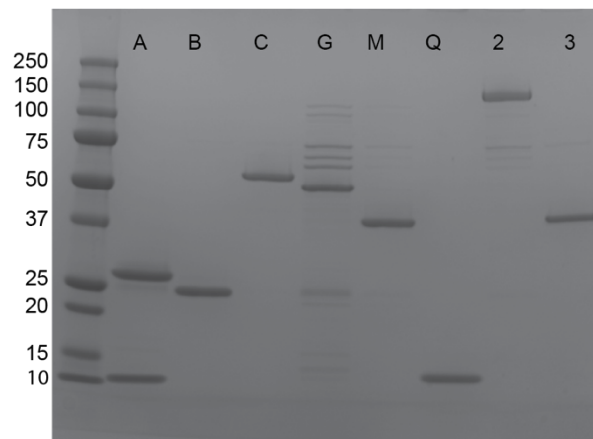


Supplementary Figure 12: MS/MS spectra of both regioisomers of synthetic **5** and **4**, and comparison with derivatized spent media from *S. coelicolor* dob-pDualP. **a)** Fragmentation pattern of DBCO-acid derivatized *S. coelicolor* dob-pDualP spent media matches that of synthetic **5**. EIC ($m/z = 418.1761 \pm 5$ ppm) of the DBCO-acid derivatized *S. coelicolor* dob-pDualP spent media compared to a synthetic standard. **b)** Fragmentation pattern of DBCO-acid derivatized *S. coelicolor* dob-pDualP spent media matches that of synthetic **4**. **4** was prepared through incubation of **4-OMe** with PLE. EIC ($m/z = 462.1660 \pm 5$ ppm) of the DBCO-acid derivatized *S. coelicolor* dob-pDualP spent media compared to a synthetic standard.



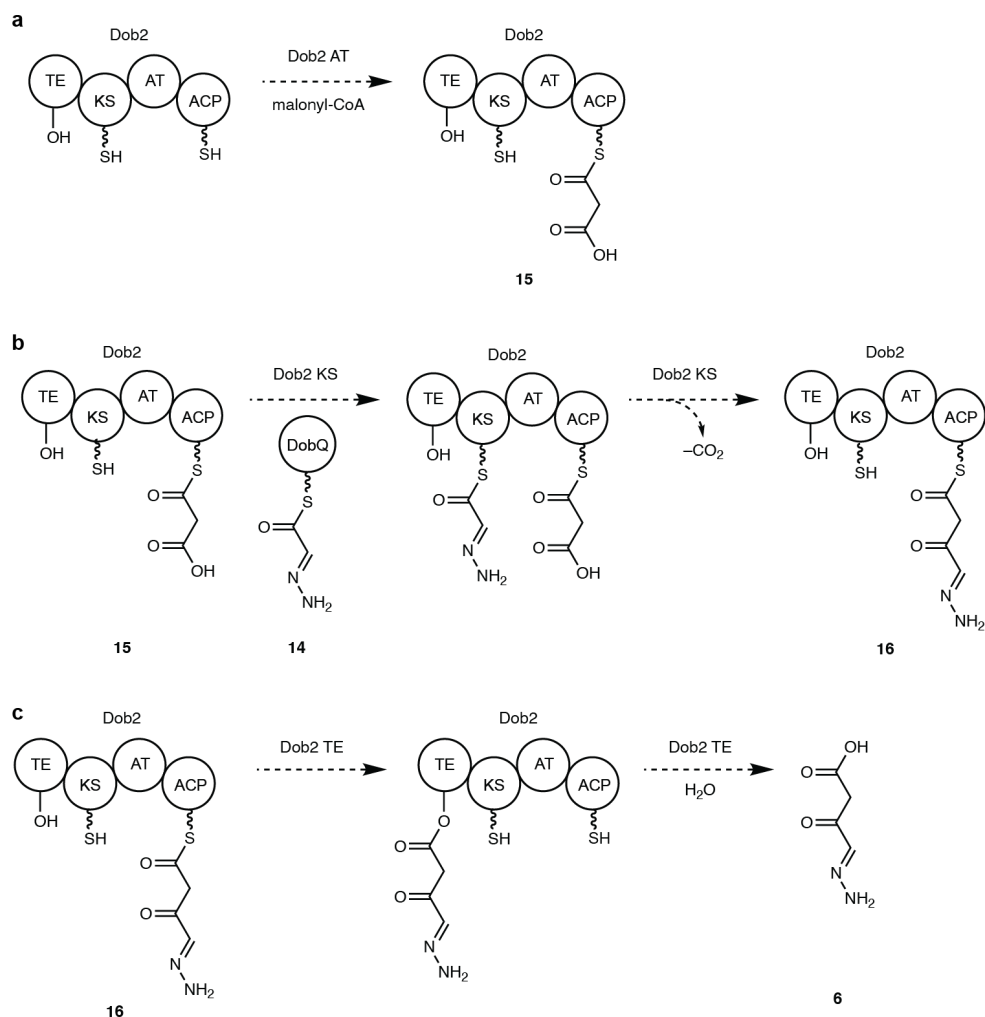
Supplementary Figure 13: Approximation of **5 in *S. coelicolor* *dob*-pDualP spent medium. **a)** Standard curve of **5** at 100 nM, 1 μM, 10 μM in ISP4 medium. Peak areas from DBCO-derivatized *S. coelicolor* *dob*-pDualP spent medium are highlighted in blue. Standards were run in biological triplicates. The dashed line indicates the 95% CI. Error bars indicate the SEM. **b)** Concentrations calculated from raw peak areas of *S. coelicolor* *dob*-pDualP spent medium. *S. coelicolor* *dob*-pDualP samples were run in biological triplicates. **c)** Concentration of **5** in *S. coelicolor* *dob*-pDualP supernatants. The mean and SEM are shown.**

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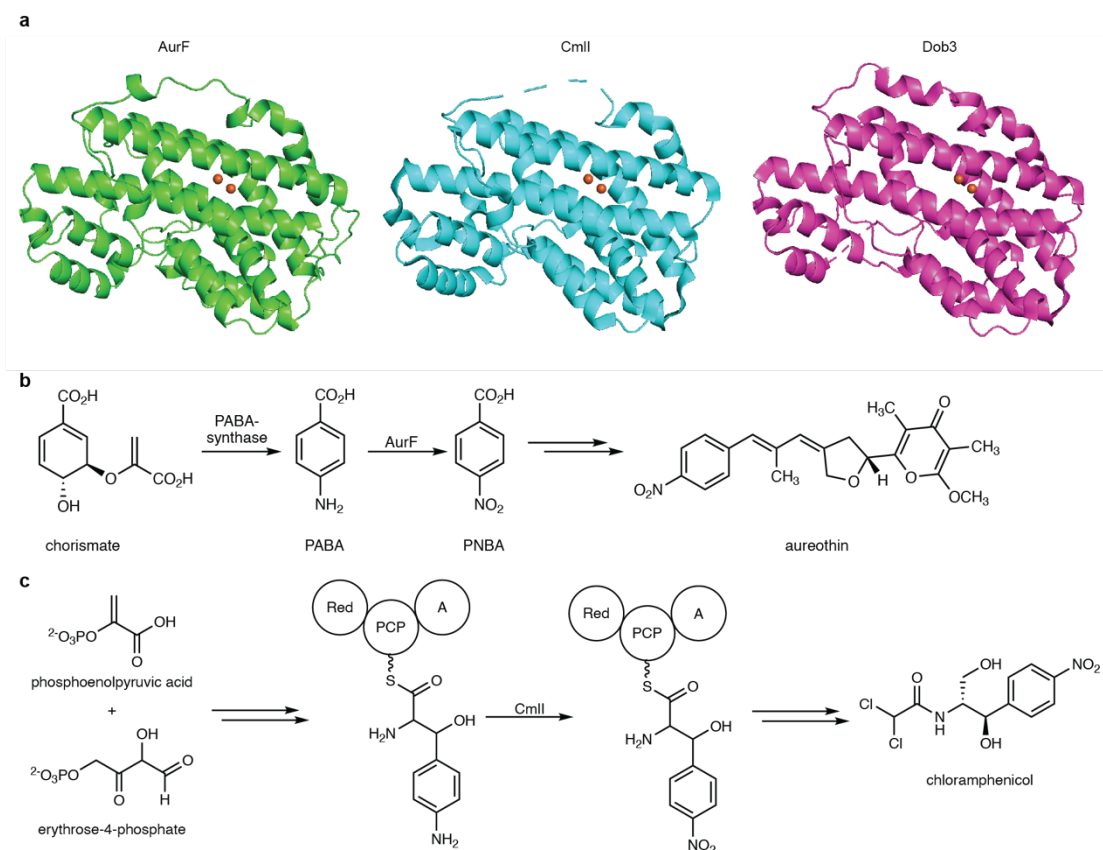


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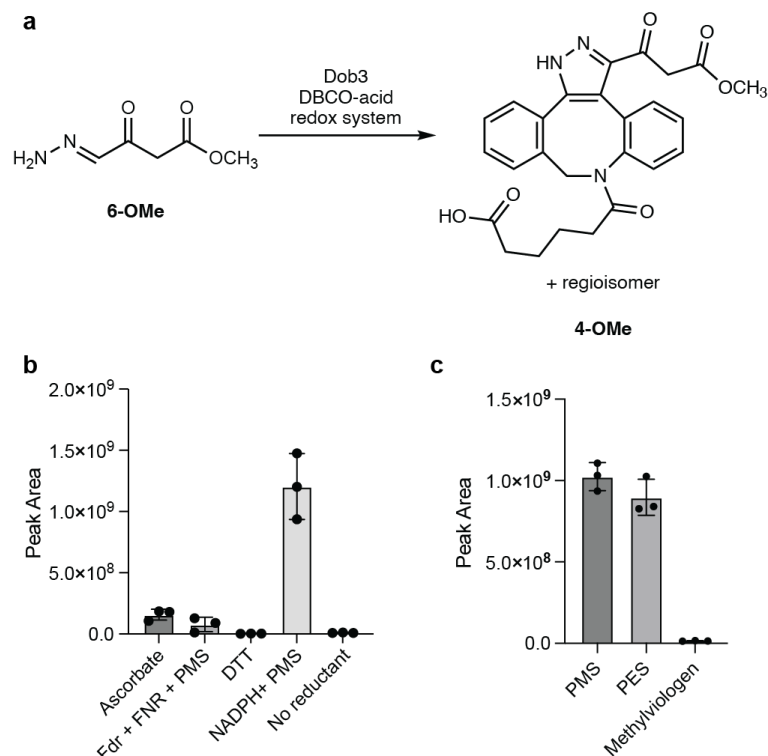
317 **Supplementary Figure 14:** SDS-PAGE gel of purified Dob biosynthetic enzymes. Molecular
318 weights for His₆-tagged constructs were calculated using ExPASy ProtParam⁴⁵: DobA (39.3 kDa;
319 12.1 kDa and 27.2 kDa after autoproteolysis at C112 to produce the mature C45 peptidase), DobB
320 (25.1 kDa), DobC (56.1 kDa), DobG (51.8 kDa), DobM (43.7 kDa), DobQ (11.5 kDa), Dob2 (133.9
321 kDa), Dob3 (40.2 kDa). Precision Plus All Blue Molecular Weight Standard (Bio-Rad) was used
322 as the ladder.



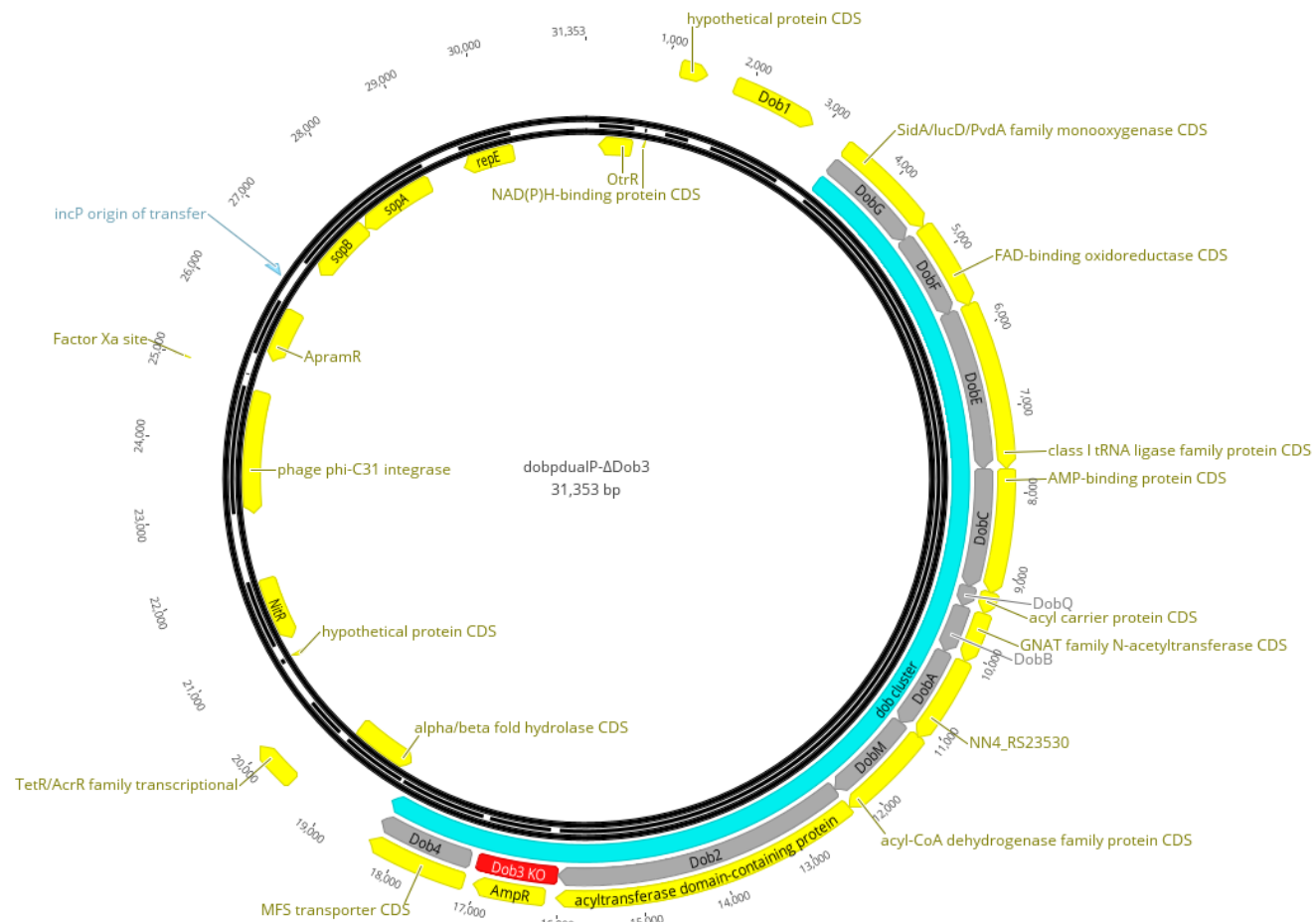
Supplementary Figure 15: Predicted functions of Dob2 domains. **a)** The Dob2 AT domain likely loads a malonyl extender unit. **b)** The KS domain likely catalyzes translocation of **14** from DobQ to a conserved Cys residue, followed by C–C bond-forming decarboxylative Claisen condensation between the malonyl extender unit and the starter unit **14** to produce **16**. **c)** The thioesterase domain likely translocates the mature PKS intermediate to a conserved serine residue (S88) prior to hydrolytic release of **6**.



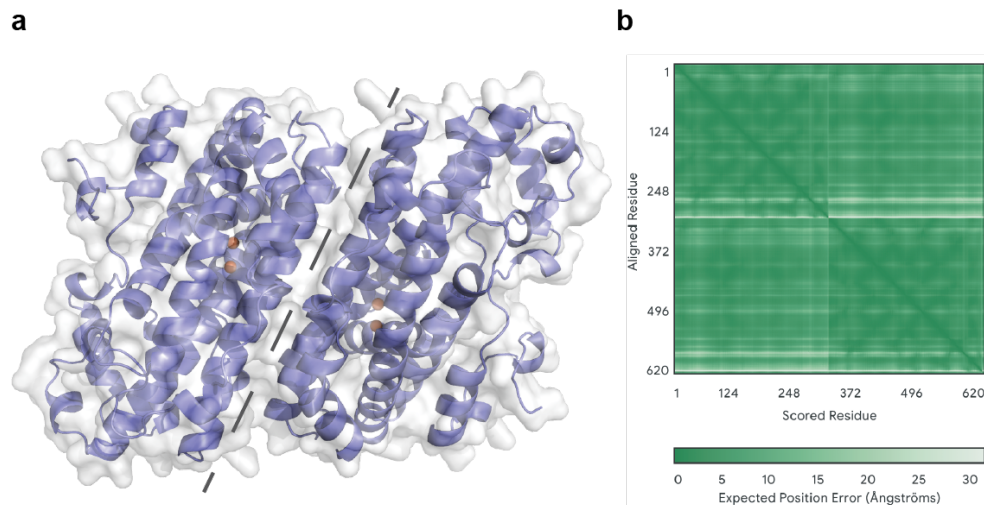
Supplementary Figure 16: Dob3 is structurally homologous to ferritin-like diiron *N*-oxygenases (FDOs) AurF and CmlI. **a)** Comparison of the AlphaFold predicted Dob3 structure to the X-ray crystal structures of AurF (PDB: 3CHH) and CmlI (PDB: 5HYH). Nitro-forming *N*-oxygenation reactions catalyzed by **b)** AurF⁴⁵ and **c)** CmlI⁸³ during the biosynthesis of aureothin and chloramphenicol, respectively.



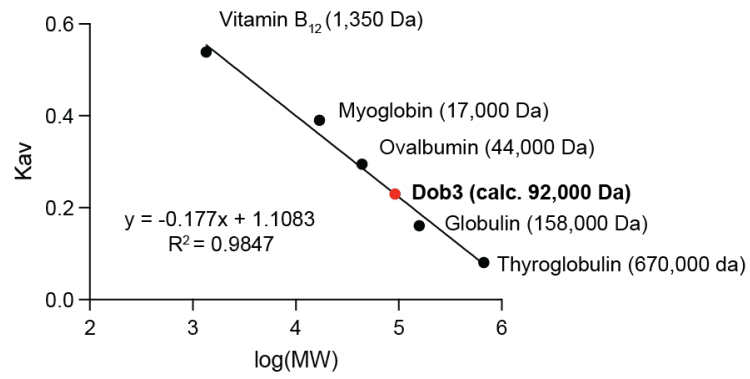
Supplementary Figure 17: Optimization of Dob3 redox system. **a)** Dob3 reaction scheme. **b)** Screening of redox systems demonstrates NADPH with PMS provides the greatest conversions. Fdr = Spinach ferredoxin, FNR = Spinach ferredoxin reductase, DTT = dithiothreitol. **c)** Screening of electron mediators in conjunction with NADPH demonstrates PMS provides the greatest conversions. PMS = phenazine methosulfate, PES = phenazine ethosulfate. All experiments were performed in biological triplicates. Error bars indicate mean $\pm$ standard deviation.



Supplementary Figure 18: Vector map of *dob*-pDualP Δ *dob3*.



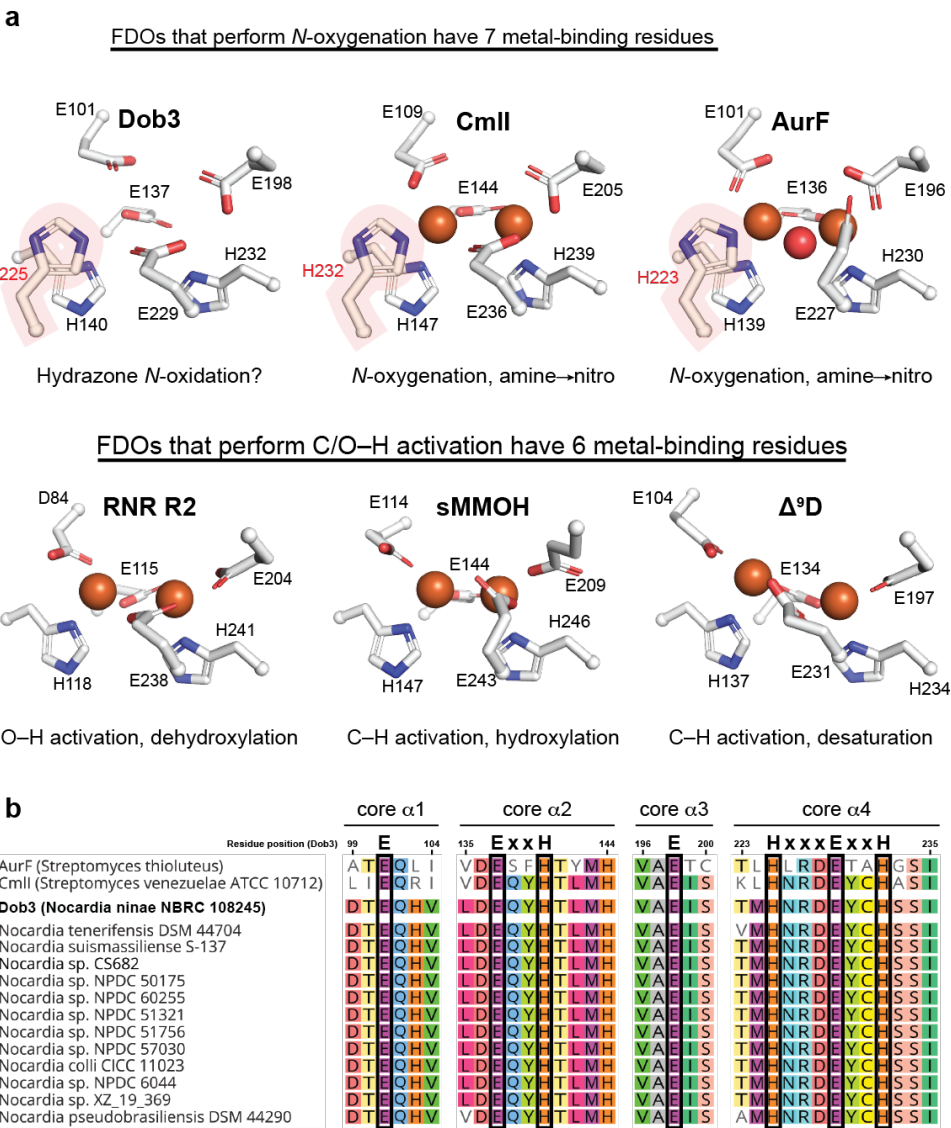
Supplementary Figure 19: AlphaFold 3 predicts that Dob3 is a homodimer. **a)** AlphaFold 3 predicted structure of the Dob3 homodimer. The dimer interface is marked with a dashed line. Residues 1-26 form a long, unfolded chain and are omitted for clarity. **b)** Expected Position Error Plot for the predicted Dob3 homodimer structure.



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353 **Supplementary Figure 20:** Size exclusion chromatography is consistent with a Dob3 homodimer

354 ($MW_{\text{dimer}} = 80.4 \text{ kDa}$, $MW_{\text{calc}} = 92.0 \text{ kDa}$).



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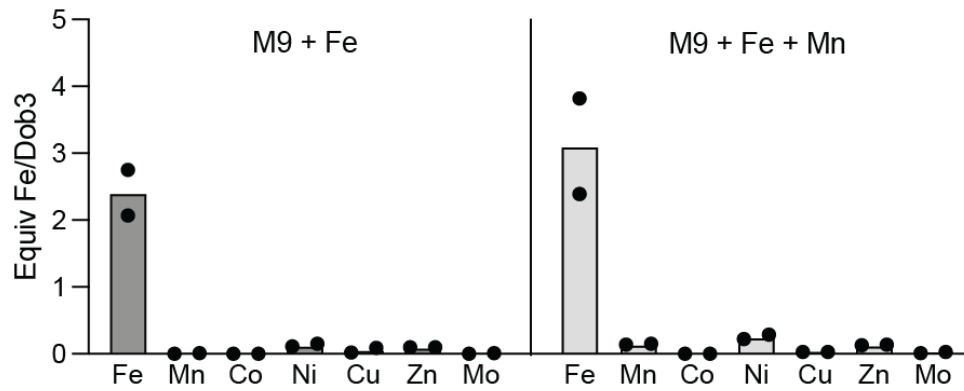
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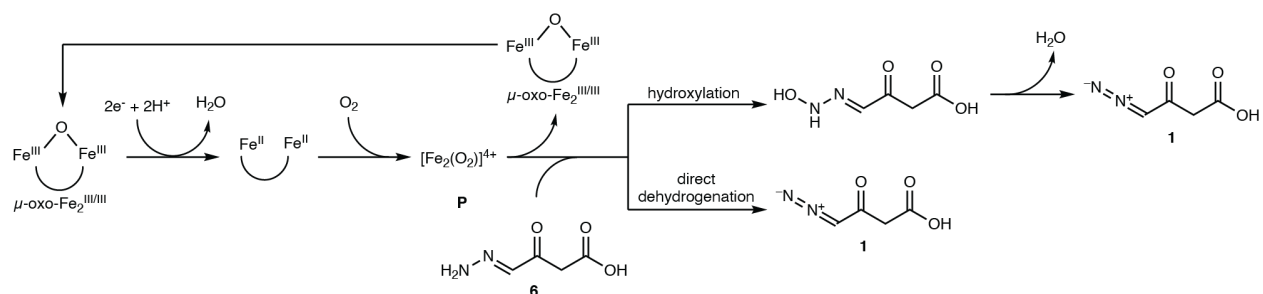
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Supplementary Figure 21: Comparison of Dob3 to known FDOs suggests its activity as an N-oxygenase. **a)** The AlphaFold 3 predicted active site structure of Dob3 compared to the X-ray crystal structures of CmlI (PDB: 5HYH), AurF (PDB: 3CHH), RNR R2 (PDB: 1PIY), sMMOH (PDB: 6YD0), and Δ⁹-stearoyl-ACP desaturase (PDB: 1AFR). **b)** Multiple sequence alignment of Dob3 and its homologs from *Nocardia* strains with the FDO N-oxygenases CmlI and AurF demonstrates conservation of Fe-binding (boxed) residues.



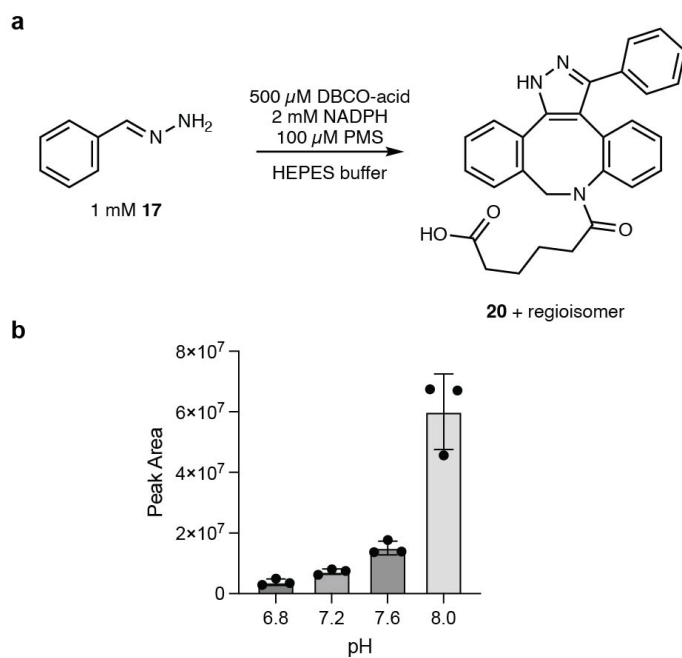
Supplementary Figure 22: ICP-MS reveals Fe is the most abundant metal associated with Dob3 when expressed in M9 medium supplemented with either Fe or Fe + Mn. Experiments were performed in biological duplicates.



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370 **Supplementary Figure 23:** Proposed mechanism of Dob3. Analogous to the mechanism of AurF,
 371 the μ -oxo-diferric cofactor is reduced to the diferrous state and subsequent oxidation by molecular
 372 oxygen yields a reactive differic-peroxo intermediate (**P**).^{84,85} Hydroxylation and subsequent
 373 dehydration, or direct dehydrogenation of **6** yields **1**.

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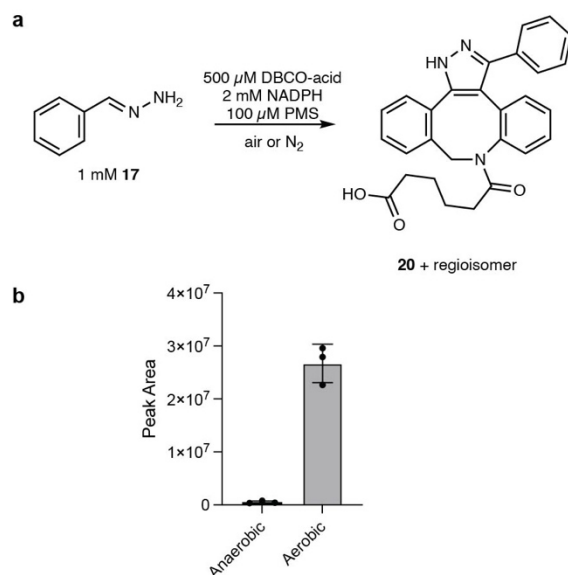
376 **Supplementary Figure 24:** pH dependence of autoxidation of **17**. **a)** Reaction scheme. **b)**

377 Combined LC–MS peak areas of both regioisomers of **20**. Experiments were performed in

378 biological triplicates. Error bars indicate mean $\pm$ standard deviation. Product identity was

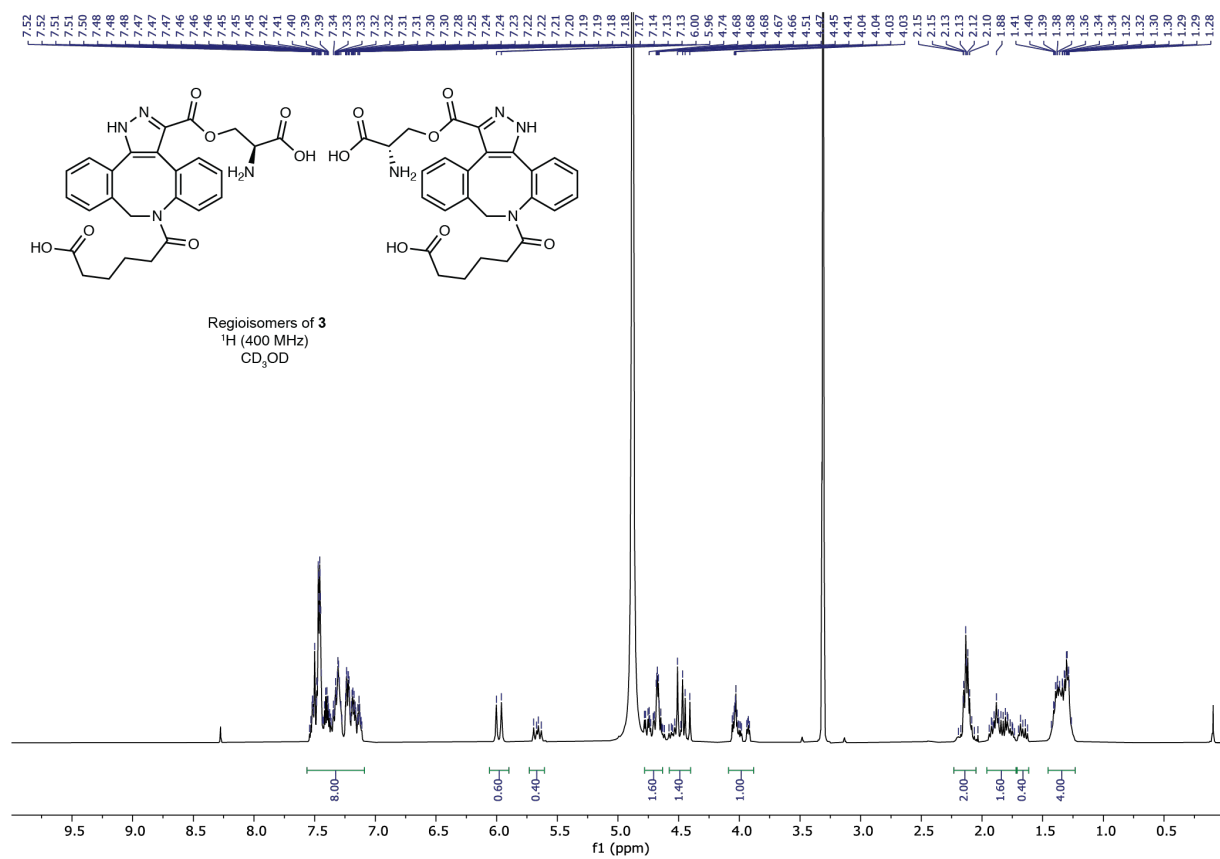
379 confirmed by comparison of LC–MS/MS data to a synthetic standard of **20**.

380



Supplementary Figure 25: Aerobic incubation of **17** with DBCO-acid, PMS, and NADPH yields more product than anaerobic incubations. **a)** Reaction scheme. Reaction mixtures were incubated at pH 8.0. **b)** Combined LC–MS peak areas of both regioisomers of **20**. Experiments were performed in biological triplicates. Error bars indicate mean $\pm$ standard deviation. Product identity was confirmed by comparison to a synthetic standard of **20**.

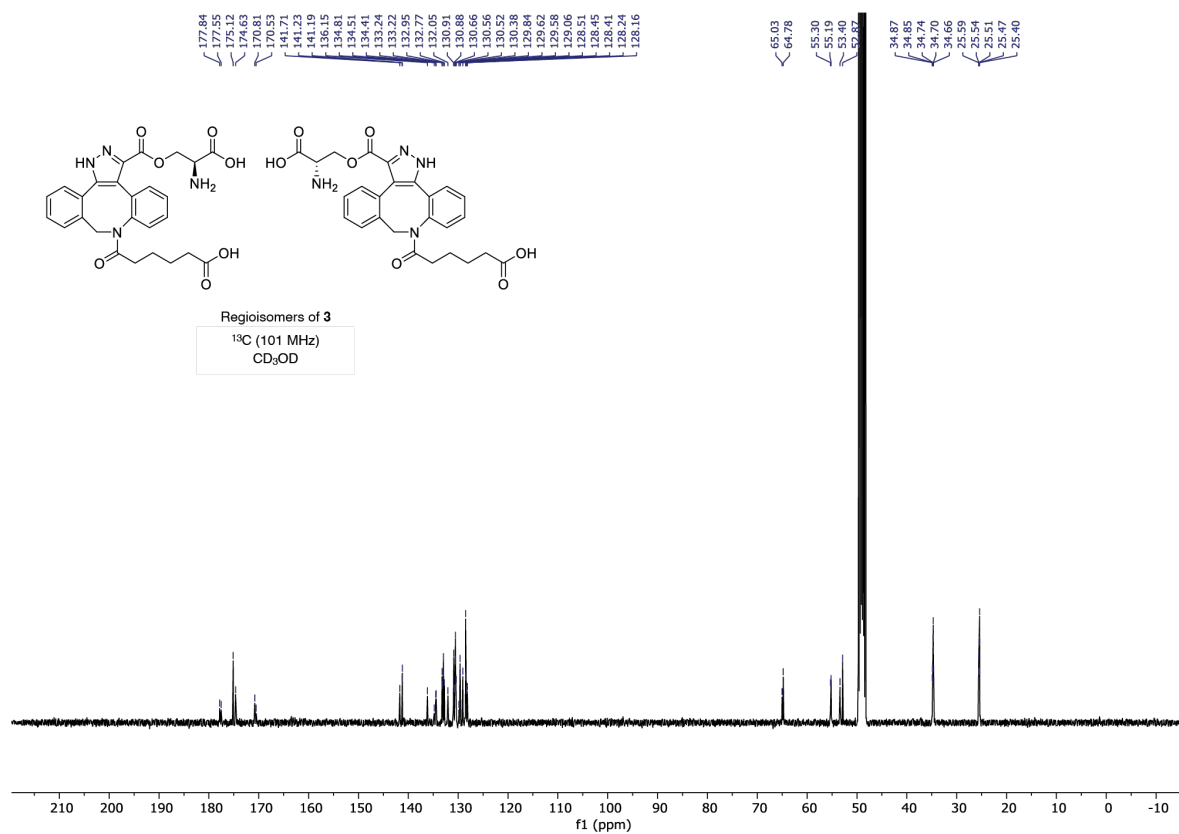
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389 Supplementary Figure 26: ^1H NMR spectrum of regioisomers of **3**.

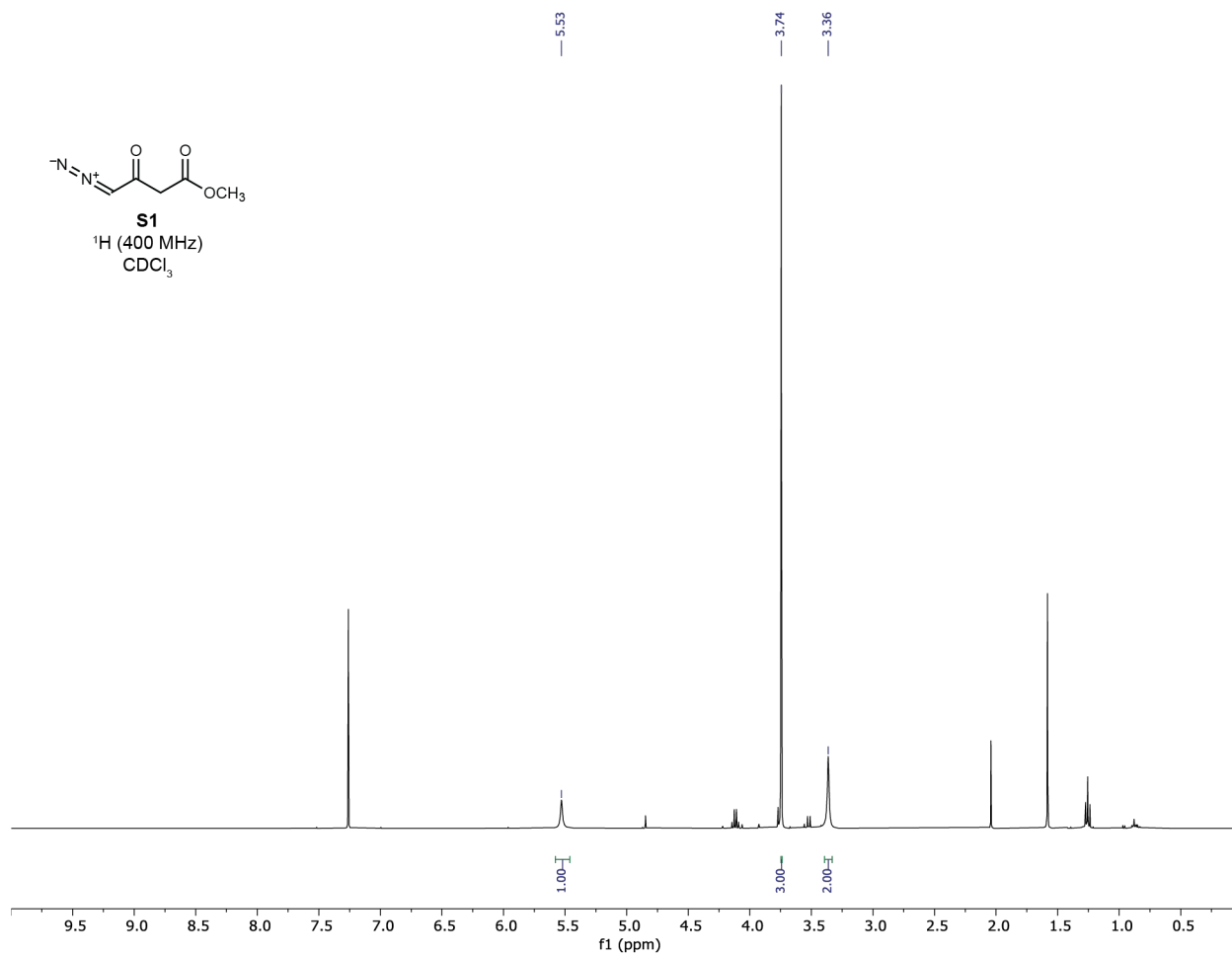
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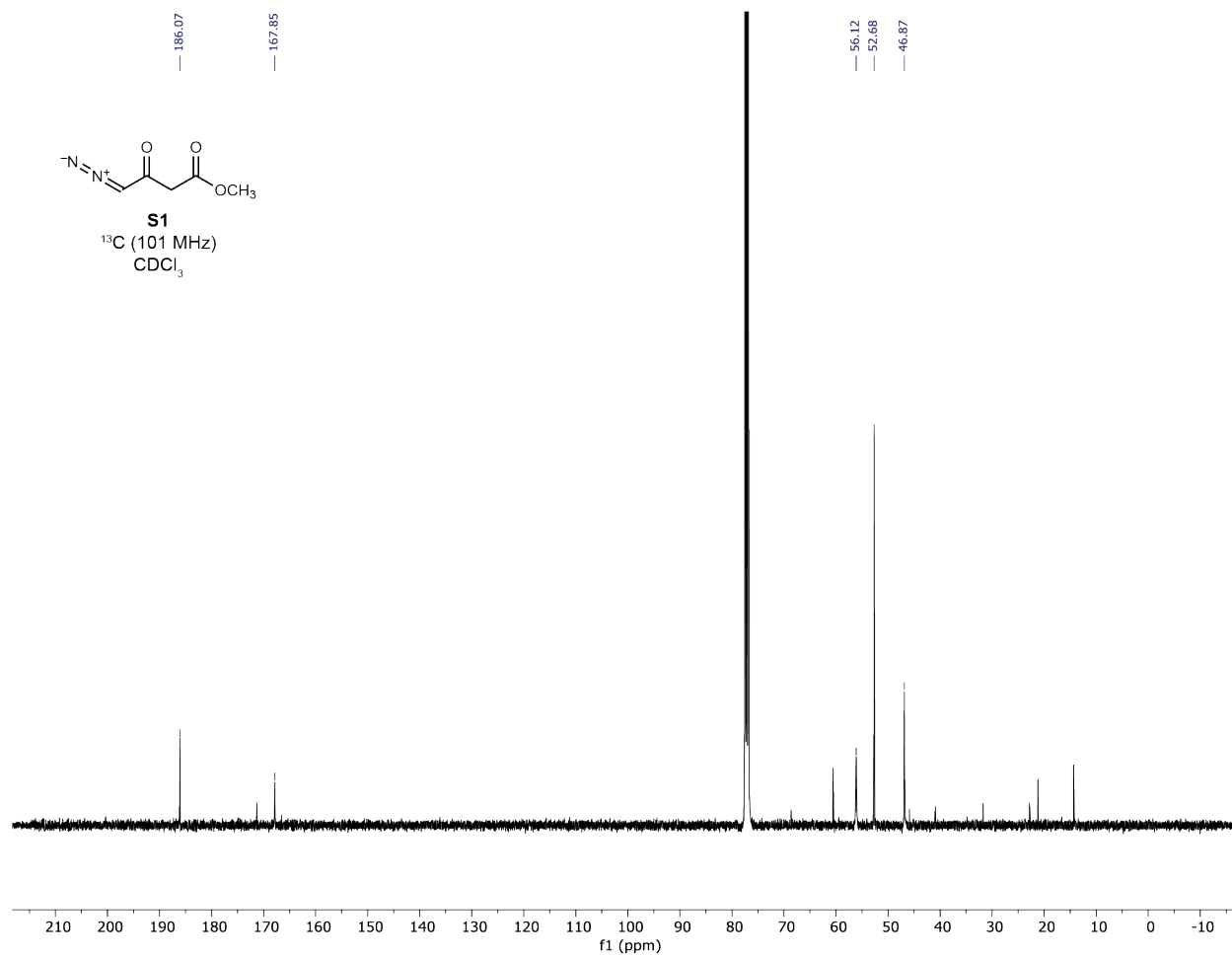
392 **Supplementary Figure 27:** ¹³C NMR spectrum of **3** regioisomers.

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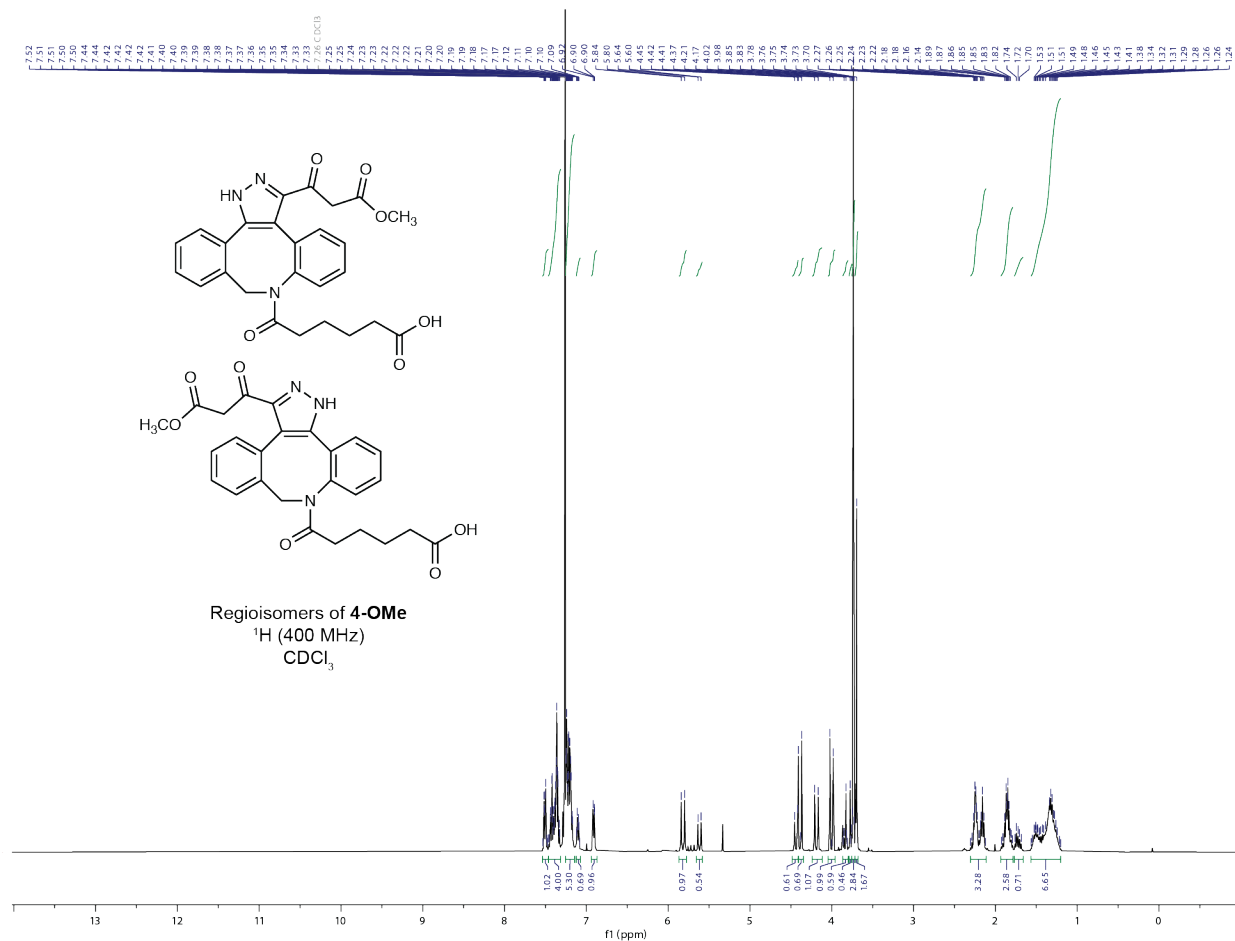
395 **Supplementary Figure 28:** ¹H NMR spectrum of **S1**.



396

397 **Supplementary Figure 29:** ^{13}C NMR spectrum of **S1**.

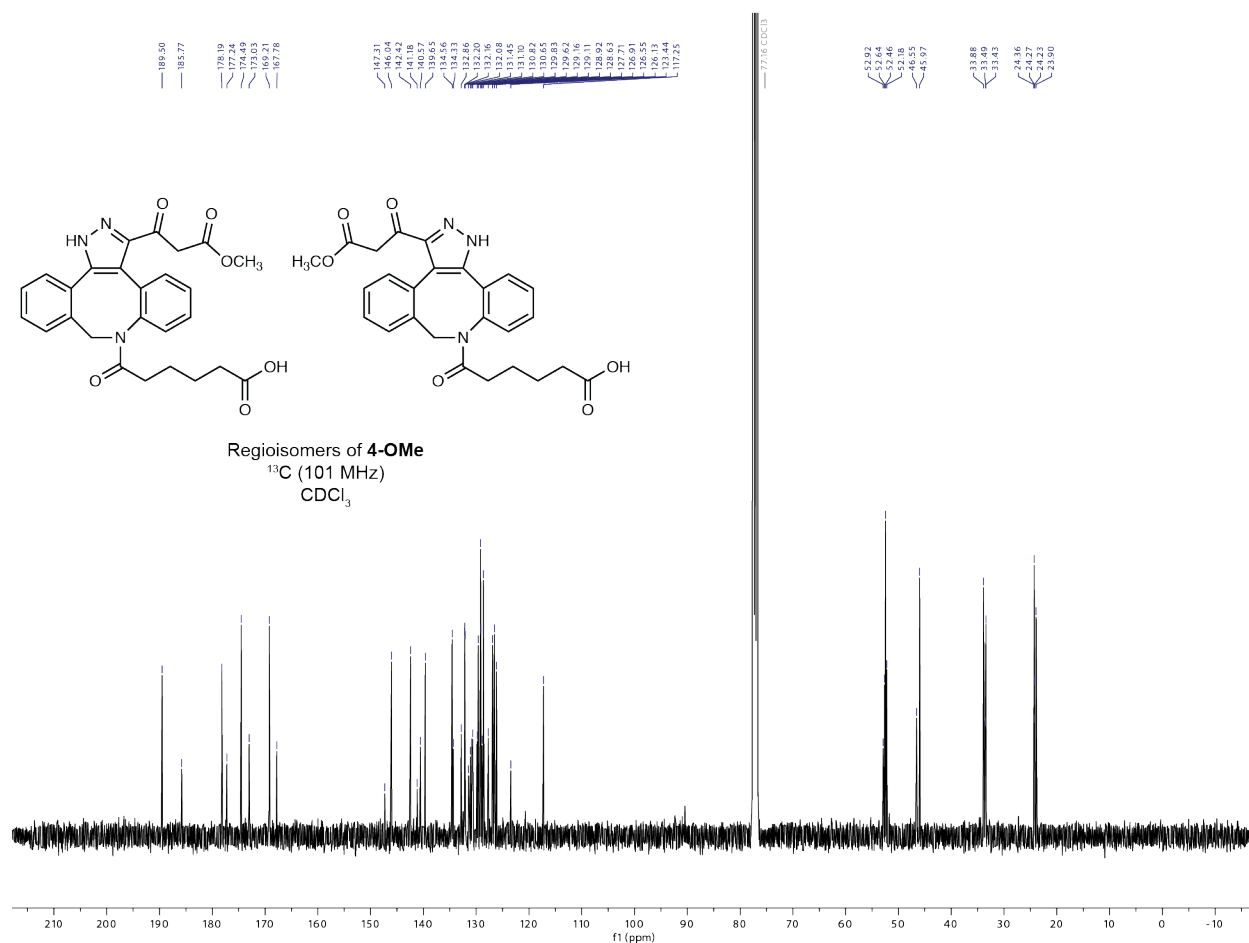
398



399

400 **Supplementary Figure 30: ¹H NMR spectrum of regioisomers of 4-OMe.**

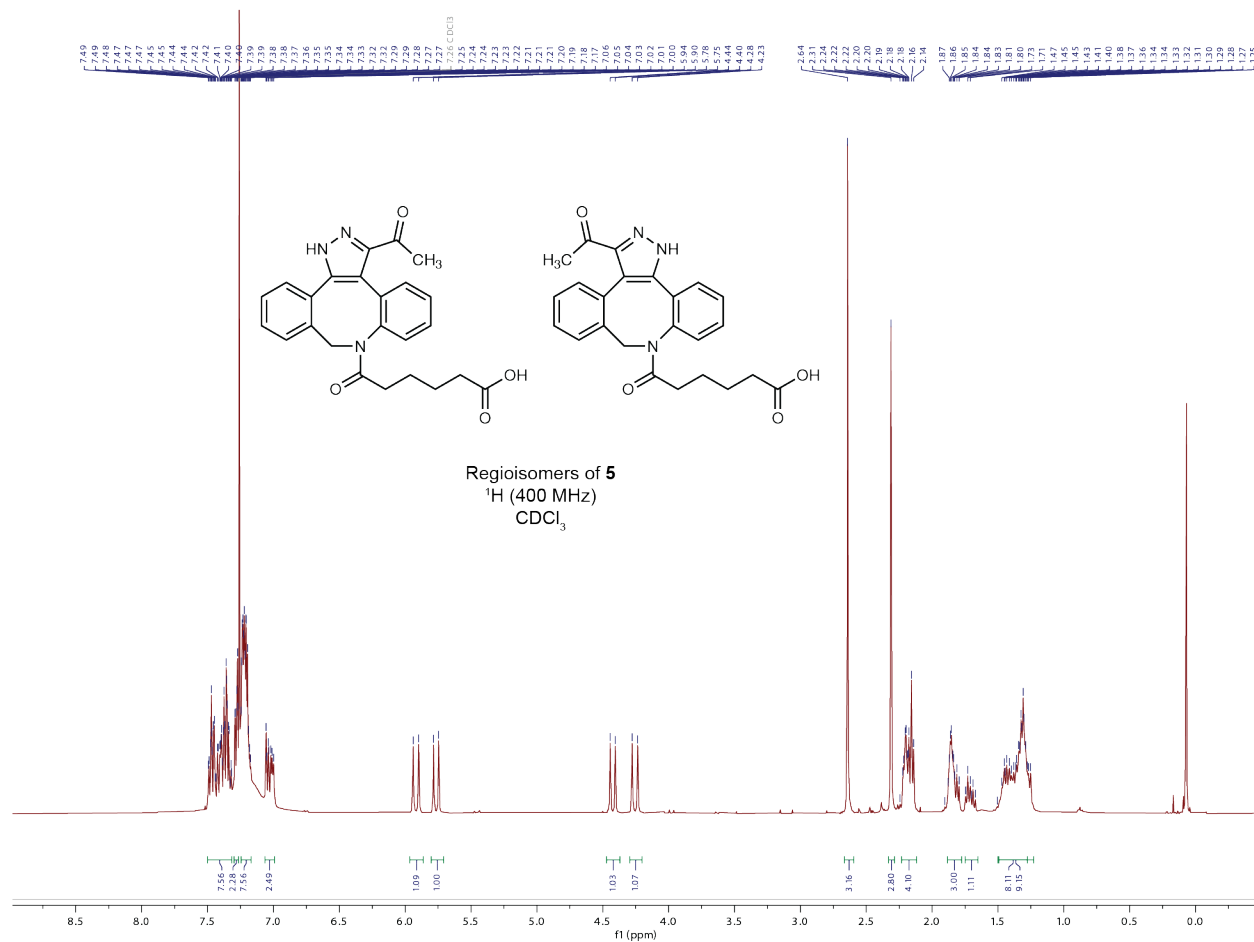
401



402

403 **Supplementary Figure 31: ¹³C NMR spectrum of regioisomers of 4-OMe.**

404



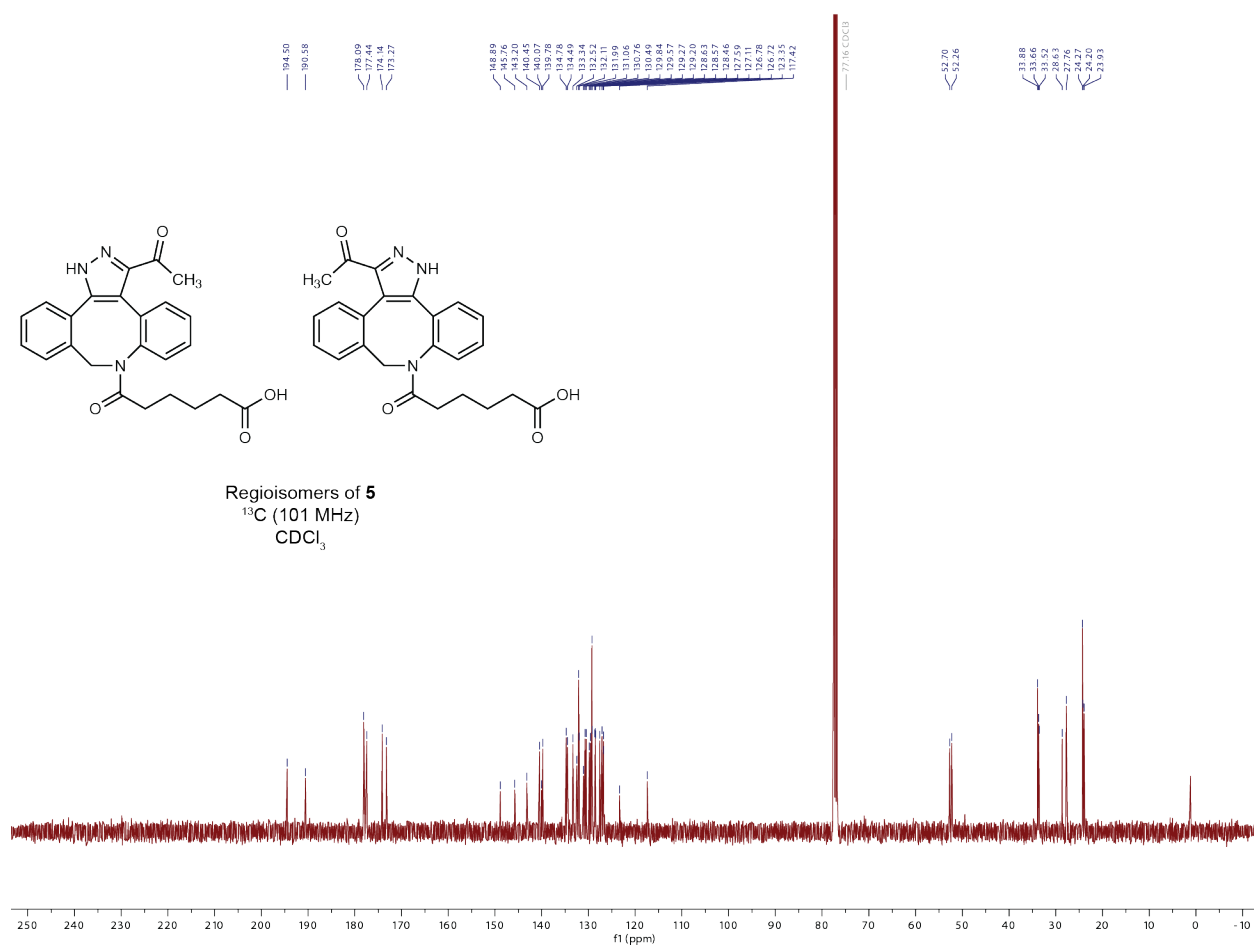
405

406

Supplementary Figure 32: ^1H NMR spectrum of regioisomers of **5.**

407

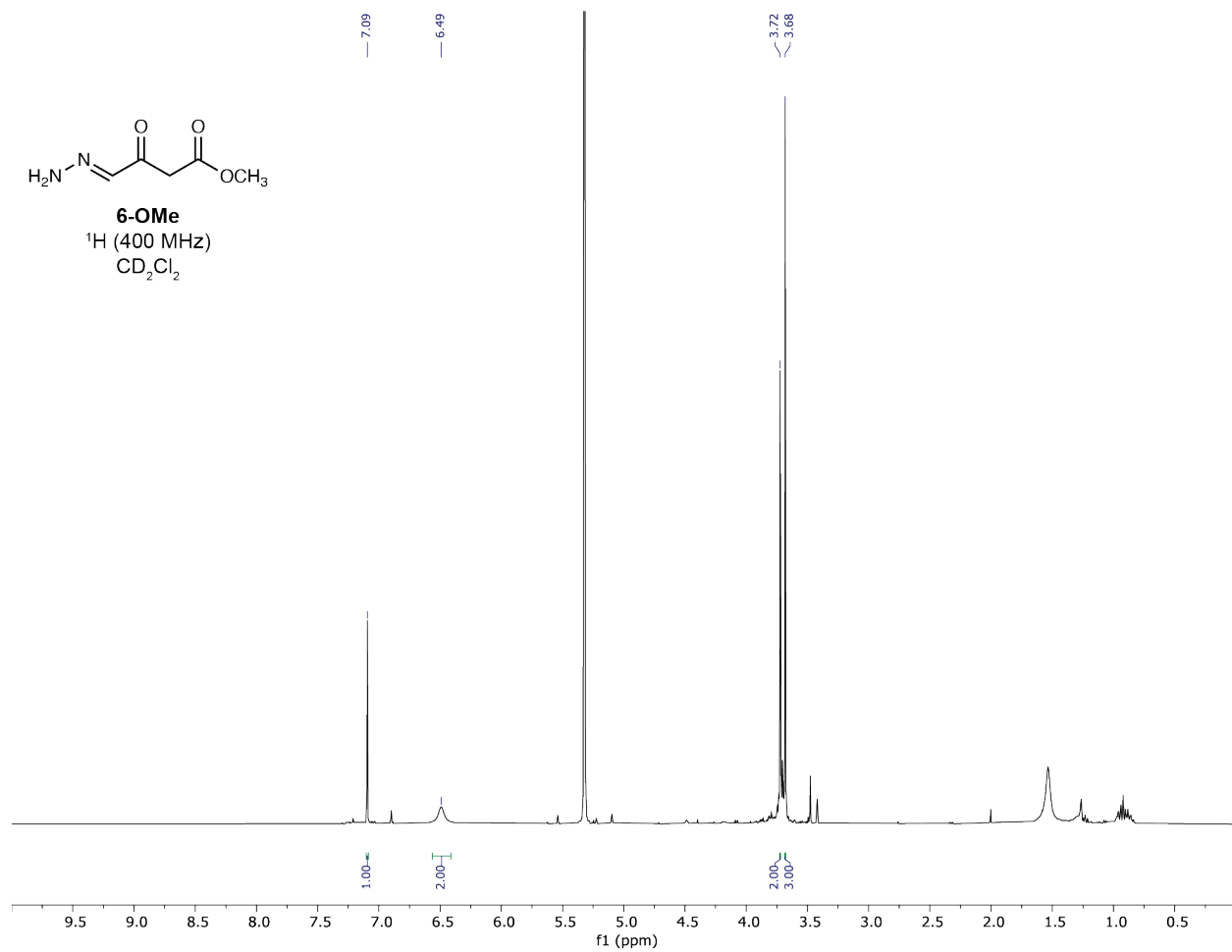
408



409

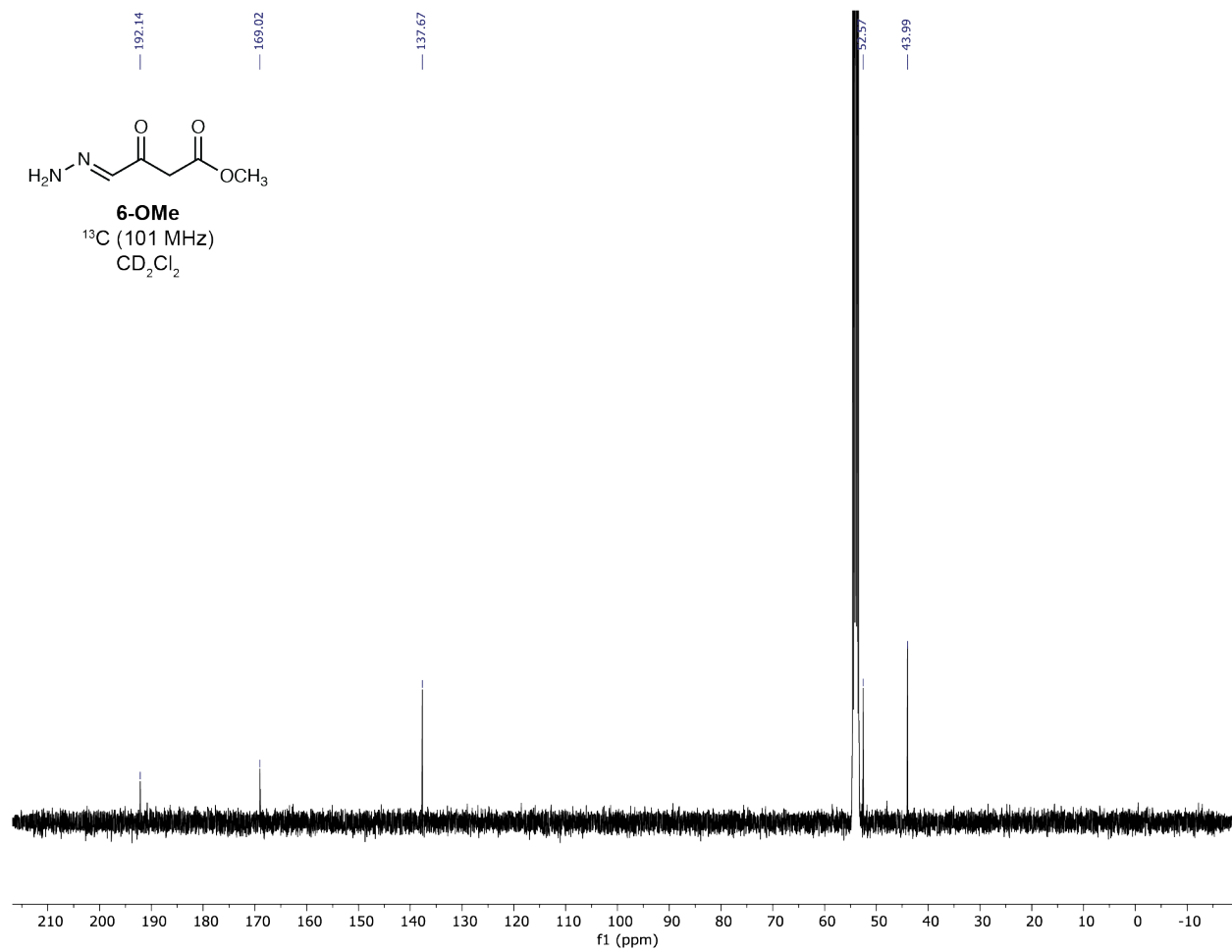
410

Supplementary Figure 33: ^{13}C NMR spectrum of regioisomers of **5**.



411

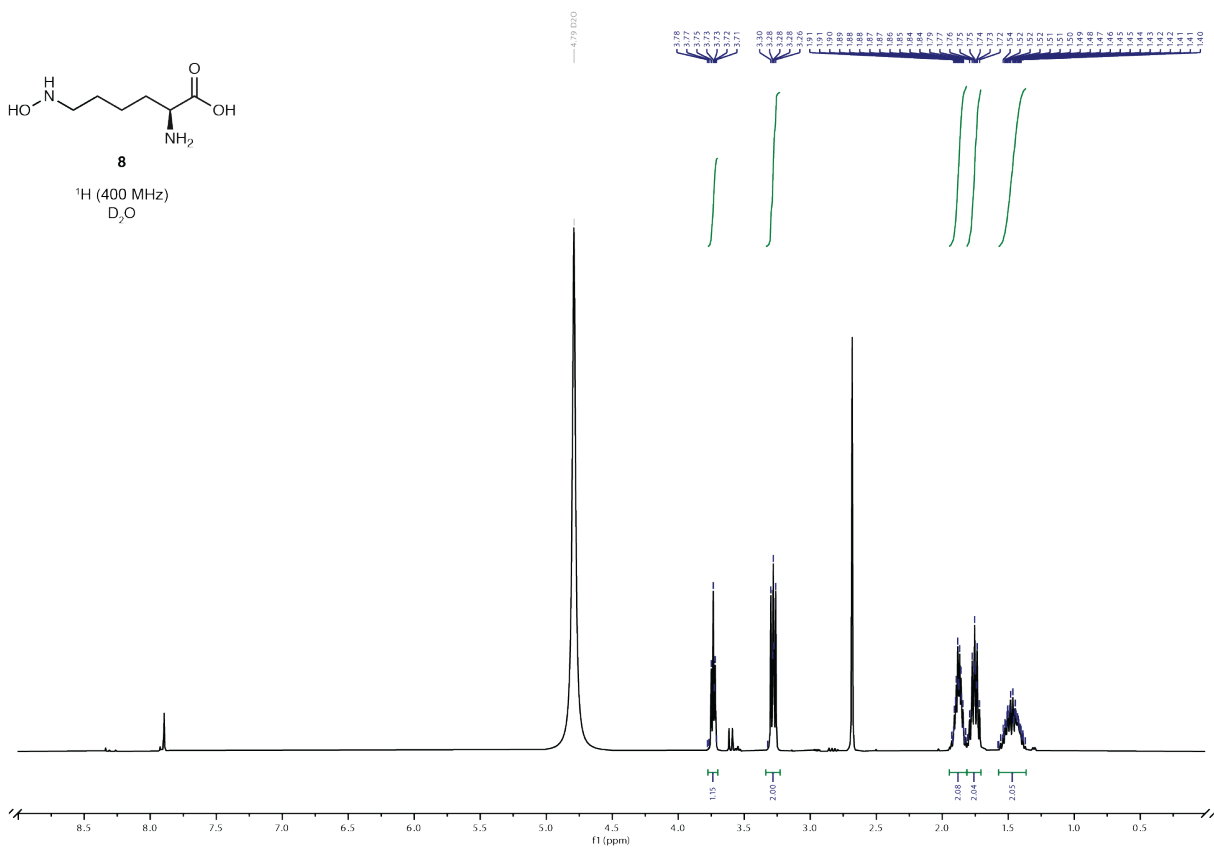
412 **Supplementary Figure 34:** ^1H NMR spectrum of **6-OMe**.



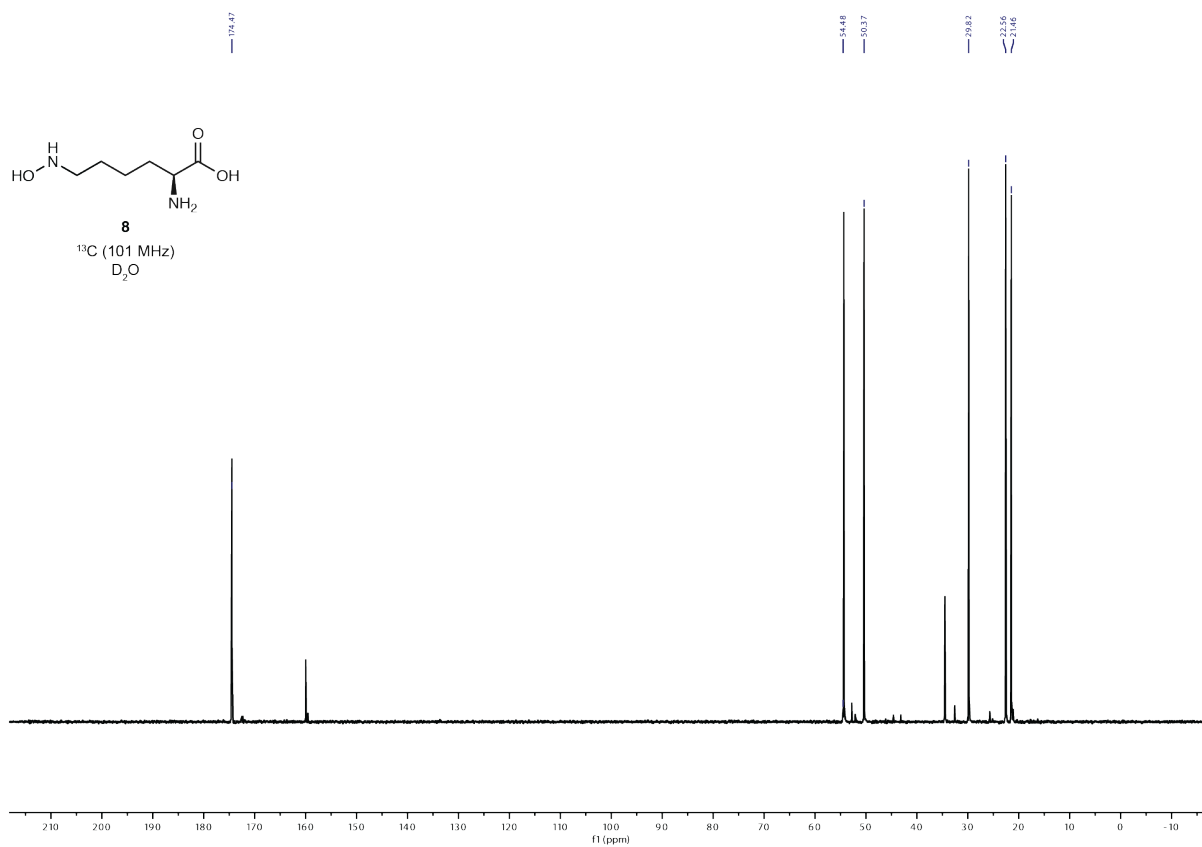
413

414 **Supplementary Figure 35:** ^{13}C NMR spectrum of **6-OMe**.

415



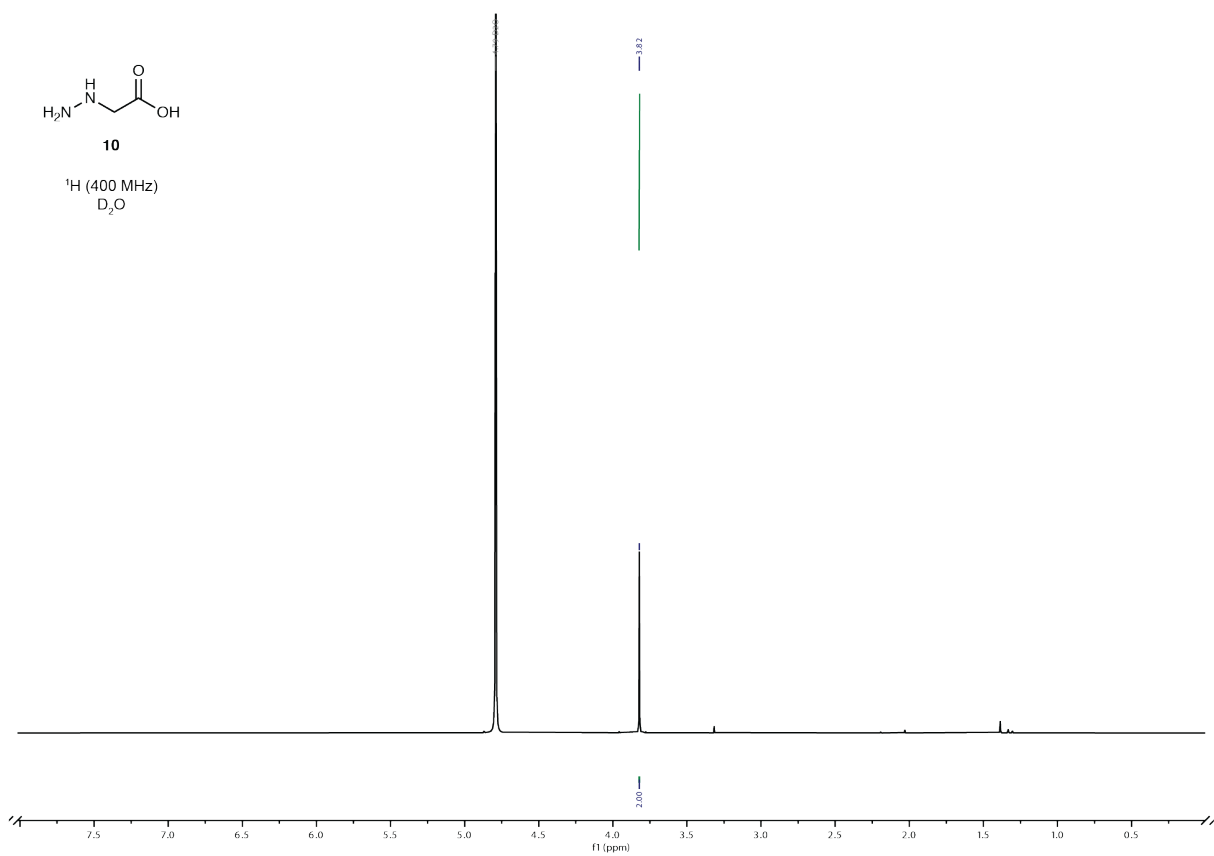
Supplementary Figure 36: ^1H NMR spectrum of **8.**



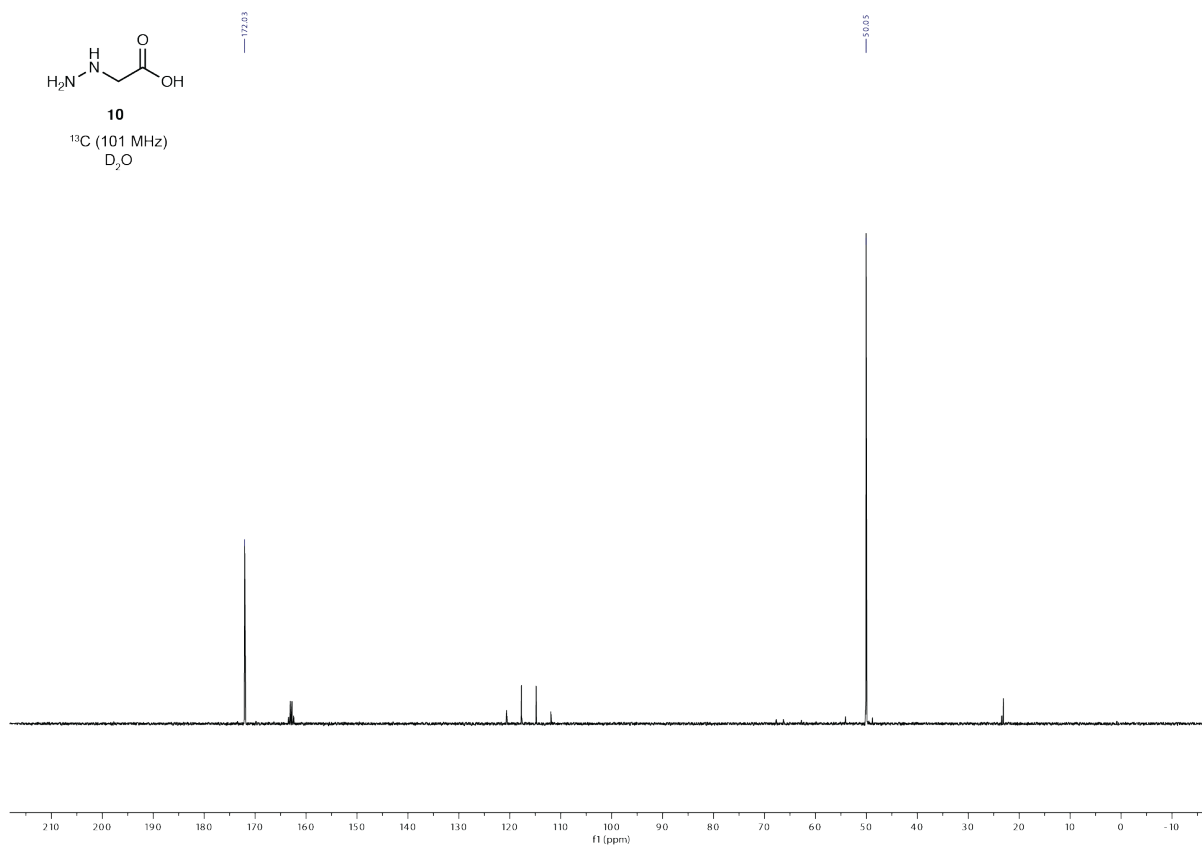
419

420

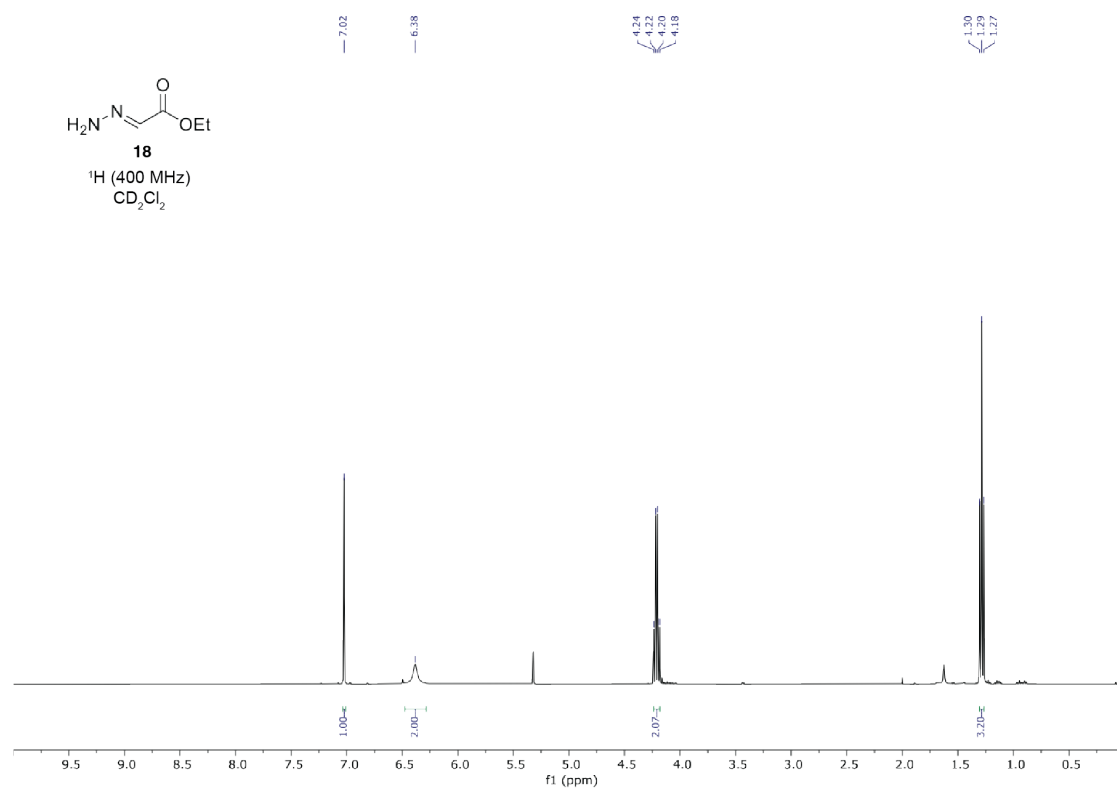
Supplementary Figure 37: ^{13}C NMR spectrum of **8**.



Supplementary Figure 38: ¹H NMR spectrum of 10.

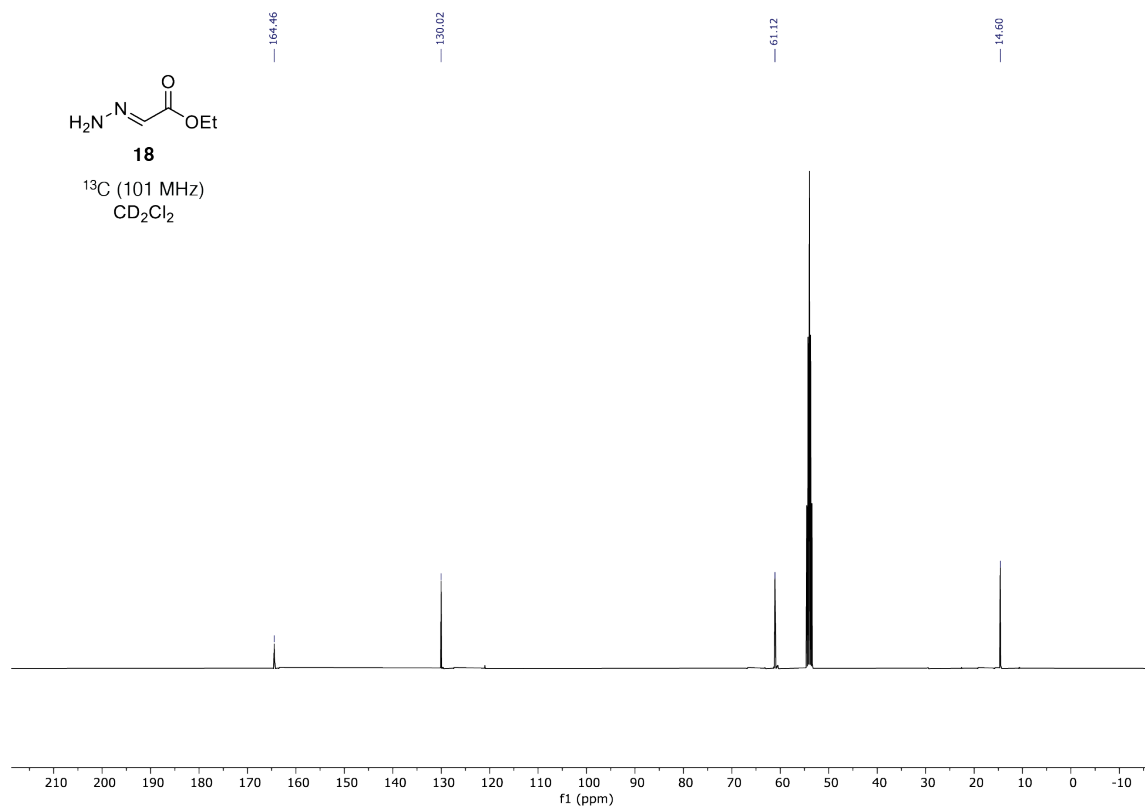


Supplementary Figure 39: ^{13}C NMR spectrum of 10.



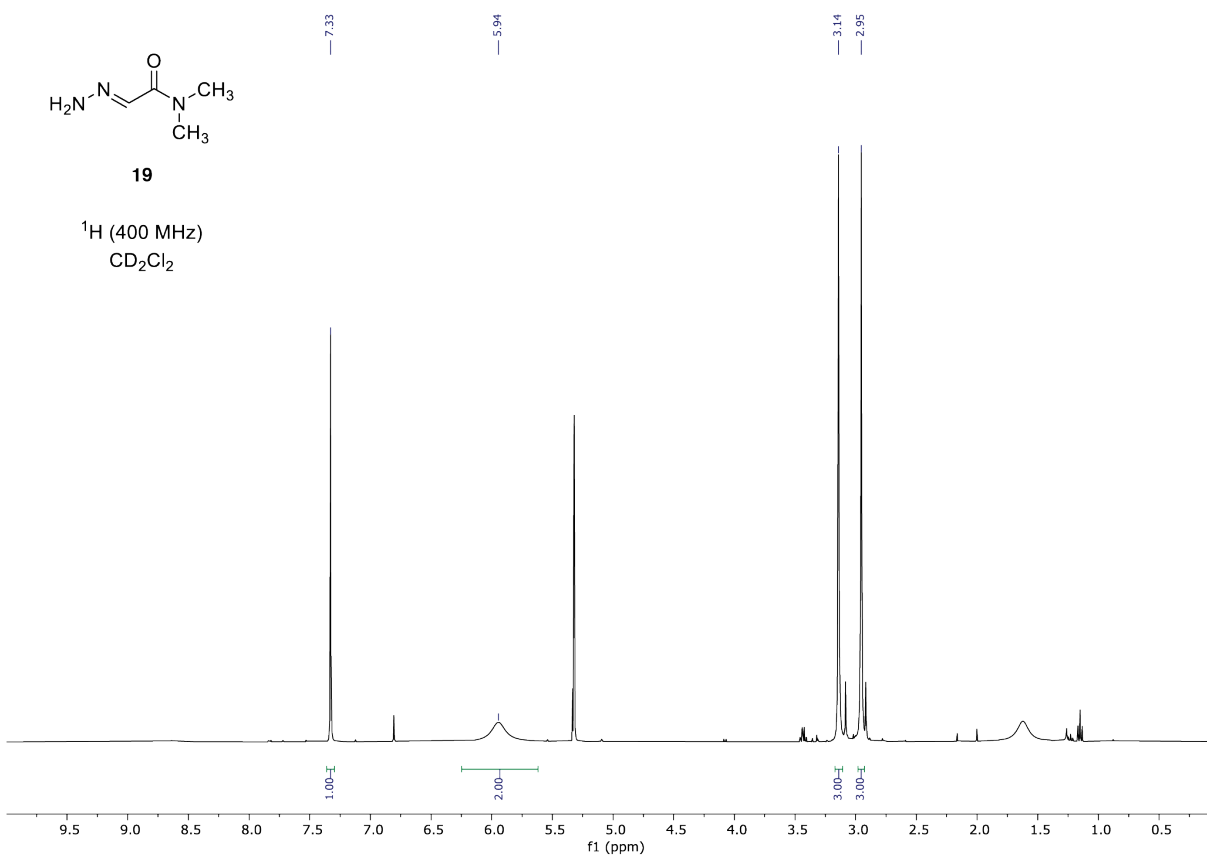
427

428 **Supplementary Figure 40:** ¹H NMR spectrum of **18**.



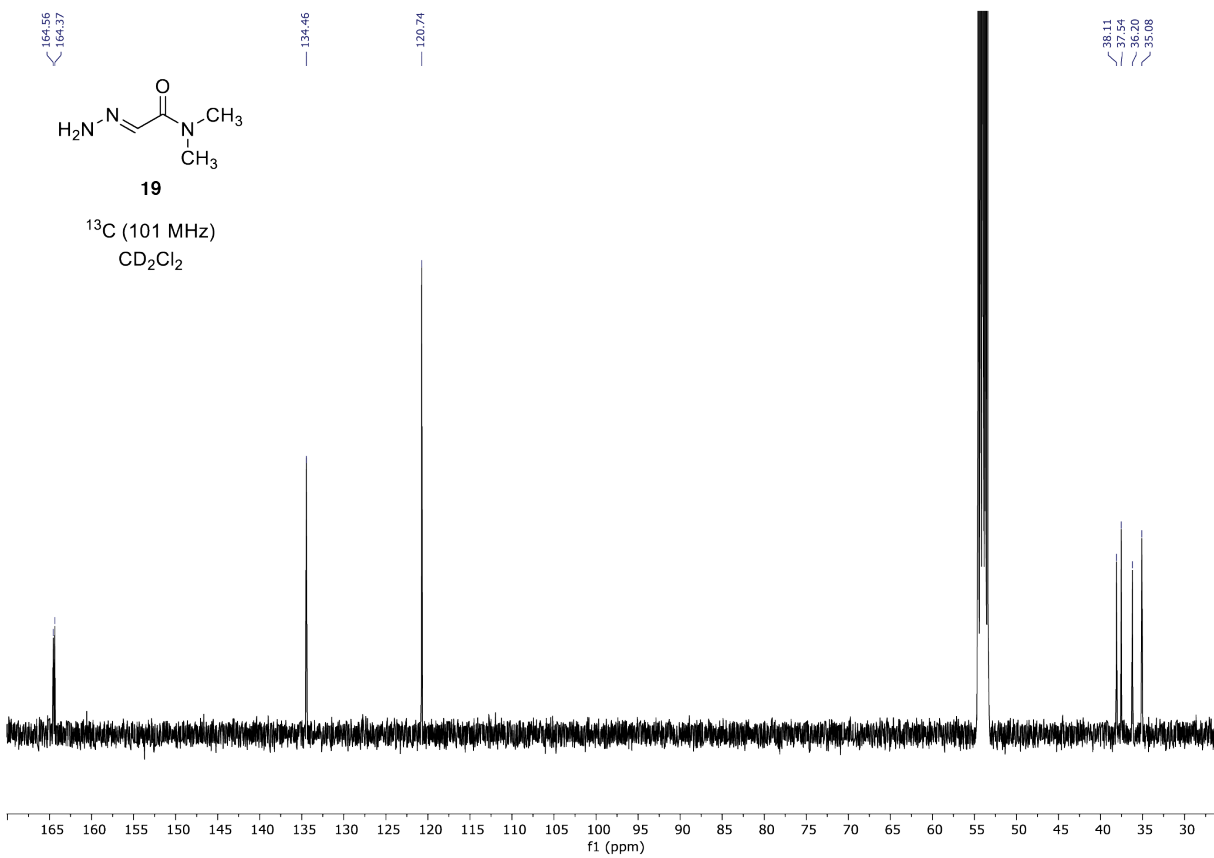
429

430 **Supplementary Figure 41:** ^{13}C NMR spectrum of **18**.



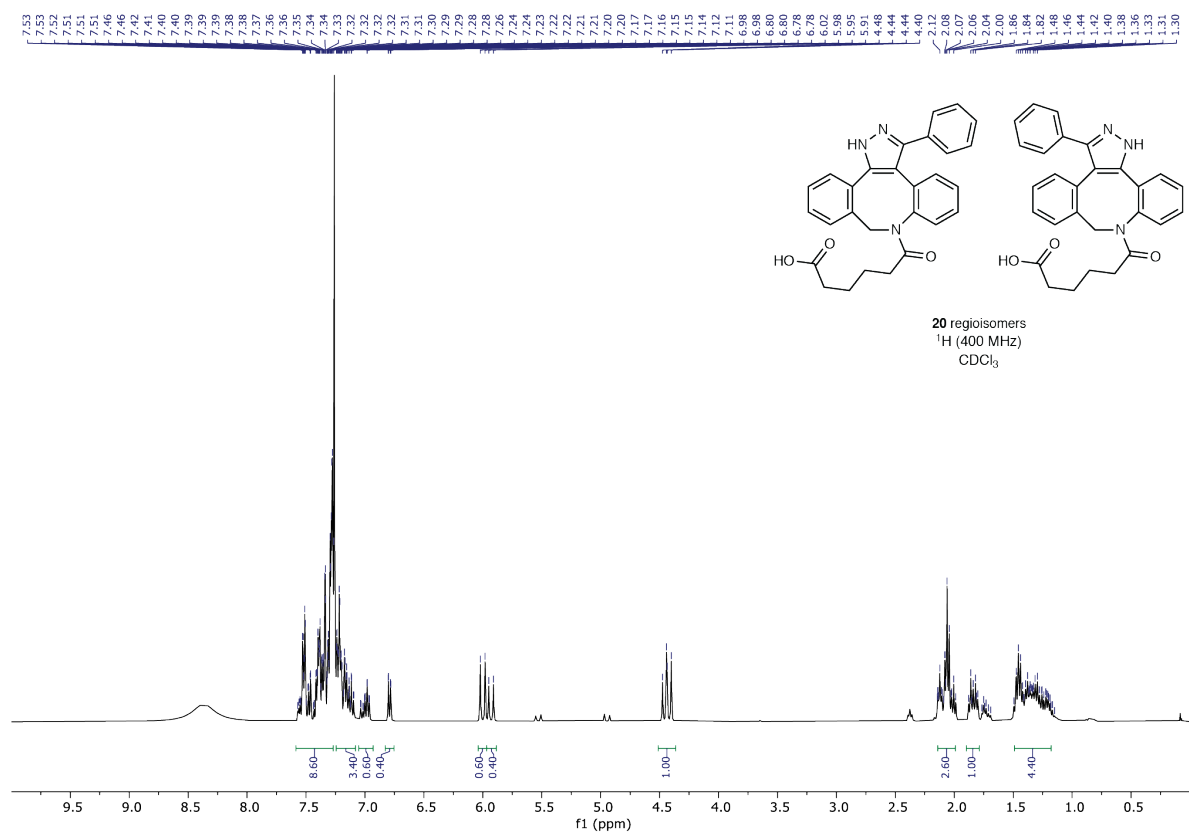
431

432 **Supplementary Figure 42: ^1H NMR spectrum of **19**.**



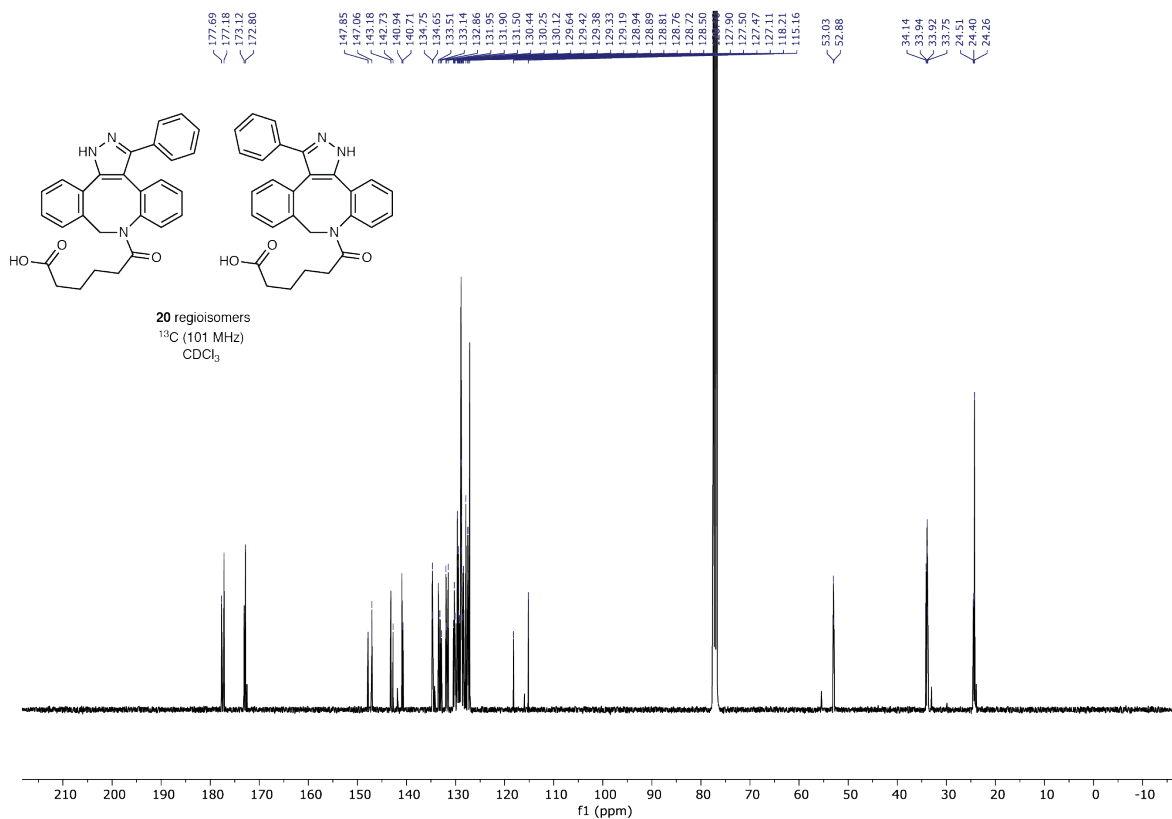
433

434 **Supplementary Figure 43:** ^{13}C NMR spectrum of **19**.



435

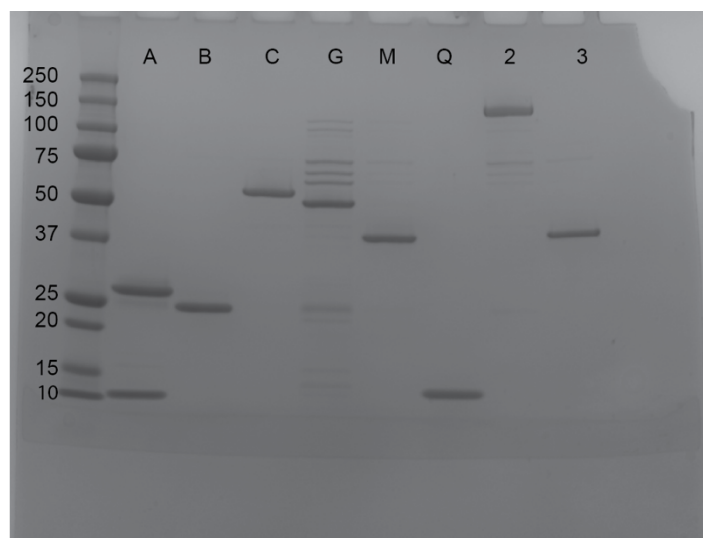
436 **Supplementary Figure 44:** ¹H NMR spectrum of **20**.



437

438 **Supplementary Figure 45:** ^{13}C NMR spectrum of **20**.

439



440

441 **Supplementary Figure 46:** Raw gel image.

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