Supporting Information

Total enzyme syntheses of napyradiomycins A1 and B1

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Experimental Procedures

Materials and methods

All chemicals used were purchased from commercial suppliers (Acros, Sigma Aldrich, Fluka, Oakwood or Alfa Aesar) and used as received unless otherwise noted. Analytical grade solvents were purchased and used without further purification. NMR spectra were recorded on a Bruker Avance III spectrometer (600 MHz) using either a 1.7 mm inverse detection triple resonance (H-C/N/D) cryoprobe or a 5 mm inverse detection triple resonance (H-C/N/D) cryoprobe, or a JEOL spectrometer (500 MHz) in the solvents indicated. Chemical shifts signals (δ) are reported in ppm and are referenced to the internal chloroform (δ = 7.26 ppm for ^{1}H, δ = 77.2 ppm for ^{13}C), or acetone signal at (δ = 2.05 ppm for ^{1}H, δ = 29.8 ppm for ^{13}C). Data for NMR spectra was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved, br = broad signal, *J* = coupling constant(s) in Hz. All *J* values were rounded to the nearest 0.1 Hz. High-resolution ESI mass spectra were recorded on an Agilent-6230 TOF LC-MS.

LC-MS was carried out on an Agilent Technologies 1200 Series system with a diode-array detector coupled to an Agilent Technologies 6530 Accurate-Mass Q-TOF mass spectrometer in negative ion mode. Analytes were separated by reversed-phase chromatography on an Agilent Technologies Eclipse XDB-C18, 5μm-150 x 4.6 mm column at a flow rate of 0.75 mL/min with a mobile phase combination of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B) using a gradient as follows: 10% to 100% (B), 0 to 20 min; 100% (B), 20 to 24 min; 100% to 10% (B), 24 to 27 min; 10% (B), 27 to 30 min. Semi-preparative HPLC was carried out on an Agilent Technologies 1200 Series system with a multiple wavelength detector using the methods described in the respective sections.

Chemical synthesis

All organic substrates (THN (**1**),[1] GPP,[2] DMAPP[2]) and napyradiomycin biosynthetic intermediates (**4**,[3] **5**,[4] **6**,[4] and **7**[4]) were synthesized following established literature protocols and matched previous spectral characterization.

Cloning, heterologous expression, and purification of previously characterized Nap enzymes

NapH1,[5] NapT8,[4] and NapH3[4] were cloned, expressed, and purified as previously described.

Cloning of *Streptomyces* sp. CNQ-525 *napH4* and *napT9*

The open reading frame encoding *Streptomyces* sp. CNQ-525 NapH4 (see Figure S1 for complete gene sequence used in this study) was amplified from chromosomal DNA by PCR using the following primers: 5'-ATAGGATCCATGACGACATCCGGACACACC-3' (forward), and 5'-AATACTCGAGGCCGTCGCCCTCGCCCTTG-3' (reverse) at an annealing temperature of 64 °C. The resulting PCR product was digested with the restriction enzymes XhoI and NdeI, and subsequently ligated into the similarly digested vector pASK-IBA43plus (IBA) for expression of a NapH4 containing an N-terminal His_6- tag and a C-terminal Strep-tag II. For expression in *Streptomyces*, the *napH4* gene, with flanking tags described above, was amplified from pASK-IBA43plus:*napH4* using the following primers:5'-TGAAAGGAATGAGCATATGGCTAGCAGAGGATCGC -3' (forward), 5'-GAACTAGTGCTAGCAAGCTTATTATTTTTCGAACTGCGGGTG-3' (reverse). The amplified fragments were assembled with the vector pHSA81[6] (previously linearized by digestion with NdeI and HindIII) using Gibson Master Mix (New England Biolabs) and the corresponding Gibson cloning protocol provided by the manufacturer.

The open reading frame encoding *Streptomyces* sp. CNQ-525 NapT9 (protein accession number: ABS50490) was amplified from chromosomal DNA by PCR using the following primers: 5'-ATACATATGTCCGGAGCTACTGGGGCGG-3' (forward), and 5'-ATAGGATCCTCACGAGGCTTGGCCTTCTTCCGC-3' (reverse) at an annealing temperature of 70 °C.

The resulting PCR product was digested with the restriction enzymes NdeI and BamHI, and subsequently ligated into the similarly digested vector pET28a (Novagen) for expression of His_6-NapT9.

Expression of NapH4 in *Streptomyces lividans TK23*

The pHSA81:*napH4* vector was introduced into *S. lividans* TK23 by the standard protoplast-PEG method.[7] Spores of *S. lividans* TK23 harboring pHSA81:*napH4* were then spread onto a Bennett's maltose plate containing 40 μg/ml of thiostrepton, and grown at 30 °C until sporulation was apparent. Approximately 4 cm^2 cuttings of the sporulated plate were used to inoculate 10-50 mL flasks (containing springs for added aeration, one cutting/flask) of yeast extract/malt extract liquid medium (1.0% glucose, 34% sucrose, 0.3% yeast extract, 0.3% malt extract, 0.5% bacto peptone, 0.1% $MgCl_2 \cdot 6H_2O$, pH 7.0) including 10 μg/ml of thiostrepton at 28 °C for ~5 days (until cells became dense). Cells were harvested by centrifugation (5000 × *g*) for 20 min, washed once with buffer A (see NapH4 purification for buffer components), then centrifuged again as before. The pellet was then used directly for purification as described.

Expression of NapT9 in *Escherichia coli*

E. coli BL21-Gold(DE3) cells (Agilent Technologies) containing the pET28a:*napT9* vector were grown in 4 L of TB broth containing 50 μg/mL kanamycin at 37 °C until an OD_{600} of approximately 0.5, at which time the temperature was lowered to 18 °C. Cells were then grown to an OD_{600} of approximately 0.6-0.8 and expression was induced by addition of 0.1 mM IPTG (final concentration). Cells were grown overnight at 18 °C, harvested the next day by centrifugation (5000 × *g*), and the pellets reserved and frozen at -80 °C.

Purification of NapH4

The cell pellet from ~500 mL of growth was resuspended in ~100 mL of a solution containing 50 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 40 mM imidazole (buffer A), with additional 1 mM phenylmethanesulfonyl fluoride and 1 mg/mL lysozyme added (both final concentrations, added as solids). The solution was then sonified on ice with constant stirring using a Branson digital sonifier (40% amplitude), 15 sec pulse on/45 seconds pulse off, for 12 total min "on" time. The lysate was centrifuged for 30 min at 18000 × *g* to pellet insoluble material and the supernatant was passed through a 0.8 μm filter. The cleared lysate was then loaded onto a 5 mL HisTrap FF column (GE Healthcare) equilibrated prior in buffer A. The column was washed with 50 mL of buffer A, and the target protein was eluted in a linear gradient of 0-100% buffer B (buffer A containing 0.5 M imidazole) over 75 mL. Fractions containing the target protein were identified by SDS-PAGE, pooled, and concentrated to <2 mL total volume using an Amicon Ultra-15 10 kDa cutoff concentrator (Millipore) by centrifugation at 3500 × *g,* 4 °C. The concentrated protein was then loaded onto a Superdex 200 gel filtration column (16 cm × 60 cm, GE Healthcare) equilibrated prior in 25 mM HEPES-NaOH pH 8.0, 300 mM NaCl, and 10% glycerol, and eluted at a constant flow rate of 1.0 mL/min. Fractions containing the target protein were identified using SDS-PAGE, pooled, and concentrated as described above. Protein aliquots were frozen on dry ice and stored at -80 °C. The protein concentration was determined using the Bradford method using bovine serum albumin as a standard.

Purification of NapT9

The cell pellet from ~3 L of growth was resuspended in ~100 mL of a solution containing 50 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 25 mM imidazole (buffer A2), with additional 1 mM phenylmethanesulfonyl fluoride added (final concentration, added as solid). The solution was then sonified on ice with constant stirring using a Branson digital sonifier (40% amplitude), 15 sec pulse on/45 seconds pulse off, for 12 total min "on" time. The lysate was centrifuged for 30 min at 18000 × *g* to pellet insoluble material. The cleared lysate was then loaded onto a 5 mL HisTrap FF column (GE Healthcare) equilibrated prior in buffer A2. The column was washed with 50 mL of buffer A2, and the target protein

was eluted in a linear gradient of 0-100% buffer B2 (buffer A2 containing 0.5 M imidazole) over 75 mL. Fractions containing the target protein were identified by SDS-PAGE, pooled, and concentrated to <2 mL total volume using an Amicon Ultra-15 10 kDa cutoff concentrator (Millipore) by centrifugation at 3500 × *g,* 4 °C. The concentrated protein was then loaded onto a Superdex 75 gel filtration column (16 cm × 60 cm, GE Healthcare) equilibrated prior in 25 mM HEPES-NaOH pH 8.0, 300 mM NaCl, and 10% glycerol, and eluted at a constant flow rate of 1.0 mL/min. Fractions containing the target protein were identified using SDS-PAGE, pooled, and concentrated as described above. Protein aliquots were frozen on dry ice and stored at -80 °C. The protein concentration was determined using the Bradford method using bovine serum albumin as a standard.

Monochlorodimedone (MCD) assay on NapH4 – pH experiments

This procedure followed previously established literature protocols.[3,8] The absorbance at 290 nm of a 1 mL solution of 50 mM MES-NaOH, pH 6.0, 200 mM KCl and 10 μM sodium orthovanadate in a quartz cuvette was initially set to zero (Cary 60 UV-Vis spectrophotometer, Agilent). To a 1 mL solution of 50 mM buffer (either MES-NaOH, pH 6.0 or HEPES-NaOH, pH 8.0), 200 mM KCl, 50 μM monochlorodimedone, and 10 μM sodium orthovanadate, His_6-NapH4-Strep (10 μg) was added and transferred to a quartz cuvette. The absorbance at 290 nm of the solution was recorded every 1 second using a kinetic scanning program for 2 minutes to obtain a baseline absorbance. At 2 minutes, 1 mM hydrogen peroxide (final concentration) was added and monitored for 5 additional minutes. After no noticeable decrease in absorbance was observed, 200 mM KBr (final concentration) was added and monitored for an additional 8 minutes.

Monochlorodimedone (MCD) assay on NapH4 – vanadium dependency

To a 1 mL solution of 50 mM MES-NaOH, pH 6.0, 200 mM KCl, 200 mM KBr, 50 μM monochlorodimedone, and 1 mM hydrogen peroxide, His_6-NapH4-Strep (10 μg) was added and transferred to a quartz cuvette. The absorbance at 290 nm of the solution was recorded every 1 second using a kinetic scanning program for 2 minutes to obtain a baseline absorbance. After no noticeable decrease in absorbance was observed, 10 μM sodium orthovanadate (final concentration) was added and monitored for 8 additional minutes.

NapH4 *in vitro* activity and requirement assays

Unless noted, assays (0.1 mL) contained 50 mM HEPES-NaOH (pH 8.0) or 50 mM MES (pH 6.0), 150 mM KCl, 0.1 mM Na_3VO_4, 2.0 mM H_2O_2, 0.25 mM napyradiomycin A1 (**2**), and initiated by addition of 5 μM NapH4. Reactions were allowed to incubate for 30 min at room temperature, and quenched by EtOAc extraction (1.0 mL, twice). The solvent was evaporated, and the residue was brought up in 0.1 mL MeOH. 25 μL was injected onto the LC-MS and analyzed as described in the analysis section. All control reactions were conducted with water in place of enzyme and all standards were dissolved in MeOH to the same concentration present in the respective assays prior to direct analysis on the LC-MS.

Preparative scale reaction of napyradiomycin B1 (3)

A 5.0 mL reaction containing 50 mM HEPES-NaOH pH 8.0, 150 mM KCl, 0.1 mM M Na_3VO_4, 4.0 mM H_2O_2, 5% glycerol, and 2.0 mg of napyradiomycin A1 (**2**) dissolved in 100 μL DMSO was initiated by addition of ~2 mg of NapH4. The reaction was then gently stirred at room temperature for ~3 h. The reaction was quenched by extraction with EtOAc (3x) and the solvent was removed. The crude mixture was resuspended in methanol and purified by reversed-phase chromatography on a semi-preparative HPLC using a Phenomenex Luna C18(2), 5μm-250 x 10 mm column at a flow rate of 2.5 mL/min with a mobile phase combination of water + 0.1% trifluoroacetic acid (A) and acetonitrile + 0.1% trifluoroacetic acid (B) using a 90% (B) isocratic elution. The solvent was removed and the compound was further purified by silica flash chromatography (2:1 hexanes:EtOAc) to remove HPLC grease and concentrated *in vacuo*, isolating **3** (1.9 mg, 89%) as a light orange solid.

NapT9 and NapT9/NapH1 coupled *in vitro* activity and requirement assays

Unless noted, assays (0.1 mL) contained 50 mM HEPES-NaOH pH 8.0, 150 mM KCl, 10 mM $MgCl_2$, 1.0 mM geranyl pyrophosphate ammonium salt (dimethylallyl pyrophosphate ammonium salt or isopentenyl pyrophosphate trilithium salt used as indicated), 5.0 mM dithiothreitol (DTT), 1.0 mM THN (**1**), and were initiated by addition of 20 μM NapT9. Reactions conducted without $MgCl_2$ also contained 5.0 mM ethylenediaminetetraacetic acid (EDTA). Reactions were allowed to incubate for 1 h at room temperature, and quenched by EtOAc extraction (1.0 mL, twice). If analyzed by LC-MS, the residue from the reaction was suspended in 0.1 mL MeOH. Otherwise the residue was resuspended in 2 μL DMSO for the subsequent NapH1 reaction. NapH1 assays (0.1 mL) contained 50 mM HEPES-NaOH pH 8.0, 150 mM KCl, 0.1 mM Na_3VO_4, 7.0 mM H_2O_2, and the NapT9 reaction extract resuspended in 2 μL DMSO, and were initiated by the addition of 20 μM NapH1. The reaction was then allowed to incubate at room temperature for 2 h and was quenched by EtOAc extraction as described above. For the NapH1 reactions using synthetic 4-geranyl THN (**4)** as a substrate, assays (0.1 mL) contained 50 mM HEPES-NaOH pH 8.0, 150 mM KCl, 2.0 mM H_2O_2, 0.1 mM Na_3VO_4, and 0.5 mM 4-geranyl THN (**4**), and were initiated by addition of 20 μM NapH1. The reaction was then allowed to incubate at room temperature for 2 h and then quenched by EtOAc extraction as described above. For LC-MS analysis, the residue was brought up in 0.1 mL MeOH, and 25 μL was injected and analyzed as described in the analysis section. All control reactions were conducted with water in place of enzyme, and all standards were dissolved in MeOH to the same concentration present in the respective assays prior to direct analysis on the LC-MS.

Preparative scale reaction of NapT9/NapH1 product 5

A 10 mL solution containing 50 mM HEPES-NaOH pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, and 5.0 mM DTT was sparged with argon for 30 min, followed by addition of geranyl pyrophosphate trisammonium salt (0.095 g, 0.26 mmol), and THN (**1**) (0.010 g, 0.052 mmol) dissolved in 100 μL DMSO. The reaction was initiated by addition of ~7 mg of NapT9 and gently stirred at room temperature for ~24 h under a constant stream of argon. Next, 7.0 mM H_2O_2, 0.1 mM Na_3VO_4, and ~2 mg of NapH1 were added and the reaction was allowed to proceed for an additional 8 h. The reaction was quenched by extraction with EtOAc (3x) and the solvent was removed. The crude mixture was resuspended in methanol and purified by reversed-phase chromatography on a semi-preparative HPLC using a Phenomenex Luna C18(2), 5μm-250 x 10 mm column at a flow rate of 2.5 mL/min with a mobile phase combination of water + 0.1% trifluoroacetic acid (A) and acetonitrile + 0.1% trifluoroacetic acid (B) using a gradient as follows: 10% to 100% (B), 0 to 20 min; 100% (B), 20 to 27 min; 100% to 10% (B), 27 to 30 min; 10% (B), 30 to 33 min. The solvent was removed, and the compound was further purified by silica flash chromatography (3:1 DCM:MeOH). The solvent was once again removed resulting in ~1-2 mg of compound.

General procedure for the one-pot preparation of napyradiomycin biosynthetic intermediates and compounds 5, 2, and 3.

Step 1: The initial starting solution for one-pot napyradiomycin assays (50 mM HEPES-KOH (pH 8.0), 150 mM KCl, 5% glycerol, 10 mM $MgCl_2$, 5 mM DTT) was portioned into 1 mL aliquots in 2 mL microcentrifuge tubes and bubbled with Ar gas for 20 seconds. This was followed by the sequential addition of: 50 μM His_6-NapT9; 5.5 mM GPP; 5.0 mM THN (as a freshly prepared DMSO solution); and 0.1 units of *E. coli* inorganic pyrophosphatase (New England Biolabs). The reaction mixture was covered with Ar gas, sealed with parafilm and left at room temperature for 14 h.

Step 2: Following overnight incubation, aqueous solutions of 0.1 mM Na_3VO_4, 10 μM His_8-NapH1, and 5 mM H_2O_2 were sequentially added to the reaction mixture. After 30 minutes, an additional 20 mM H_2O_2 was added, and the reaction mixture was incubated for an additional 5.5 h.

Step 3: To the reaction mixture was sequentially added: 5.5 mM DMAPP; 30 μM His_6-NapT8; 50 μM His_6-NapH3; 10 μM His_8-NapH1; 10 μM His_6-NapH4-Strep; and 20 mM H_2O_2. This solution was then incubated for 4 h. Individual reactions were pooled together, quenched with an equal volume of 1%

v/v formic acid in acetonitrile, and vortexed to mix thoroughly. Precipitated protein was pelleted by centrifugation (4000 x *g*, 5 minutes, 25 °C), and rinsed with additional 1% formic acid in acetonitrile. The decanted supernatant was pooled and concentrated *in vacuo* to remove acetonitrile. The reaction mixture was adjusted to pH ~3 and extracted with chloroform (3 x 25 mL) and ethyl acetate (3 x 25 mL). Pooled organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. Napyradiomycin biosynthetic intermediates (**5**, **2**, or **3**) were purified by reversed-phase chromatography on a semi-preparative HPLC using a Phenomenex Luna C8(2) 5μm-250 x 10 mm column at a flow rate of 3 mL/min using the following method: 50% B (3 min), 50 – 100% B (10 min), 100% B (7 min), 100 – 50% B (2 min), 50% B (3 min), where A = 0.1% aqueous trifluoroacetic acid and B = 0.1% trifluoroacetic acid in acetonitrile. Isolated compounds (9.5, 17.5, and 17.9 min retention times for **5**, **2**, and **3** respectively) were concentrated *in vacuo* and further purified by a small pipette silica column to remove HPLC grease as described in previous sections.

(*S*,*E*)-3-chloro-1-(3,7-dimethylocta-2,6-dien-1-yl)-1,4,5,7-tetrahydroxynaphthalen-2(1*H*)-one (**5**):
THN (**1**) (5.8 mg, 30 μmol, divided over 6 x 1 mL) was subjected to only steps 1 and 2 of the general procedure, isolating **5** (5.1 mg, 45%) as a light brown solid. Characterization closely matched that of synthetic **5**[3,4]: $[\alpha]_D^{26}$ = +4.1 (*c* 0.5 MeOH); ^{1}H NMR (600 MHz, d_6-acetone) δ 8.72 (s, 1H), 6.59 (d, *J* = 2.2 Hz, 1H), 6.14 (d, *J* = 2.1 Hz, 1H), 5.03 (t, *J* = 7.3 Hz, 1H), 4.81 (t, *J* = 8.0 Hz, 1H), 2.96 – 2.92 (m, 1H), 2.46 (dd, *J* = 13.3, 7.5 Hz, 1H), 1.92 (dt, *J* = 7.7, 7.7 Hz, 2H), 1.78 (t, *J* = 8.2 Hz, 2H), 1.62 (s, 3H), 1.56 (s, 3H), 1.27 (s, 3H); HRMS (ESI) calculated for $C_{20}H_{22}{}^{35}ClO_5$ 377.1161, found 377.1159.

napyradiomycin A1 (**2**):
THN (**1**) (9.6 mg, 50 μmol, divided over 10 x 1 mL) was subjected to all steps of the general procedure except omitting His_6-NapH4-Strep, isolating **2** (5.4 mg, 22%) as a light orange solid. Characterization closely matched that of isolated **2**[9] (see Tables S1 and S2 for comparison): $[\alpha]_D^{26}$ = +47 (*c* 0.5 EtOH); ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.83 (s, 1H), 7.16 (d, *J* = 2.5 Hz, 1H), 6.70 (d, *J* = 2.4 Hz, 1H), 6.14 (br s, 1H), 4.94 – 4.87 (m, 1H), 4.71 (t, *J* = 7.9 Hz, 1H), 4.43 (dd, *J* = 11.7, 4.2 Hz, 1H), 2.70 (d, *J* = 8.2 Hz, 2H), 2.48 (dd, *J* = 14.2, 4.3 Hz, 1H), 2.42 (dd, *J* = 14.2, 11.7 Hz, 1H), 1.66 – 1.60 (m, 7H), 1.51 (s, 3H), 1.50 (s, 3H), 1.32 (s, 3H), 1.18 (s, 3H); HRMS (ESI) calculated for $C_{25}H_{29}{}^{35}Cl_2O_5$ 479.1398, found 479.1394.

napyradiomycin B1 (**3**):
THN (**1**) (9.6 mg, 50 μmol, divided over 10 x 1 mL) was subjected to all steps of the general procedure, isolating **3** (4.6 mg, 18%) as an orange solid. Characterization closely matched that of isolated **3**[9] (see Tables S1 and S2 for comparison): $[\alpha]_D^{26}$ = -46 (*c* 0.5 MeOH); ^{1}H NMR (600 MHz, $CDCl_3$) δ 12.04 (s, 1H), 7.14 (d, *J* = 2.5 Hz, 1H), 7.10 (br. s, 1H), 6.73 (d, *J* = 2.5 Hz, 1H), 4.78 (d, *J* = 1.4 Hz, 1H), 4.75 (s, 1H), 4.45 (dd, *J* = 12.0, 4.0 Hz, 1H), 3.80 (dd, *J* = 11.6, 4.4 Hz, 1H), 2.64 (dd, *J* = 15.5, 8.7 Hz, 1H), 2.52 (dd, *J* = 14.1, 3.9 Hz, 1H), 2.35 (dd, *J* = 14.1, 12.0 Hz, 1H), 2.24 (ddd, *J* = 13.2, 4.2, 4.2 Hz, 1H), 2.03 (ddd, *J* = 12.7, 4.6, 3.5 Hz, 1H), 1.99 (d, *J* = 8.6 Hz, 1H), 1.92 (td, *J* = 13.3, 13.3, 4.9 Hz, 1H), 1.75 – 1.68 (m, 1H), 1.61 (d, *J* = 15.2 Hz, 1H), 1.38 (s, 3H), 1.19 (s, 3H), 0.71 (s, 3H), 0.58 (s, 3H); HRMS (ESI) calculated for $C_{25}H_{28}{}^{35}Cl_3O_5$ 513.1008, found 513.1010.

ATGACGACATCCGGACACACCTCTGTCTCAGACTTCTCGCTGGGCCGCAGATCGCTGCTGGTCGGCGGCG
CCTCGGCCGCGTCGCTGTCCATGCTGTCGTTGTCCCGCCCCGCTGCCGCCGCCACCGGCGATGAGCCGCC
CGAGCTGCCCGACCTGGACAAGGACAACTACATCAAGTGGGCCCAGCCCACCGGCGCGCGGACCGGTCAG
TCGGCCAGCGACGCGTTCATCGGCTCGATGGACGTGACCCTCATCCTGTTCATGAACCGCGCGGCGATGC
TGGCGCTGTTCGACGCGCTGGCGCCGTACCACGAGACGGCGGTCGGCATCCACAGCCAGATCCCGCGCCG
CCCCTCCAGCGAGTCGGCGACCAACCGGAACATGAACATCGCCTGTATCCACGCTCAGAACGCCATCTGG
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GAAGAACGACGGCATGAACGTCCTCGGCCACGAGGGCGGCCGCAAGTACAACCCCCGGCCCTGGGCGGAC
TACACCGGCTACGTGCCGACGAACACCGCCTTCAAGCTGAACAACCCCTCGCGCTGGCAGCCGCAGCTCC
AGGCCCACAACGGCCGCCGCGCCGGCGGCGGCCCCGGCGACCTGGGCATCTACGTCACCCAGCACTTCGT
GACCCCGCAGATCGCGGTGACCCTGCCGCACATCTTCAAGGACCCGACCGCGTTCCCGCTTCCCCGGCCC
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CGCTCGACGACGAGCGCAAGGCGCTCGCCGAGATCATGGAGAACAAGCTCTGGGGCATCGGCCACTCGTC
CATCGAGATCGGCCTGAAGAACGACCAGAACAACGAGCTGGGCGTGCACGGCTGGGCGCAGTGGATGCTC
CAGCACATCCTGGCGACCTTCGACACGCTGATCGCCGCCTGGGGATACAAGCGCAAGTACGAAGCCGTGC
GGCCGATCACCGCGGTCAGGCACGTGTACGGCAACCGCAAGATCCGCGCCTGGGGCGGCGTCGGCATGGG
CACGGTCGACGACATCCCGGCCAACGAGTGGGCCGGCTACCTGCCGGTGGGCGACCACCCGGAGTACCCG
TCGGGCTCTACCACCGTGGGCTCCGCCGCCTCGCAGGCGGCGCGGCGCTTCTTCGACTCCGACGACCTGA
ACTGGGAGTTCGACTTCGAGGTCGGCAAGTCCATCGTGGAGCCGGGCATCACCCCCGTCGAGAACGTCCG
GGTGAGCTTCCCCACCTGGACCGACTTCAACAAGAGGTGCGCCTACAGCCGCCTGGACGGCGGCGTGCAC
TTCAAGAAGACGGTCGAGCGGTCCATGGCGTTCGGCGAGCAGTTCGGCGACCTGGCCCACGACTTCATAC
AGCGGCACGTCAAGGGCGAGGGCGACGGCTGA

Figure S1. The gene sequence of NapH4 used in this study. A putative open reading frame in the region of the originally annotated *napH4* was predicted using GeneMark.[10] The expressed open reading frame from 23449 bp to 25020 bp (encoding NapH4) in the *Streptomyces* sp. CNQ-525 *nap* cluster resulted in soluble, functional protein used in this study.

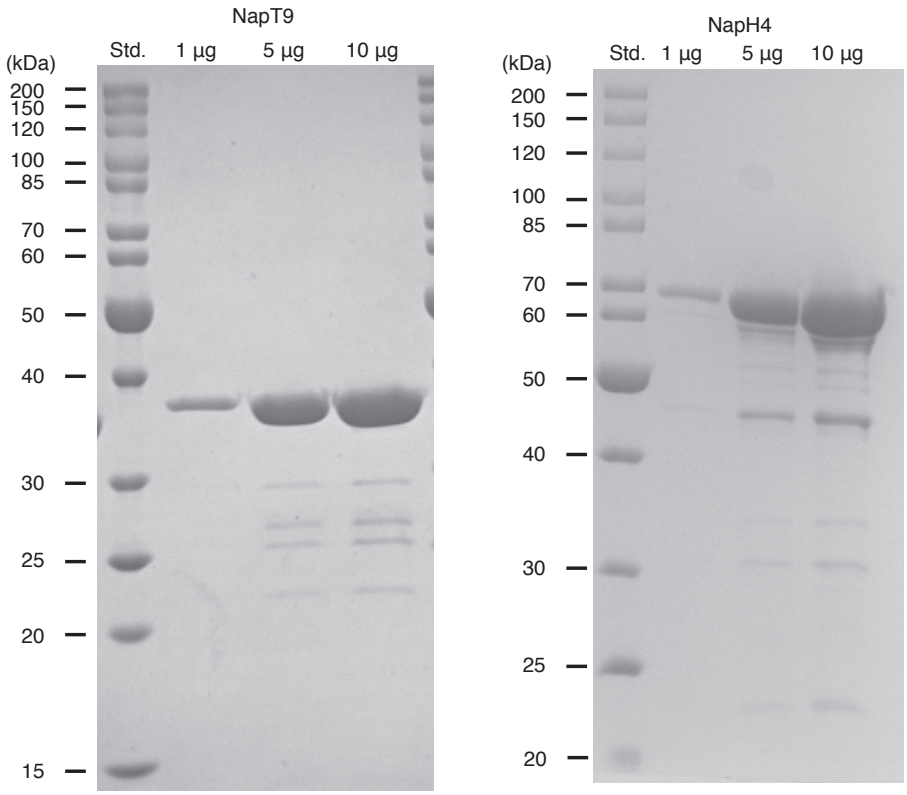


Figure S2. SDS-PAGE gels (12%) of purified NapT9 and NapH4. The bands are consistent with the predicted molecular weights of His_6-NapT9 (35.0 kDa), and His_6-NapH4-Strep (62.7 kDa). Both proteins were judged to be >80% pure based on band intensities.

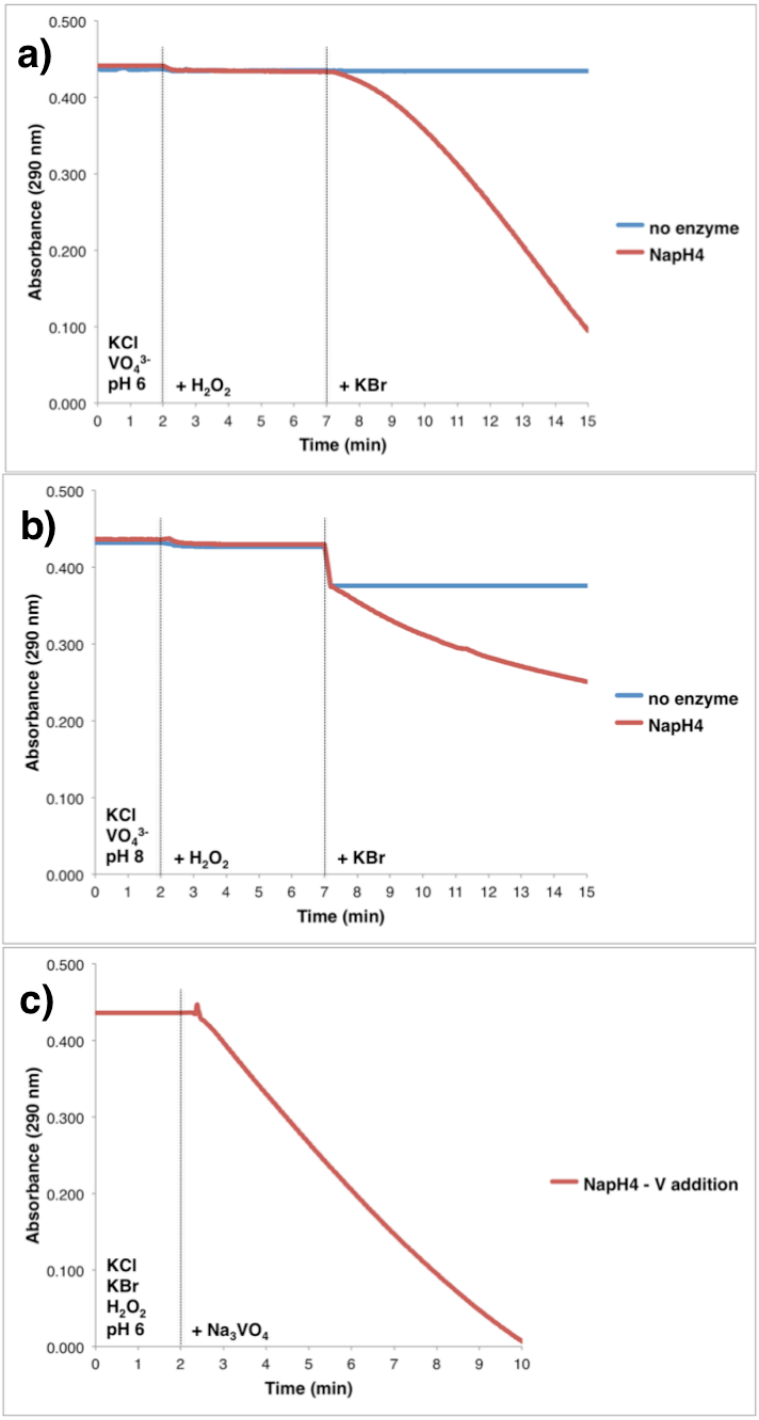
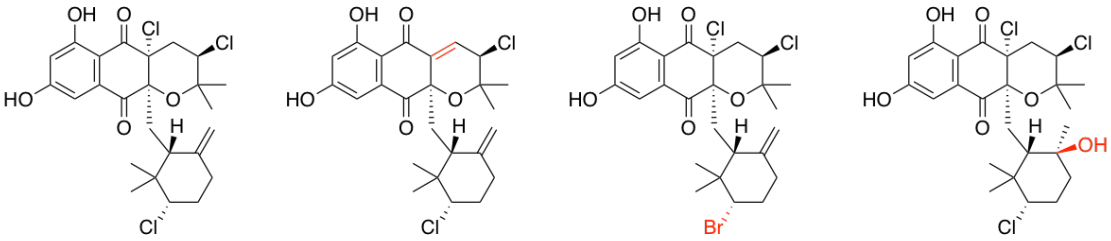


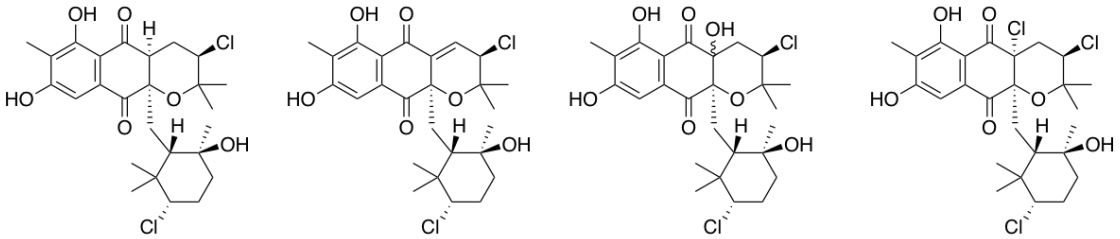
Figure S3. Monochlorodimedone assays on recombinant His_6-NapH4-Strep. Consistent with other characterized *Streptomyces* VCPO enzymes,[3,8] NapH4 shows oxidative activity in this assay only following the addition of bromide ions and at both pH 6.0 (trace a) and pH 8.0 (trace b), albeit at a relatively faster rate at the lower pH. Also consistent with other characterized *Streptomyces* VCPOs, vanadium does not appear to be bound in the active site following heterologous expression, and upon sodium orthovanadate addition to the reaction mixture, negligible time is required to initiate oxidative catalysis (trace c).

a) Napyradiomycin B molecules isolated from *Streptomyces ruber* (formerly *Chainia rubra*):



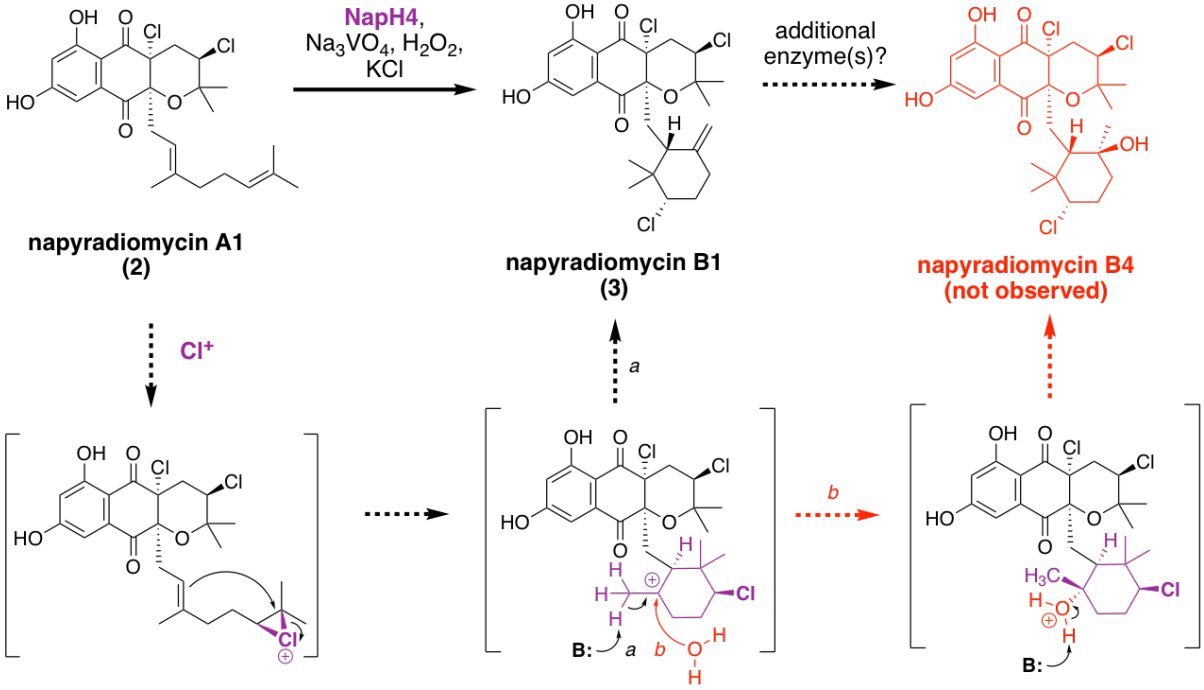
napyradiomycin B1 (3) | napyradiomycin B2 | napyradiomycin B3 | napyradiomycin B4

b) Napyradiomycins isolated from *Streptomyces* sp. CNQ-525 containing the *nap* gene cluster



major compound

c)



napyradiomycin A1 (2) | napyradiomycin B1 (3) | napyradiomycin B4 (not observed)

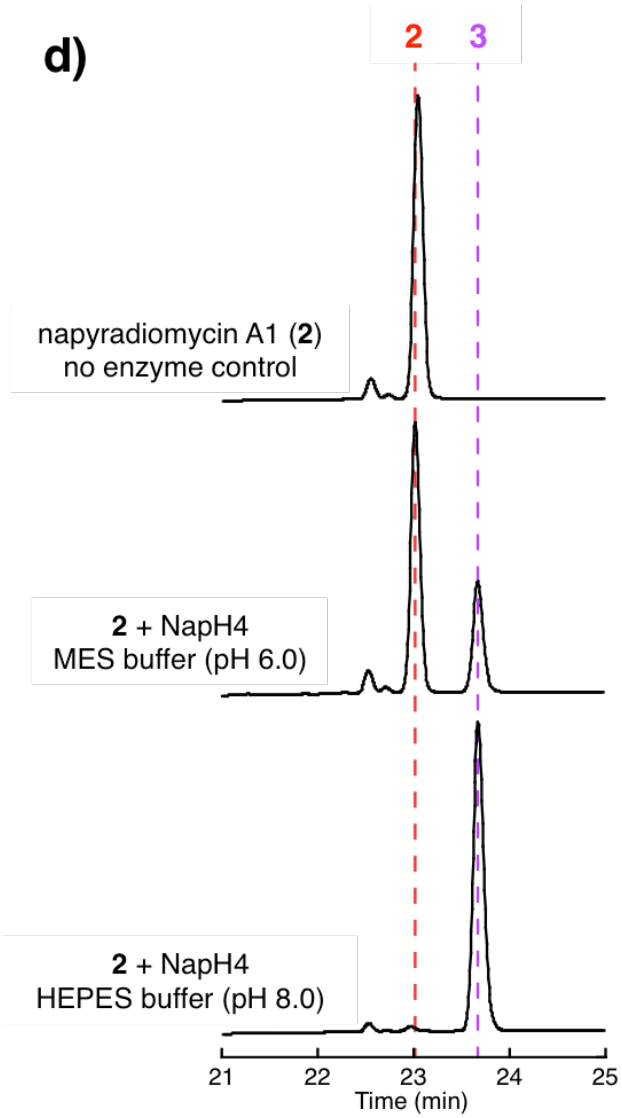


Figure S4. Isolated napyradiomycin B molecules with proposed NapH4 mechanism and pH dependence. **a**) Napyradiomycin B molecules isolated from *Streptomyces ruber* (formerly *Chainia rubra*),[9,11] with structural differences from napyradiomycin B1 (**3**) highlighted in red. **b**) Napyradiomycin molecules isolated from *Streptomyces* sp. CNQ-525 from the *nap* biosynthetic gene cluster, with all compounds having a chlorinated cyclohexanol moiety in the formerly geranyl chain.[12] **c**) Proposed NapH4 mechanism for the formation of either napyradiomycin B1 (**3**) or napyradiomycin B4 via chloronium-induced terpenoid cyclization of the geranyl moiety of napyradiomycin A1 (**2**). In our *in vitro* assay conditions, only deprotonation (path a) to **3** instead of hydration (path b) to napyradiomycin B4 was observed. **d**) Reversed-phase HPLC UV chromatograms (254 nm) of a reaction with NapH4 and **2** in either HEPES pH 8.0 or MES pH 6.0 as the buffer. Based on the stop-point assays, the enzyme is more active when assayed at pH 8.0, however the only product observed at either pH is **3**.

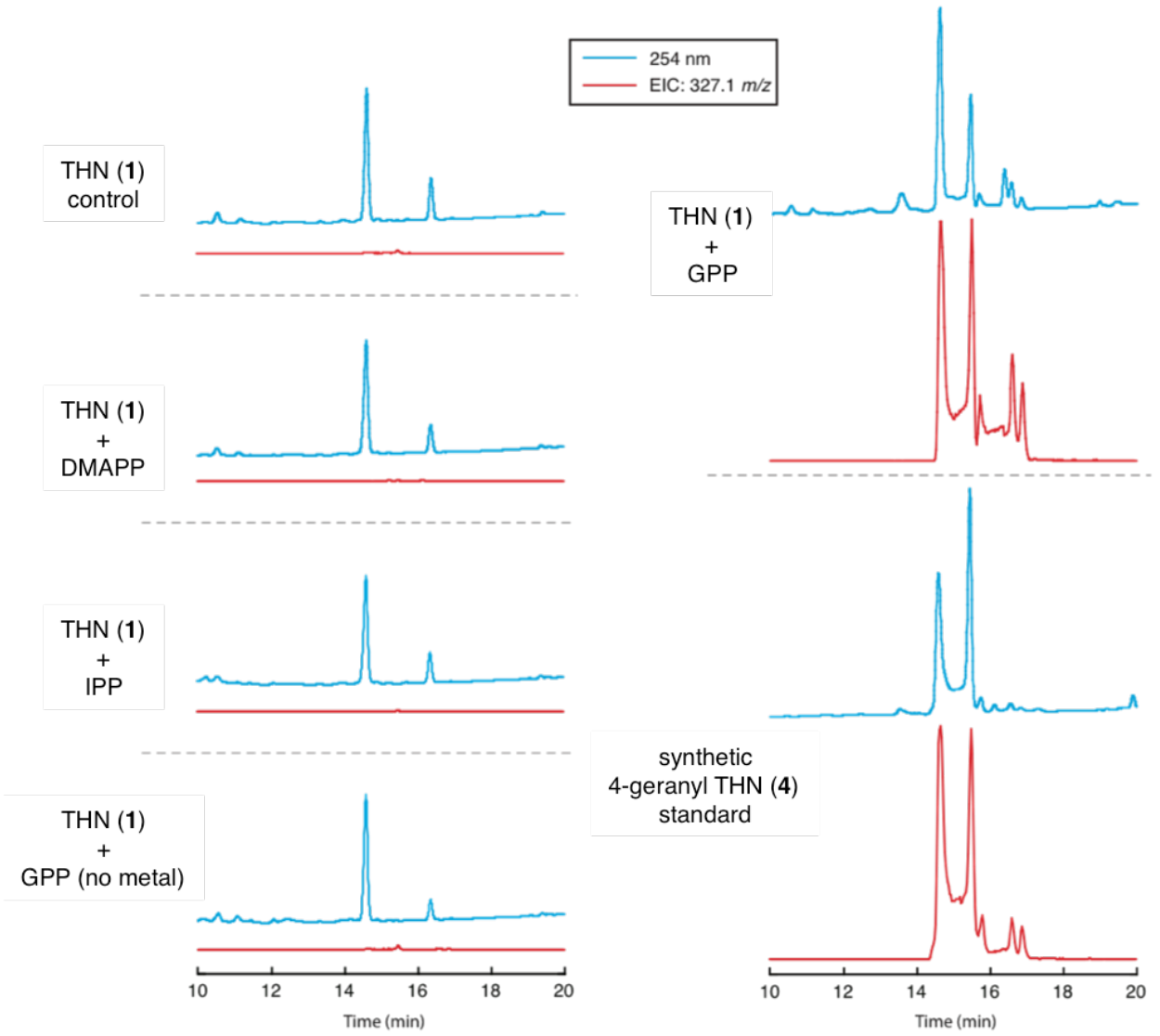
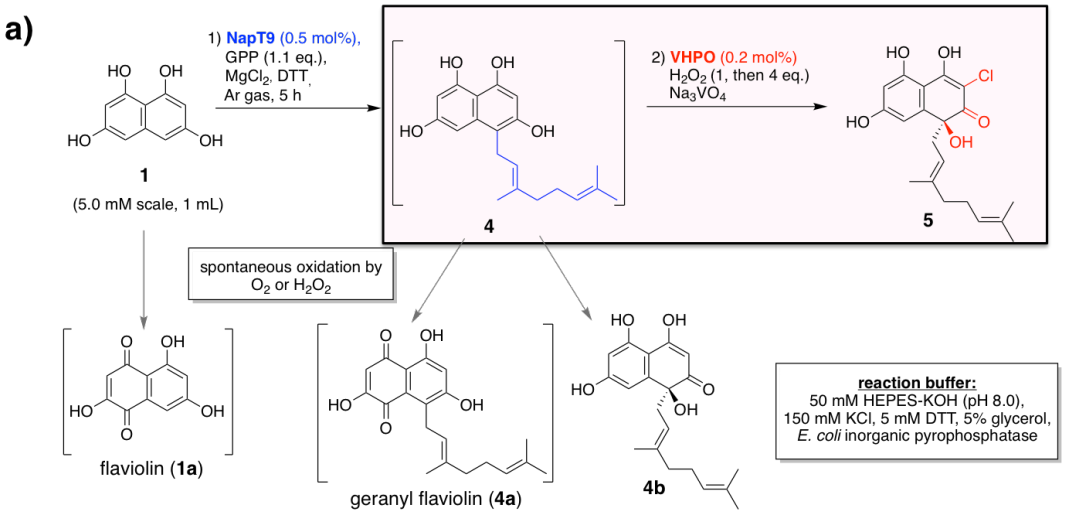
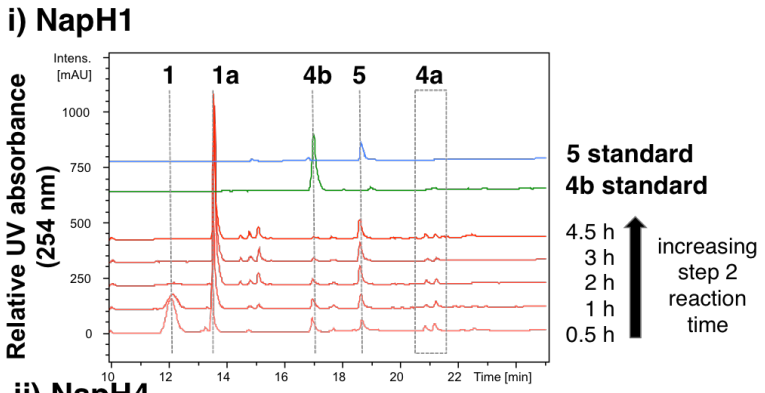
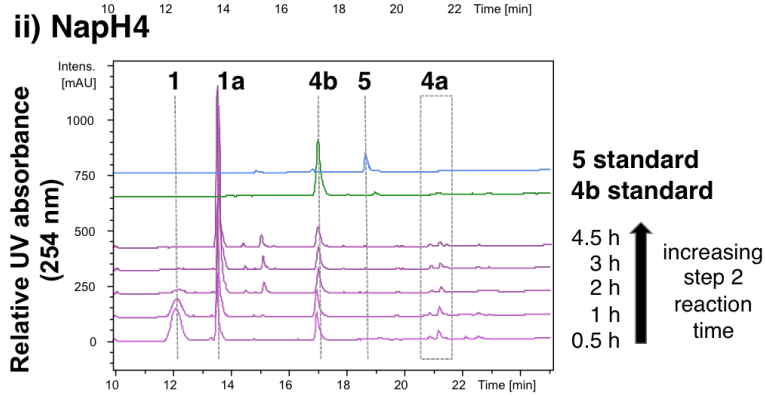
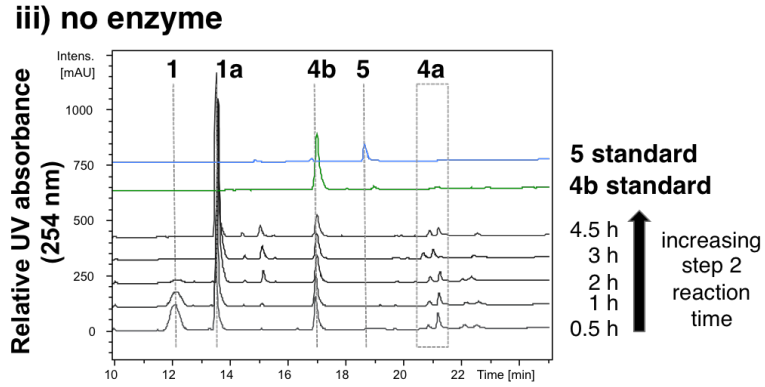


Figure S5. NapT9 requirements and substrate specificity. Reversed-phase HPLC UV chromatogram (254 nm, blue trace) and negative mode extracted ion chromatogram ($[M-H]^-$: 327.1 ± 0.1 *m/z*) of NapT9-catalyzed prenylation of THN (**1**) when incubated with various isoprenoid pyrophosphates (DMAPP, IPP, or GPP) and in the absence of exogenous Mg^{2+}, and comparison to synthetic standard 4-geranyl THN (**4**).


a)
1) NapT9 (0.5 mol%),
GPP (1.1 eq.),
MgCl2, DTT,
Ar gas, 5 h
2) VHPO (0.2 mol%)
H2O2 (1, then 4 eq.)
Na3VO4
1
(5.0 mM scale, 1 mL)
4
5
spontaneous oxidation by
O2 or H2O2
flaviolin (1a)
geranyl flaviolin (4a)
4b
reaction buffer:
50 mM HEPES-KOH (pH 8.0),
150 mM KCl, 5 mM DTT, 5% glycerol,
E. coli inorganic pyrophosphatase


i) NapH1
Relative UV absorbance (254 nm)
Intens. [mAU]
1
1a
4b
5
4a
Time [min]
5 standard
4b standard
4.5 h
3 h
2 h
1 h
0.5 h
increasing step 2 reaction time


ii) NapH4
Relative UV absorbance (254 nm)
Intens. [mAU]
1
1a
4b
5
4a
Time [min]
5 standard
4b standard
4.5 h
3 h
2 h
1 h
0.5 h
increasing step 2 reaction time


iii) no enzyme
Relative UV absorbance (254 nm)
Intens. [mAU]
1
1a
4b
5
4a
Time [min]
5 standard
4b standard
4.5 h
3 h
2 h
1 h
0.5 h
increasing step 2 reaction time

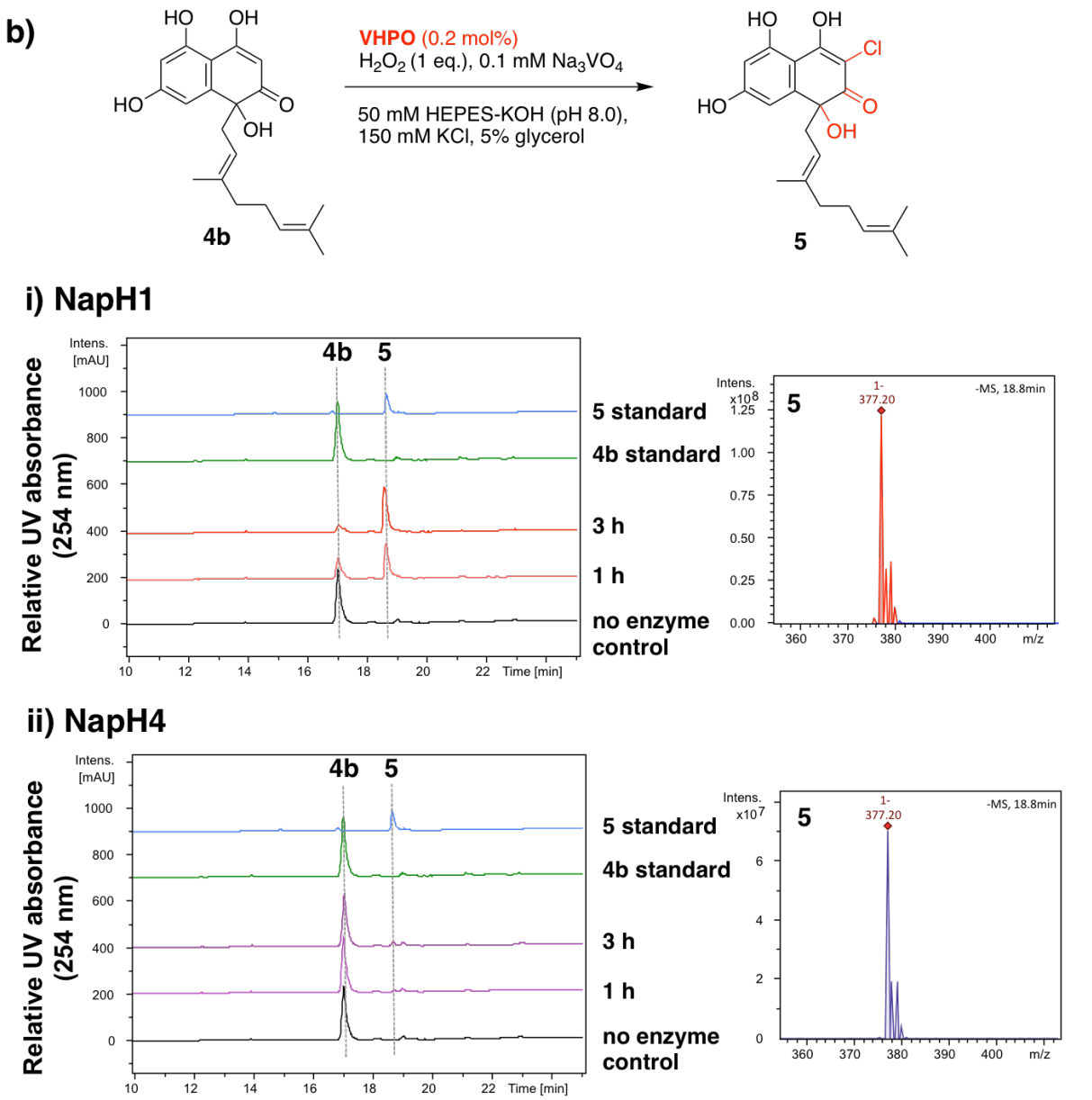
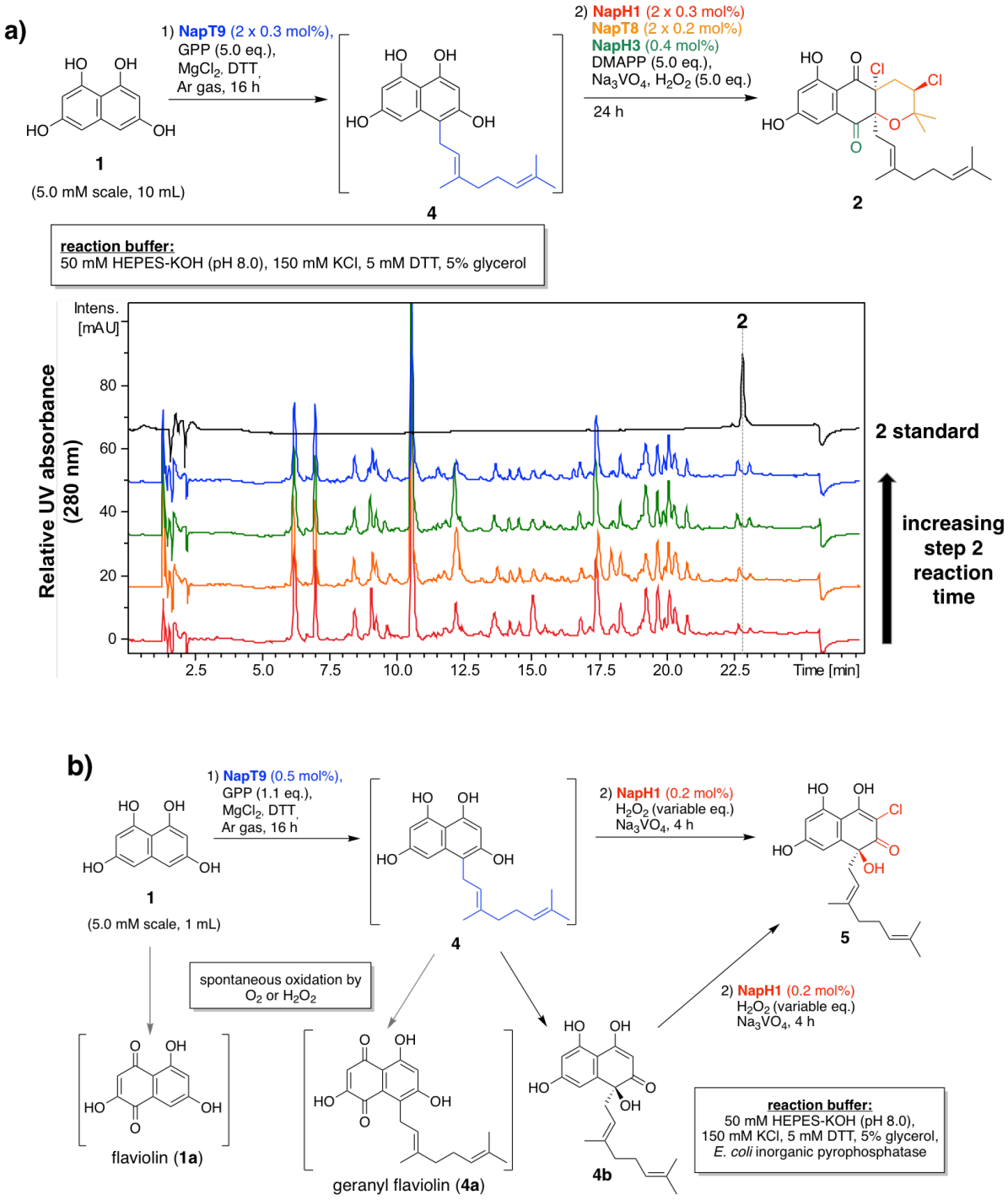


Figure S6. Comparison of NapH1 and NapH4 conversion of 4-geranyl THN (**4**) to **5**. **a**) RP-HPLC UV chromatograms (254 nm) of the conversion of *in situ* NapT9-generated **4** into **4a**, **4b**, and **5** over time in the presence of i) NapH1; ii) NapH4; or iii) no VCPO (negative control). The oxidation of **4** to **4a** and **4b** occurs in the presence or absence of VCPO enzyme, indicating a spontaneous conversion under the reaction conditions; however only in the presence of NapH1 is chlorination to **5** observed in any appreciable quantity. **b**) RP-HPLC UV chromatograms (254 cm) of the conversion of synthetic racemic **4b**[4] to **5** over time in the presence of i) NapH1; or ii) NapH4. Although both VCPOs were capable of catalyzing the formation of **5**, NapH1 had a dramatically higher *in vitro* preference for chlorinating **4b** and is therefore believed to be the responsible biosynthetic enzyme for this step.


a)
1) NapT9 (2 x 0.3 mol%),
GPP (5.0 eq.),
MgCl2, DTT,
Ar gas, 16 h
1
(5.0 mM scale, 10 mL)
4
2) NapH1 (2 x 0.3 mol%)
NapT8 (2 x 0.2 mol%)
NapH3 (0.4 mol%)
DMAPP (5.0 eq.),
Na3VO4, H2O2 (5.0 eq.)
24 h
2
reaction buffer:
50 mM HEPES-KOH (pH 8.0), 150 mM KCl, 5 mM DTT, 5% glycerol
Intens. [mAU]
Relative UV absorbance (280 nm)
Time [min]
2 standard
increasing step 2 reaction time
b)
1) NapT9 (0.5 mol%),
GPP (1.1 eq.),
MgCl2, DTT,
Ar gas, 16 h
1
(5.0 mM scale, 1 mL)
4
2) NapH1 (0.2 mol%)
H2O2 (variable eq.)
Na3VO4, 4 h
5
spontaneous oxidation by O2 or H2O2
flaviolin (1a)
geranyl flaviolin (4a)
4b
2) NapH1 (0.2 mol%)
H2O2 (variable eq.)
Na3VO4, 4 h
reaction buffer:
50 mM HEPES-KOH (pH 8.0),
150 mM KCl, 5 mM DTT, 5% glycerol,
E. coli inorganic pyrophosphatase

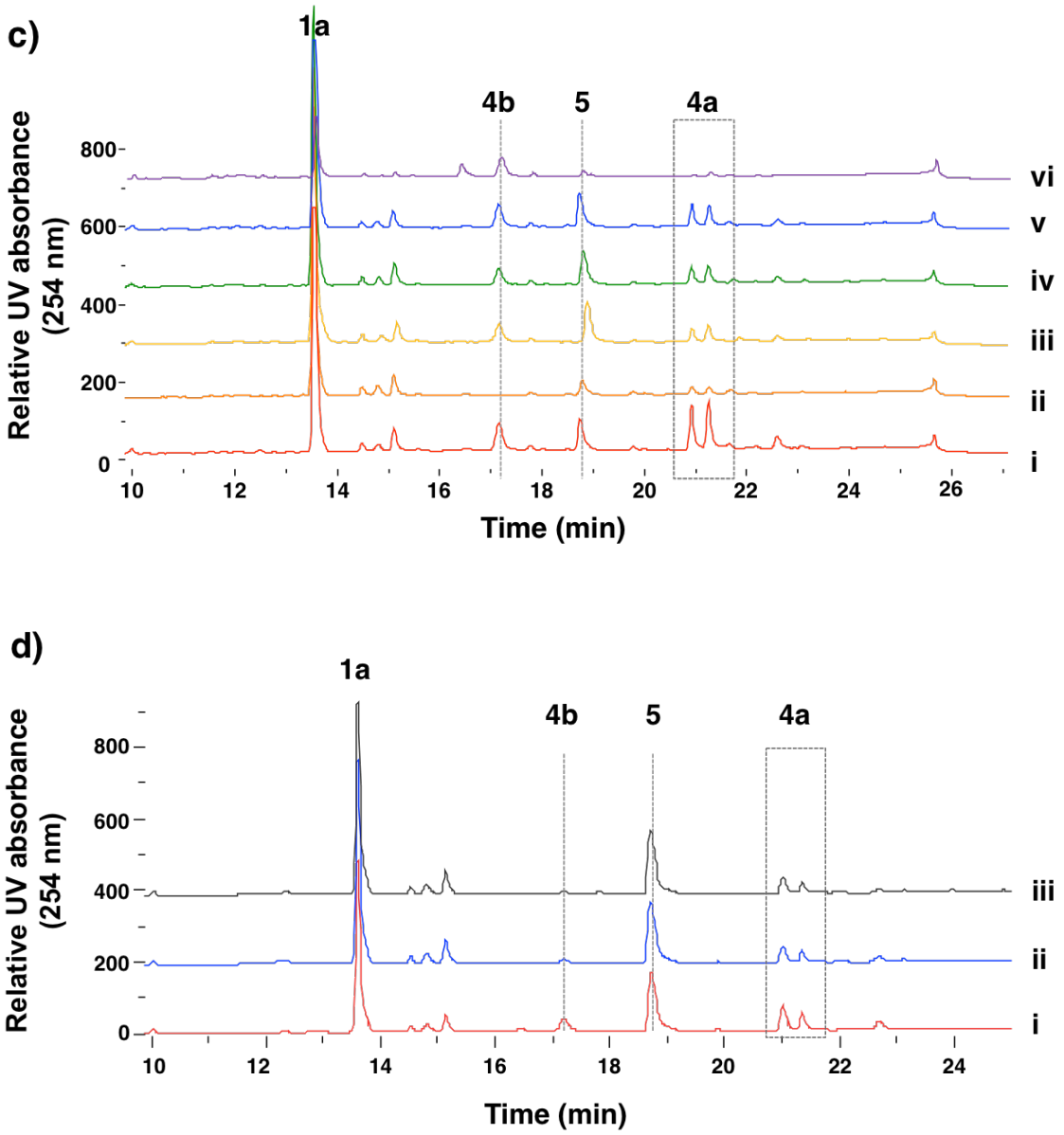


Figure S7. One-pot optimization to napyradiomycin molecules and biosynthetic intermediates. **a)** Initial one-pot effort to synthesize napyradiomycin A1 (**2**). Trace amounts of **2** (the right shoulder of the peak at ~22.7 min) were obtained from a complex reaction mixture. **b)** Subsequent optimization strategy of NapT9 and NapH1 steps, to maximize production of **5**, while minimizing oxidized side products flaviolin (**1a**), geranyl flaviolin (**4a**), and des-chlorinated **5** (**4b**). **c)** RP-HPLC chromatograms (254 nm) showing NapT9/NapH1 reaction optimization varying the conditions listed in b) by adding/removing reagents and/or adjusting the quantity or timing of H_2O_2 addition: i) 10 mM H_2O_2 added at once; ii) no DTT in reaction; iii) 5 mM H_2O_2 added, then 5 mM H_2O_2 added after 30 minutes; iv) same conditions as trace iii with added *E. coli* inorganic pyrophosphatase (New England Biolabs); v) same as trace iii except NapH1 was pre-incubated with H_2O_2 before addition; vi) 5 mM *tert*-butyl hydrogen peroxide (TBHP) added, then 5 mM TBHP added after 30 minutes. Conditions from (iv) were deemed to be the most appropriate for subsequent optimization. **d)** NapT9/NapH1 reactions varying the quantity of added H_2O_2: i) 5 mM H_2O_2, then 5 mM H_2O_2 added after 30 minutes; ii) 5 mM H_2O_2, then 10 mM H_2O_2 added after 30 minutes; iii) 5 mM H_2O_2, then 20 mM H_2O_2 added after 30 minutes. Conditions from (iii) were used for subsequent one-pot optimization of additional Nap enzymes.

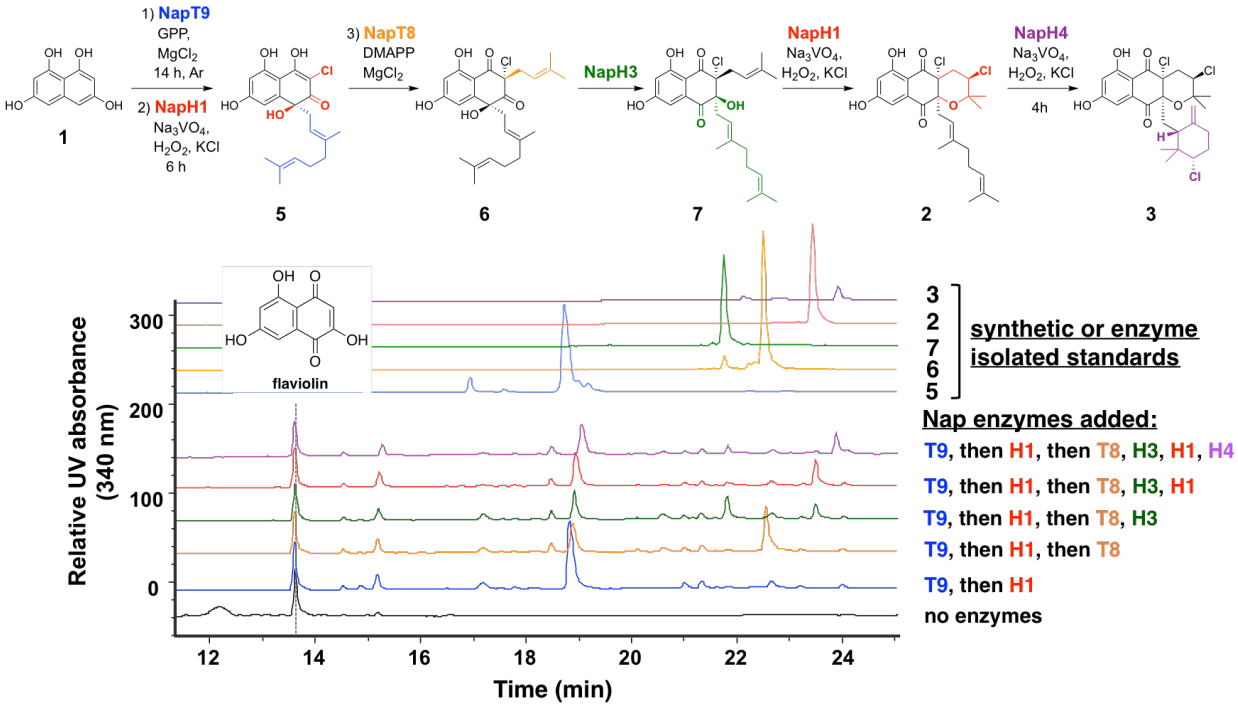


Figure S8. Optimized one-pot napyradiomycin biosynthesis and comparison to isolated standards. RP-HPLC chromatograms (340 nm) showing optimized one-pot reactions incorporating up to five napyradiomycin enzymes and comparison to synthetic or enzyme isolated standards of **5**, **6**, **7**, **2**, and **3**.

Table S1. Sequence similarity/identity comparison of napyradiomycin VHPO homologs to other characterized meroterpenoid VHPO enzymes from the merochlorin (Mcl)[13] or marinone (MarH)[3] biosynthetic pathways.

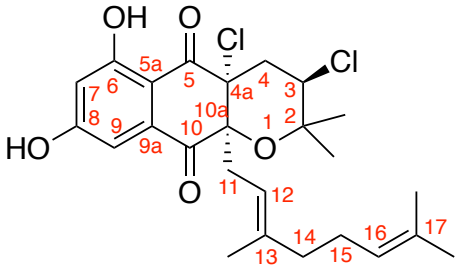
	NapH1	**NapH3**	**NapH4**	**Mcl24**[13]	**Mcl40**[13]	**MarH1**[3]	**MarH2**[3]	**MarH3**[3]
NapH1		64/50	82/69	69/54	77/65	79/64	67/52	68/53
NapH3	64/50		63/49	75/61	60/48	65/53	74/64	71/59
NapH4	82/69	63/49		67/53	80/67	78/63	64/50	67/53

Table S2. Sequence similarity/identity comparison of napyradiomycin aromatic prenyltransferases to other characterized meroterpenoid prenyltransferase enzymes from the merochlorin (Mcl),[13] naphterpin (NphB),[14] marinone (CnqP),[3,15] or furaquinocin (Fur)[16] biosynthetic pathways.

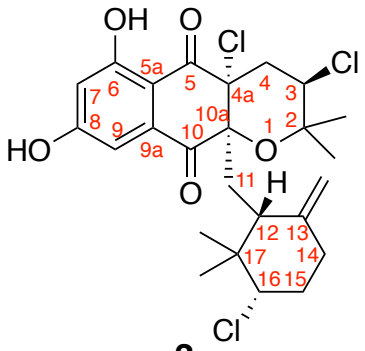
	NapT8	**NapT9**	**Mcl23**[13]	**NphB**[14]	**CnqP3**[3,15]	**CnqP4**[3,15]	**Fur7**[16]
NapT8		61/41	54/37	59/39	60/42	59/40	65/46
NapT9	61/41		66/45	55/36	56/38	64/47	60/37

NMR Data

Labeling scheme used for napyradiomycin compounds based on that used in the original isolation paper.[9]



2



3

Table S3. ^{1}H NMR comparison of one-pot enzymatically produced napyradiomycins A1 (**2**) and B1 (**3**) with naturally isolated compounds.

Assignment	2	natural 2[9]	3	natural 3[9]
2-CH_3	1.51, s	1.50, s	1.38, s	1.37, s
2-CH_3	1.18, s	1.18, s	1.19, s	1.18, s
3	4.43, dd (11.7, 4.2)	4.42, dd (11.2, 4.8)	4.45, dd (12.0, 4.0)	4.44, dd (12.0, 4.0)
4	2.48, dd (14.2, 4.3) 2.42, dd (14.2, 11.7)	2.48, dd (14.0, 4.8) 2.41, dd (14.0, 11.2)	2.52, dd (14.1, 3.9) 2.35, dd (14.1, 12.0)	2.52, dd (13.6, 4.0) 2.34, dd (13.6, 12.0)
6-OH	11.83, s	11.84, s	12.04, s	-
7	6.70, d (2.4)	6.73, d (2.4)	6.73, d (2.5)	6.72, br s
8-OH	6.14, br s	3.6,^ br s	7.10, br s	-
9	7.16, d (2.5)	7.22, d (2.4)	7.14, d (2.5)	7.14, br s
11	2.70, d (8.2)	2.70, br d (8.0)	2.64,# dd (15.5, 8.7) 1.61, d (15.2)	1.99,# br d (8.4) 1.61, br d (15.6)
12	4.71, t (7.9)	4.70, br t (8.0)	1.99,# d (8.6)	2.64,# dd (15.6, 8.4)
13-CH_3/CH_2	1.32, s	1.31, s	4.78, d (1.4) 4.75, s	4.78, s 4.75, s
14	1.67-1.60, m	1.6, m	2.24, ddd (13.2, 4.2, 4.2) 1.94, ddd (13.3, 13.3, 4.9)	2.25, ddd (12.4, 3.6, 3.6) 1.93, ddd (12.4, 12.4, 3.6)
15	1.67-1.60, m	1.6, m	2.03, dddd (12.7, 4.6, 3.5, 3.5) 1.75-1.68, m	2.03, dddd (12.4, 4.0, 3.6, 3.6) 1.72, dddd (12.4, 12.4, 11.6, 3.6)
16	4.92-4.88, br s	4.89, br s	3.80, dd (11.6, 4.4)	3.78, dd (11.6, 4.0)
17-CH_3	1.50, s	1.50, s	0.58, s	0.57, s
17-CH_3	1.63, s	1.62, s	0.71, s	0.69, s

NMR experiments run in $CDCl_3$

^8-OH 6.21 in synthetic reference[17]

#misassignment in original isolation reference[9]

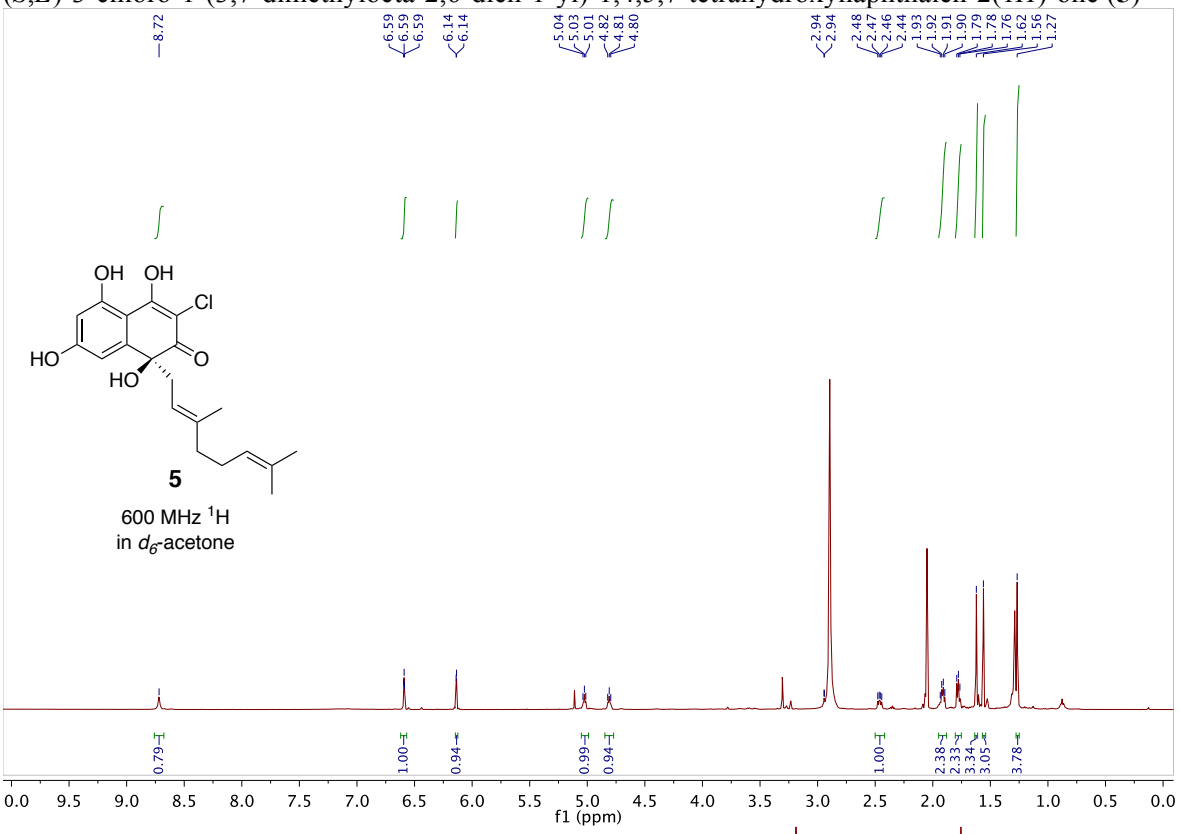
Table S4. ^{13}C NMR chemical shift comparisons of enzymatically produced napyradiomycins A1 (**2**) and B1 (**3**) with naturally isolated compounds.

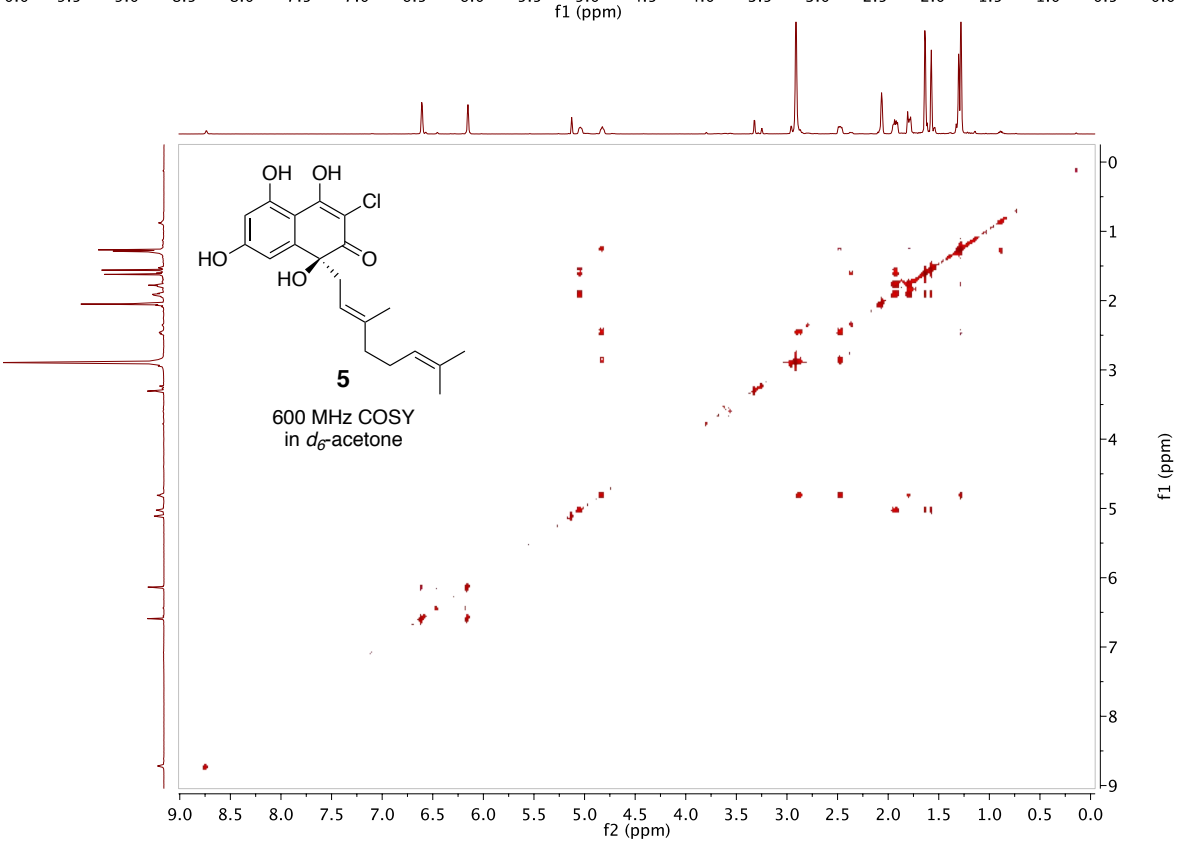
Assignment	2	natural 2[9]	3	natural 3[9]
2	78.7	(79.0)	78.8	(80.9)
2-CH_3	28.9	28.8	28.9	29.0
2-CH_3	22.4	22.3	22.4	22.4
3	58.8	58.8	58.6	58.7
4	42.8	42.8	42.6	42.7
4a	78.9	(78.8)	80.8	(78.8)
5	193.7	(193.7)		(193.5)
5a	110.3	110.2	108.9	108.8
6	164.5	(163.9)	164.9	(164.2)
7	109.4	109.6	109.4	109.5
8	163.4	(164.8)	164.1	(165.5)
9	107.5	107.8	108.4	108.5
9a		135.3		135.1
10	195.9	(196.2)	193.5	(193.8)
10a	83.5	83.6	84.2	84.3
11	41.3	41.3	34.8	35.6
12	115.0	114.9	45.7	45.9
13	142.9	142.8	145.3	145.3
13-CH_3/CH_2	16.6	16.5	110.3	110.2
14	39.9	39.8	35.6	35.0
15	25.6	26.0	34.4	34.5
16	123.8	123.7	70.8	70.7
17	131.8	131.8	41.8	41.8
17-CH_3	17.7	17.5	15.3	15.5
17-CH_3	25.5	25.6	26.3	26.4

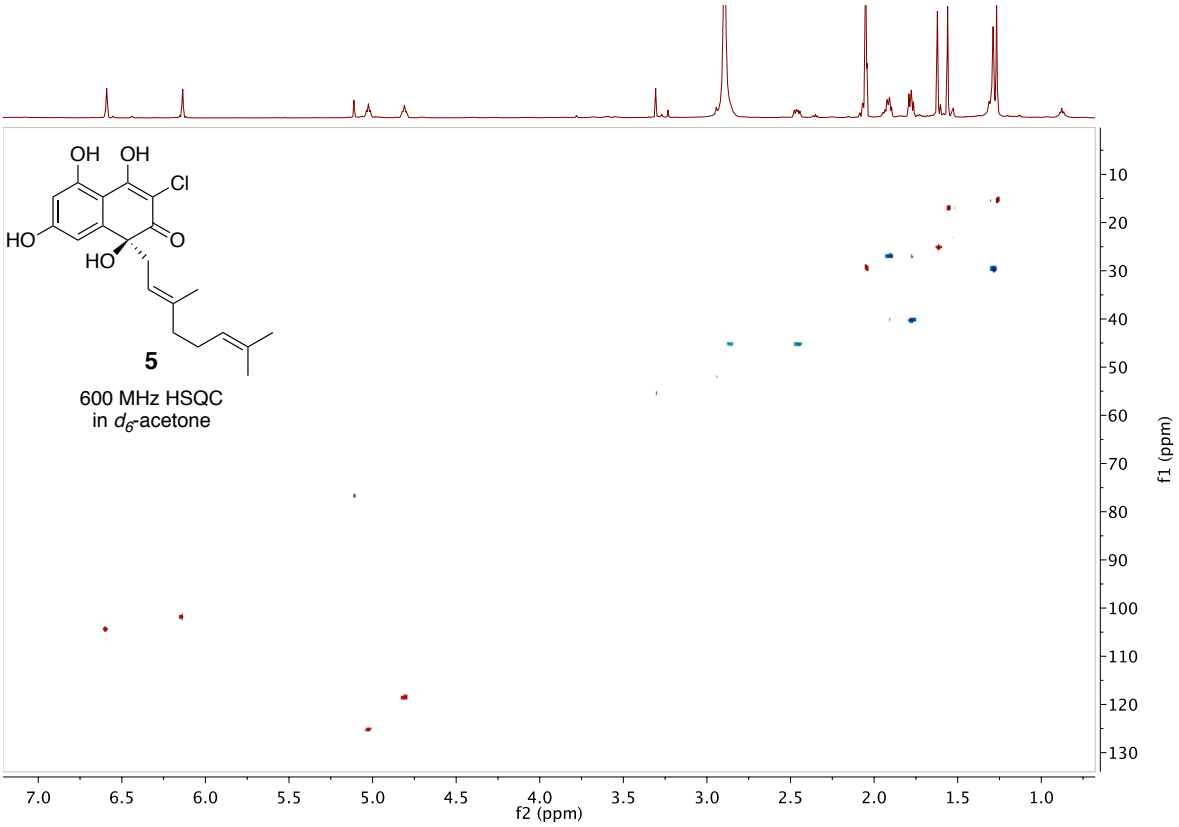
NMR experiments run in $CDCl_3$
Carbon signals for enzyme isolated **2** and **3** obtained from HSQC and HMBC experiments.
Numbers in parentheses for natural **2** and **3** indicate unclear assignment[9]

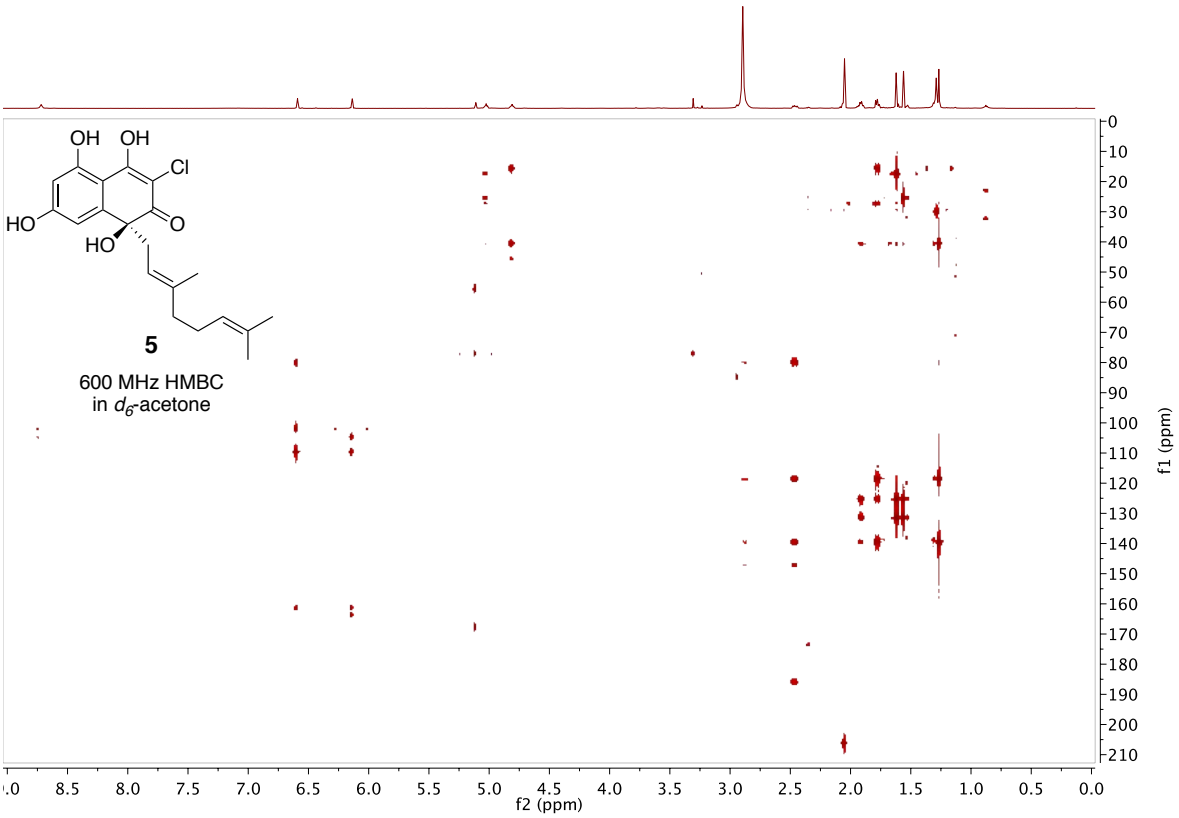
NMR spectra of isolated one-pot enzymatic products:

(*S*,*E*)-3-chloro-1-(3,7-dimethylocta-2,6-dien-1-yl)-1,4,5,7-tetrahydroxynaphthalen-2(1*H*)-one (**5**)

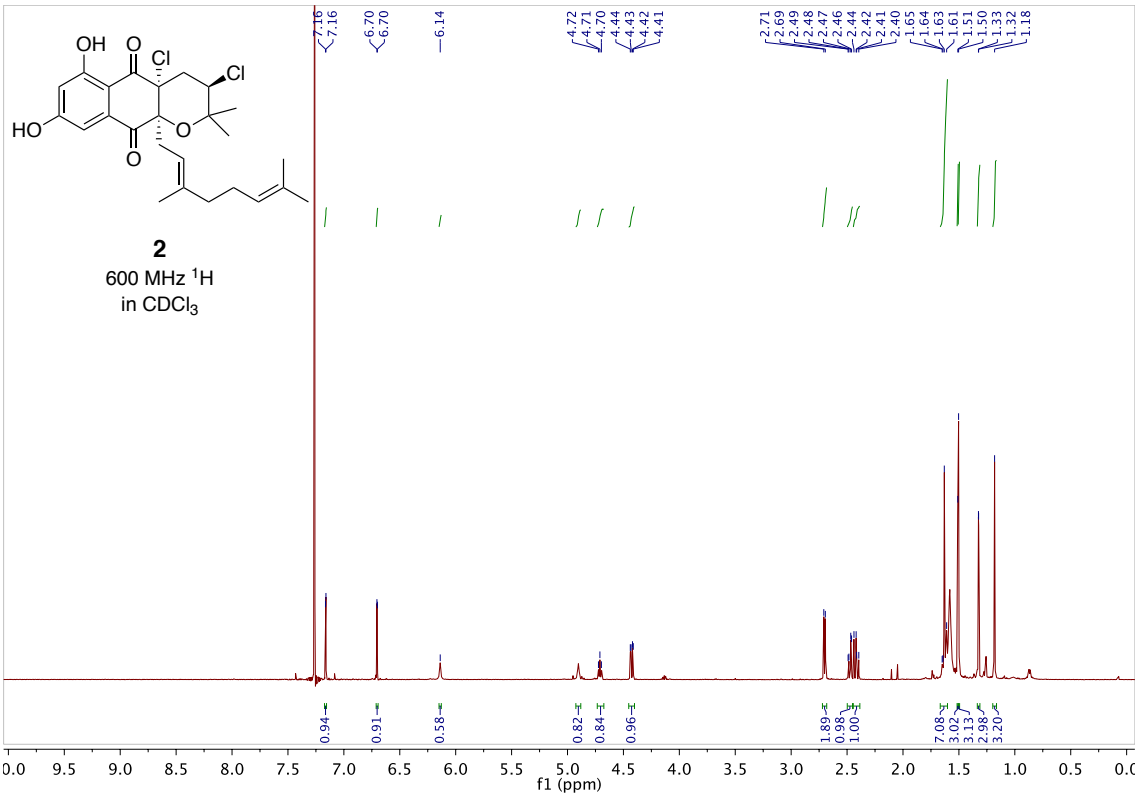


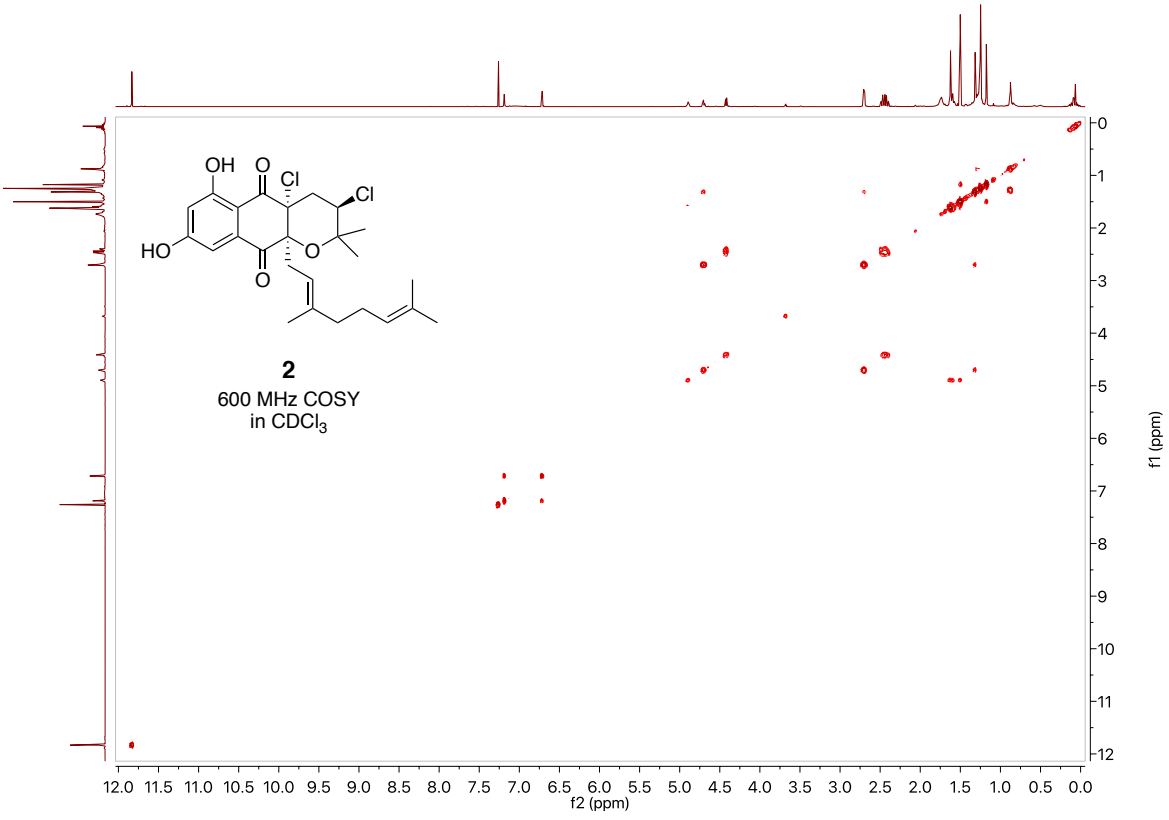


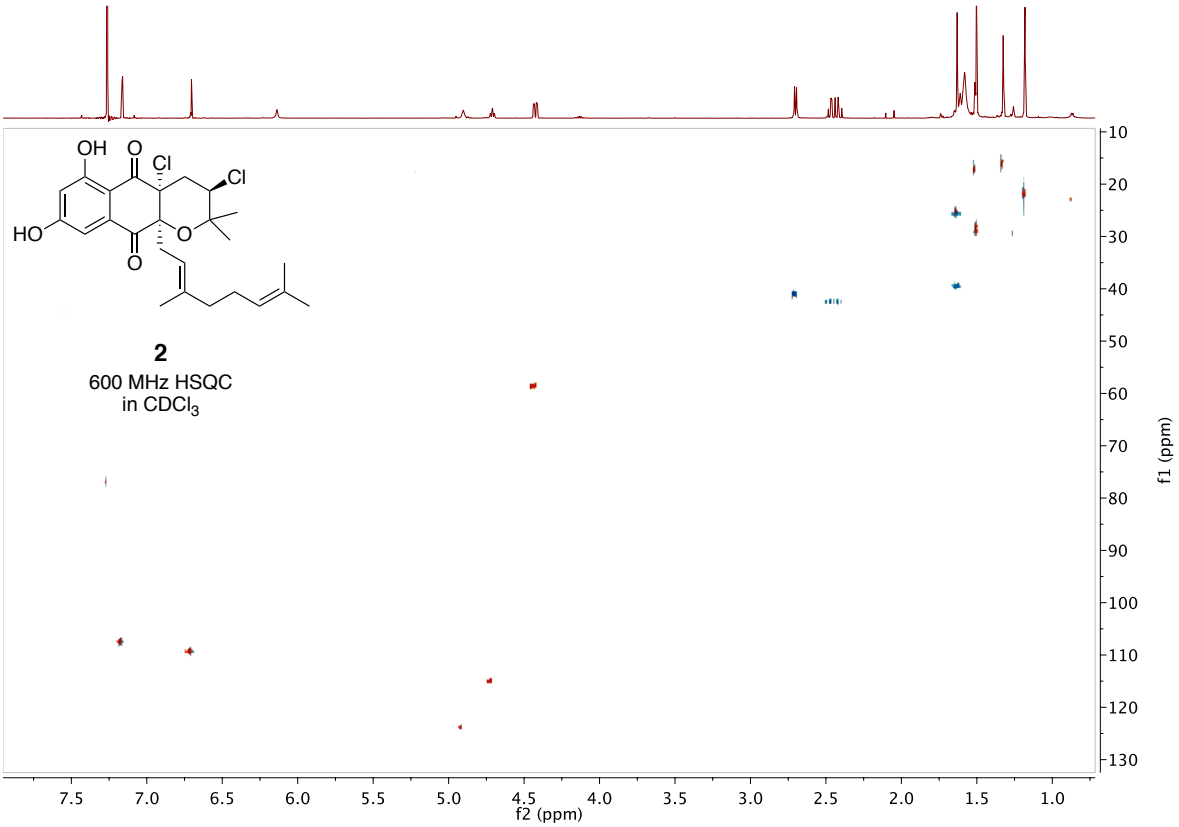

OH
OH
Cl
HO
O
HO
5
600 MHz HSQC
in d_6-acetone
f1 (ppm)
f2 (ppm)


OH
OH
Cl
HO
O
HO
5
600 MHz HMBC
in d_6-acetone
f1 (ppm)
f2 (ppm)

napyradiomycin A1 (**2**)



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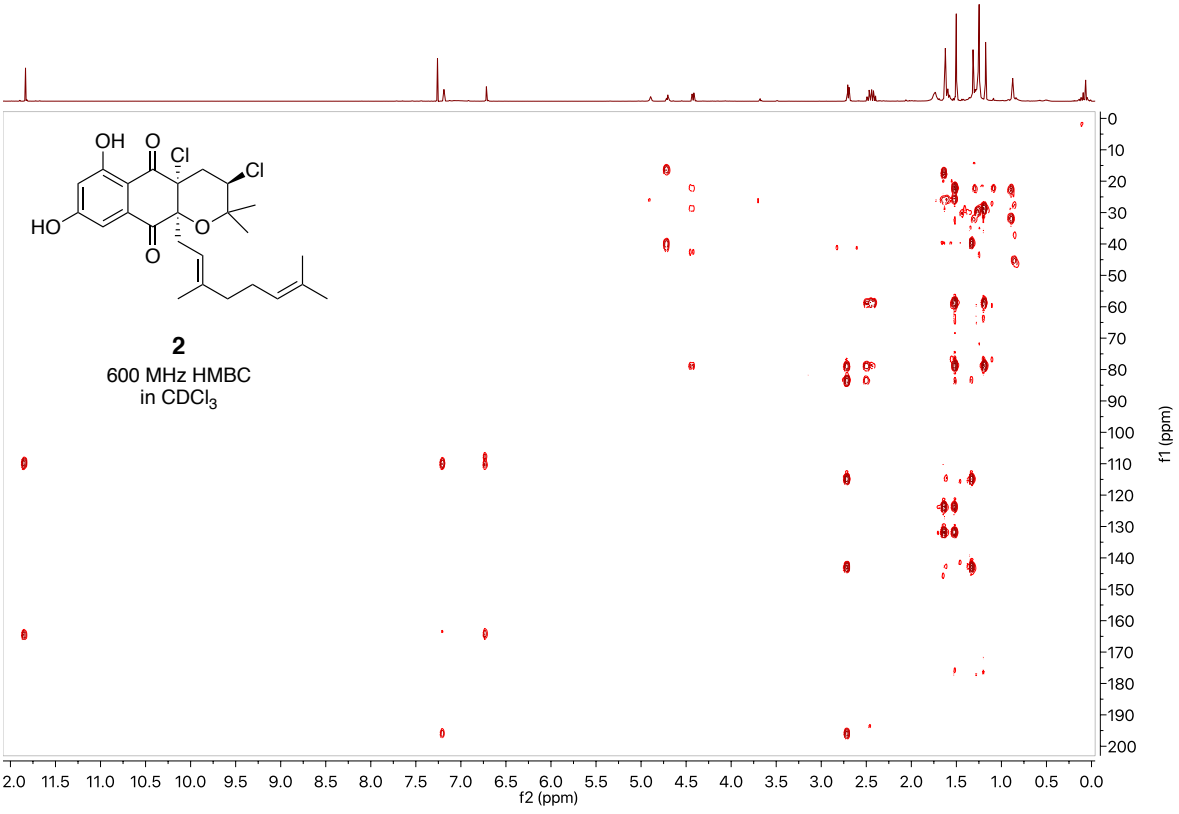
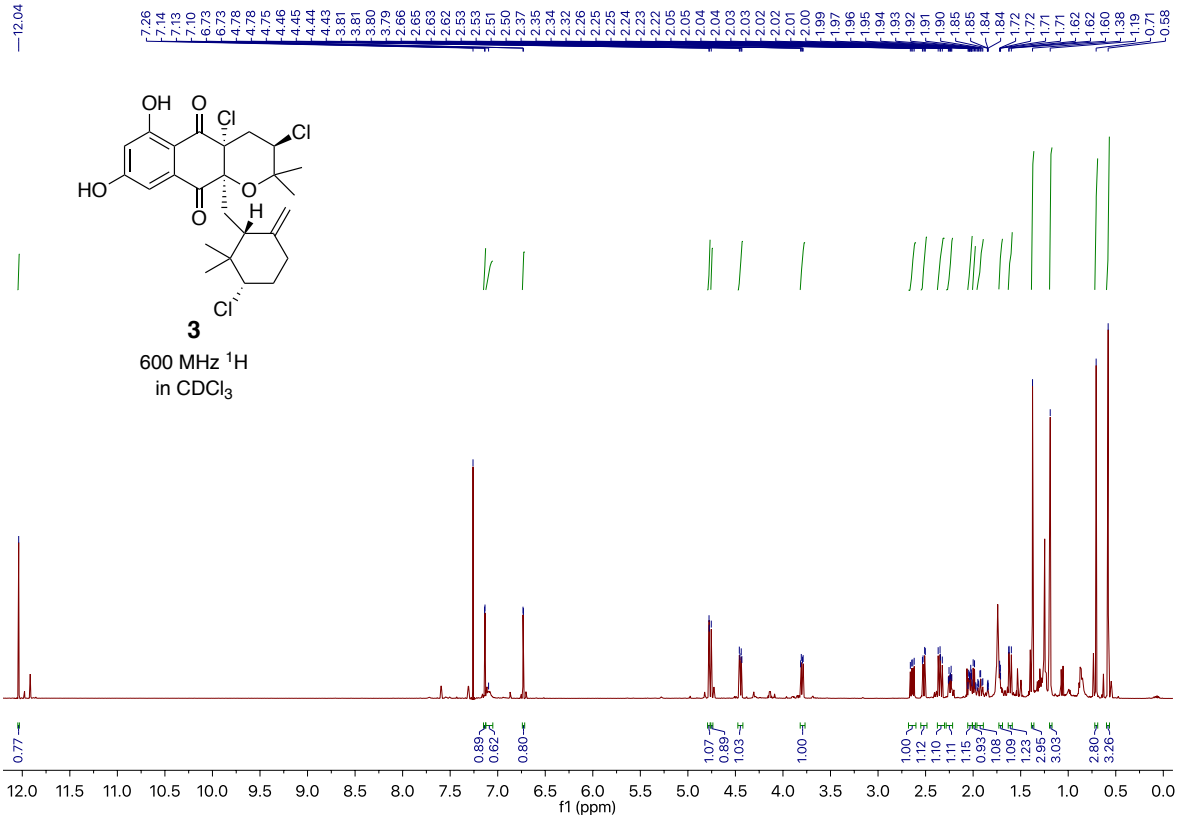
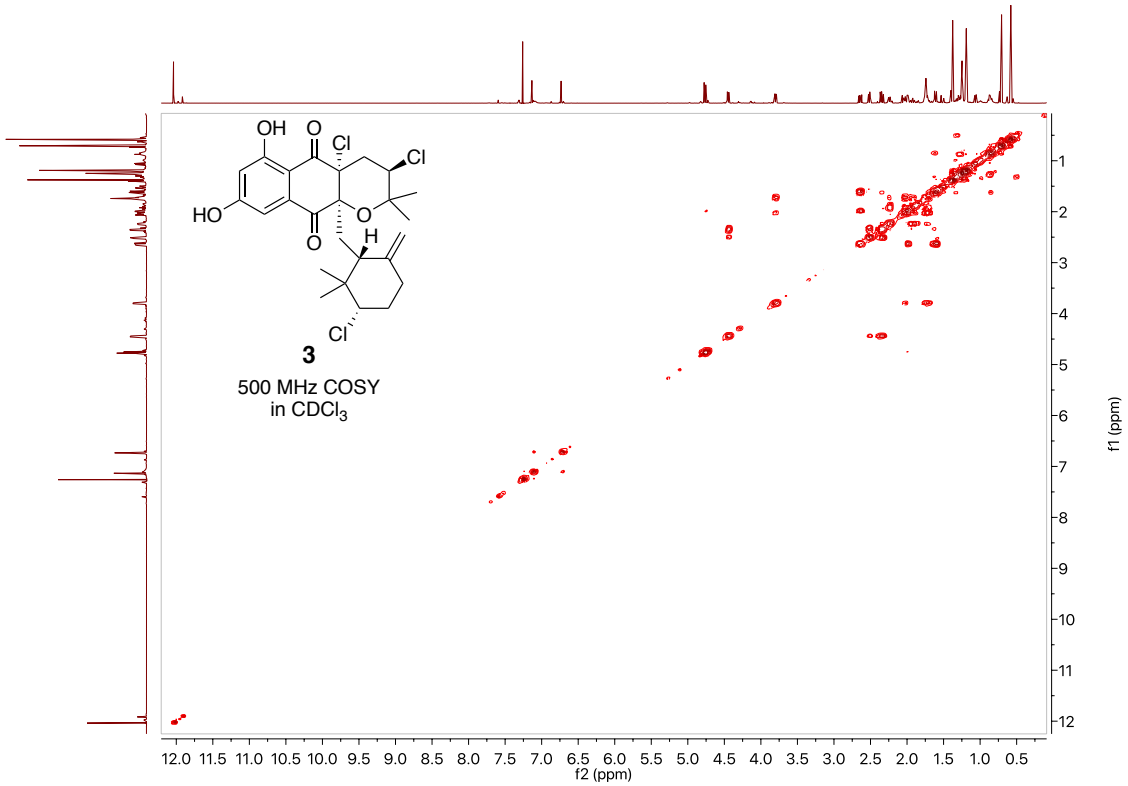
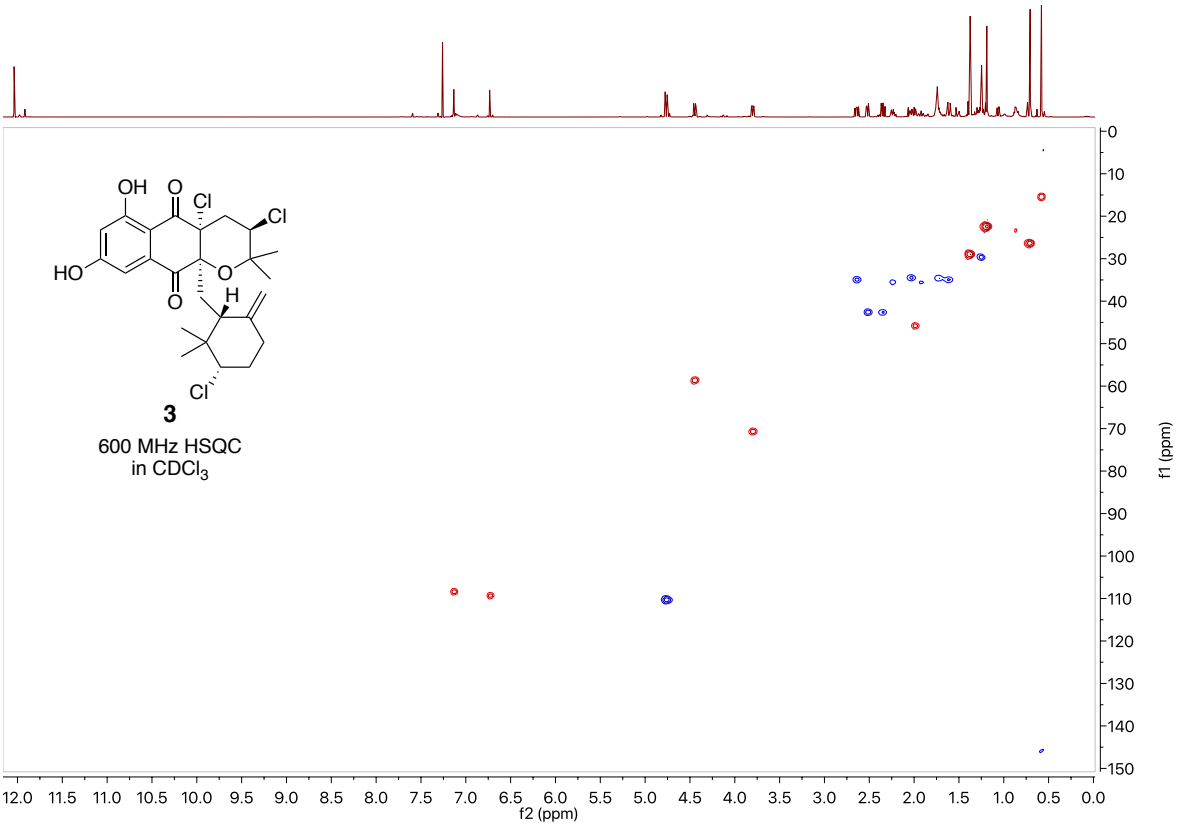
O
Cl
Cl
HO
O
O
2
600 MHz HSQC
in CDCl3
f1 (ppm)
f2 (ppm)

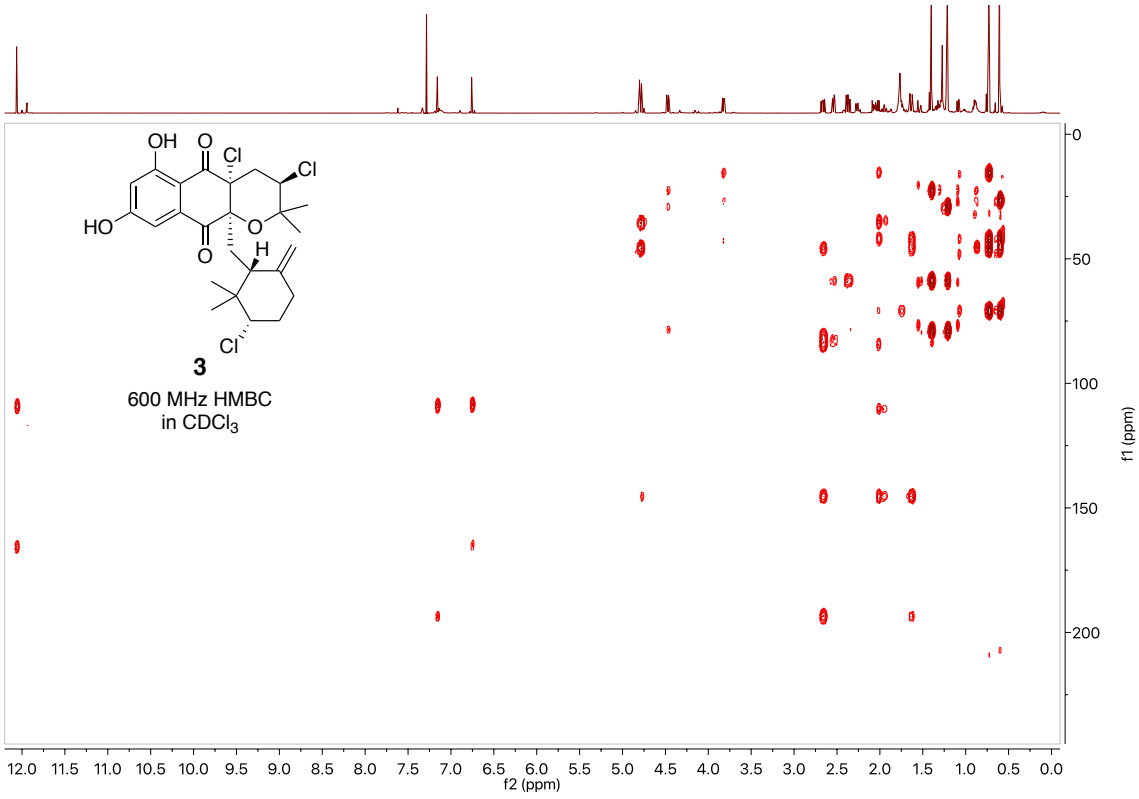

OH
O
Cl
Cl
HO
O
O
2
600 MHz HMBC
in CDCl3
f1 (ppm)
f2 (ppm)

napyradiomycin B1 (**3**)






OH
O
Cl
Cl
HO
O
O
H
Cl
3
600 MHz HSQC
in CDCl3
f1 (ppm)
f2 (ppm)


OH
O
Cl
Cl
HO
O
O
H
Cl
3
600 MHz HMBC
in CDCl3
f1 (ppm)
f2 (ppm)

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