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Total Enzyme Syntheses of Napyradiomycins A1 and B1

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Abstract

The biosynthetic route to the napyradiomycin family of bacterial meroterpenoids has been fully described 32 years following their original isolation and 11 years after their gene cluster discovery. The antimicrobial and cytotoxic natural products napyradiomycins A1 and B1 are produced using three organic substrates (1,3,6,8-tetrahydroxynaphthalene, dimethylallyl pyrophosphate, and geranyl pyrophosphate), and catalysis via five enzymes: two aromatic prenyltransferases (NapT8 and T9); and three vanadium dependent haloperoxidase (VHPO) homologues (NapH1, H3, and H4). Building upon the previous characterization of NapH1, H3, and T8, we herein describe the initial (NapT9, H1) and final (NapH4) steps required for napyradiomycin construction. This remarkably streamlined biosynthesis highlights the utility of VHPO enzymology in complex natural product generation, as NapH4 efficiently performs a unique chloronium-induced terpenoid cyclization to establish two stereocenters and a new carbon−carbon bond, and dual-acting NapH1 catalyzes chlorination and etherification reactions at two distinct stages of the pathway. Moreover, we employed recombinant napyradiomycin biosynthetic enzymes to chemoenzymatically synthesize milligram quantities in one pot in 1 day. This method represents a viable enantioselective approach to produce complex halogenated metabolites, like napyradiomycin B1, that have yet to be chemically synthesized.

The napyradiomycins are a diverse set of meroterpenoid natural products originally isolated from the actinomycete *Streptomyces ruber* (formerly *Chainia rubra*) in 1986,[1,2] and today number in excess of 50 members.[3] Initially isolated for their broad-spectrum Gram-positive antibacterial activities, additional studies of these hybrid polyketide-terpenoid compounds

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ASSOCIATED CONTENT

Supporting Information

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Materials and methods, general procedures, compound characterization and NMR spectra (PDF)

Notes

The authors declare no competing financial interest.

have identified promising anticancer and antiangiogenic activities. Select few napyradiomycins have been synthesized in a racemic manner,[4,5] and only one enantioselective synthetic route to napyradiomycin A1 has been achieved to date (Figure 1).[6] No chemical syntheses of cyclohexane-containing napyradiomycins have however been reported. The main challenge relating to the construction of the napyradiomycins, and similar molecules, is the limited methodology for stereospecific installation of their halogenated stereocenters. Recent pioneering advances have dramatically accelerated the field of asymmetric halogenation.[7] As recently reported, over 2000 natural products that possess a chiral chlorinated or brominated stereocenter have been isolated, while only 12 have been synthesized via a catalytic, enantioselective halogenation strategy.[8] Many of these chiral halogenated metabolites have biological activities with significant therapeutic and chemical interest,[9] necessitating development of viable strategies to perform this challenging chemical transformation.

Nature's methodology for installing halogenated stereo-centers has been developed through a few unique approaches.[10] Among the families of natural halogenating enzymes, the vanadium-dependent haloperoxidases (VHPOs) are remarkable biological catalysts that perform a two-electron oxidation of aqueous halide ions in the presence of vanadate (VO_4^{3-}) and H_2O_2, generating the traceless byproduct water. Moreover, these enzymes have garnered significant interest as potential biocatalysts due to their robust thermostability and oxidative activity without the need for costly cofactors or redox regenerating systems.[11] VHPOs can be divided into two major categories: the more extensively studied macroalgal and fungal VHPOs that produce diffusible hypohalous acid capable of reacting with electron-rich substrates;[12] and *Streptomyces* VHPOs that perform regio-and enantioselective halogenations, suggestive of enzymatic capture of halenium ions.[13]

In 2007, the 43 kb napyradiomycin biosynthetic gene cluster (*nap*) was reported from the marine isolate *Streptomyces* sp. CNQ-525.[14] Three VHPO homologues (*napH1*, *H3*, *H4*) were annotated within this cluster, suggestive of the first dedicated involvement of this family of enzymes in secondary metabolism.[14,15] Moreover, the *napH* genes were localized in close proximity to those capable of producing napyradiomycin biosynthetic precursors identified from stable isotope incorporation experiments, including mevalonate-derived isoprene pyrophosphates and the polyketide 1,3,6,8-tetrahydroxynaph-thalene (THN, 1).[16] Subsequent *in vitro* validation of NapH1 demonstrated its ability to facilitate a stereospecific chloronium-induced cyclization of the prenyl moiety to form a 7-methylated napyradiomycin A1 derivative.[17] Over the past decade, VHPO homologues were discovered and shown to catalyze many key bond-constructing reactions within *Streptomyces* sp. THN-derived meroterpenoid biosyntheses, including the merochlorins,[18–20] naphterpin, and marinone.[21] These diverse chemical transformations include chlorination-induced dearomatization, cyclization, and isomerization reactions that proceed in regio-and stereospecific manners. We recently reported two unique transformations within the napyradiomycin biosynthetic pathway: the Mg^{2+}-dependent NapT8 prenylation of monochlorinated 5 with dimethylallyl pyrophosphate (DMAPP); and the subsequent α-hydroxyke-tone rearrangement of NapT8 product 6 to naphthomevalin (7) via VHPO homologue NapH3.[5] This result rationalized the vicinal diprenylation motif unique to the napyradiomycins that formerly defied biosynthetic logic. While this study further expanded

the repertoire of biological activities from VHPO enzymes, the transformations for the generation of 5 from 1 and the enzymes required for the formation of the cyclized geranyl moiety in napyradiomycin B1 (3) remained unknown. Our studies aimed to understand the total biosynthetic logic of the entire pathway, while identifying these new enzymatic reactions. Herein we report the characterization of VHPO and prenyltransferase enzymology related to the biosynthesis of 3, and subsequent application of this streamlined five-enzyme pathway to enantioselectively synthesize milligram quantities of these complex napyradiomycin meroterpenoids.

Many napyradiomycins contain additional structural complexity via presumed halogenation-induced cyclization of the geranyl moiety. Because of the functional assignments of two *napH* homologues, we suspected that NapH4 catalyzes this reaction. Previous attempts to obtain soluble protein expression in *Escherichia coli* were unsuccessful;[17] however, reassignment of the *napH4* open reading frame, and subsequent recloning and heterologous production in *Streptomyces lividans* TK23 resulted in soluble NapH4 (Figures S1, S2). Application of the model monochlorodimedone assay to detect hypohalous acid formation indicated that recombinant NapH4 was properly folded and active, albeit only showing activity in the presence of bromide ions; an unusual characteristic of these stereospecific *Streptomyces* sp. VCPO enzymes (Figure S3).[13]

We initially surmised that NapH4 would produce the chlorinated cyclohexanol moiety observed in napyradiomycin B4, a 7′-demethylated variant of the major metabolite isolated from heterologous expression of the *nap* cluster (Figure S4).[14] Instead, when incubated with 2 in the presence of Na_3VO_4, KCl, and H_2O_2, NapH4 catalyzed the formation of a single product with a dehydrated mass compared to napyradiomycin B4. Following scale up, isolation, and NMR characterization, the product was identified as 3, possessing an exomethylene-containing chlorinated cyclohexane ring (Figure 2), which matched the original isolation spectra.[2] Although alteration of the reaction pH can have a significant impact on the biochemistry of *Streptomyces* VHPOs, as best exemplified with Mcl24 from the merochlorin biosynthetic pathway,[5,13,18,19] we only observed a decrease in the relative formation of 3 at a lower pH (Figure S4).

An analogous bromonium-based cyclization reaction was previously reported with marine red algal VBPO enzymes when incubated with terpenoid substrates.[22] Despite suffering from poor regioselectivities, broad substrate specificities, and the generation of multiple products in low yields, these enzymes showcased the first example of enantioselective halogenation and concomitant carbon−carbon bond formation by a VHPO. This biological reactivity has been paralleled chemically in the halonium-induced polyene cyclizations that have been employed asymmetrically via stereoselective iodonium/bromonium formations.[23] Only two racemic polyene cyclizations of chloronium ions have been reported;[24] however, these reactions generally occur in much lower yield and diastereoselectivity than bromo-and iodo-cyclizations.[25] In contrast, NapH4 catalyzes a remarkably high yielding, diastereoselective, and stereospecific chloronium-induced terpenoid cyclization.

With the completion of the pathway from 5 to 7, we next turned our attention to the start point from 1 to 5. As prenyltransferase (PTase) NapT8 had previously been assigned

function in the prenylation of 5,[5] we suspected the second predicted ABBA aromatic PTase NapT9 as the enzyme responsible for installing the geranyl moiety. This function would be analogous to the prenylation of 1 by Mcl23 and NphB (originally Orf2) in merochlorin[18] and naphterpin[26] biosyntheses, respectively. Following heterologous expression and purification of NapT9, we observed the Mg^{2+}-dependent conversion of 1 and geranyl pyrophosphate (GPP) to 4-geranyl 1,3,6,8-tetrahydroxynaphthalene (4-geranyl THN, 4) (Figures 3, S5). The reversed-phase HPLC and extracted ion chromatograms of this reaction exhibited a unique elution profile, likely due to keto−enol tautomers formed under the weakly acidic conditions.[27] This unique chromatogram mirrored that of synthetic 4,[21] but due to significant oxygen sensitivity, isolation was not attempted.

We suspected the conversion of 4 to 5 to be catalyzed by a VCPO based on related transformations in other 1-derived meroterpenoid pathways.[5,18,19,21] Incubation of *in situ* NapT9-generated 4 with *nap* VCPO enzymes in the presence of Na_3VO_4, H_2O_2, and KCl showed that NapH1 catalyzed the production of 5 as the major chlorinated product. This was further confirmed by preparative scale up and NMR characterization (Figures 3, S6). The NapH1 activity was particularly surprising given its previous role in alkene halofunctionalization to cyclize 7 to 2.[17] NapH4 was also able to form 5 albeit in a substantially lower quantity compared to NapH1 (Figure S6). Analogous to marinone biosynthetic VCPO enzyme MarH1,[21] NapH1 catalyzes the monochlorination of prenylated THN molecules even with excess H_2O_2. This diverges from the *in vitro* activities of other VCPO enzymes (MarH3,[21] Mcl24[5]) that facilitate dichlorination and subsequent α-hydroxyketone rearrangements with comparable substrates. We are presently exploring the biophysical understanding of these divergent activities.

Identification of the activities of NapT9 and NapH4, and the dual functionality of NapH1, allowed us to complete the napyradiomycin biosynthetic pathway (Scheme 1). Briefly, prenyltransferase NapT9 catalyzes the Mg^{2+}-dependent ger-anylation of 1 at the nucleophilic 4-position. Prenylated product 4 is subsequently oxidatively dearomatized and monochlorinated via NapH1 in the presence of Na_3VO_4 and H_2O_2 to afford 5. NapT8 then catalyzes a Mg^{2+}-dependent prenylation with DMAPP to form 6, which undergoes a NapH3-catalyzed α-hydroxyketone rearrangement, producing 7.[5] Next, NapH1 performs an enantioselective chlorination-induced cyclization reaction to form 2,[5,17] which is further transformed by a novel asymmetric, chlorination-induced terpenoid cyclization by NapH4 to 3.

Following complete elucidation of the biosynthetic pathway to 3, analysis of the individual enzymatic activities suggested that only two types of enzymes were present with orthogonal redox and metal cofactor requirements. We hypothesized that a controlled addition of all Nap biosynthetic enzymes to one pot could be employed to generate useful quantities of napyradiomycin family members in an enantioselective and protecting group-free strategy. *In vitro* interrogation identified maximal activity of NapH4 in HEPES-KOH, pH 8.0 (Figure S4), so this was selected as the one-pot reaction buffer for scale up and optimization. A 5 mM reaction scale (~1 mg/mL of 1) was chosen to generate maximal quantities of napyradiomycins despite the increasing hydrophobicity of these complex halogenated meroterpenoid products.

An initial division of the NapT9 reaction with subsequent H_2O_2-dependent VHPO steps was required to maximize the amount of oxygen-sensitive 4 for downstream catalysis. Initial attempts to transform 1 in one pot with excess GPP, H_2O_2, and multiple enzyme additions were successful in producing 2 albeit as a very minor component of a complex reaction mixture (Figure S7). The high abundance of oxidized byproducts of 1 and 4 in the one pot reaction mixture implied that significant optimization of the NapT9 and NapH1 reactions would be necessary to improve yields to 5. Major contributors to yield improvement (45% isolated yield of 5) included: reducing GPP to 1.1 mol equiv; adding commercial *E. coli* inorganic pyrophosphatase to minimize PTase inhibition; and sequentially adding H_2O_2 to the reaction mixture (Figure S7).

Following the two-step optimization to 5, the addition of DMAPP (1.1 equiv), H_2O_2, and additional biosynthetic enzymes at once enabled production of intermediates along the napyradiomycin biosynthetic pathway that directly correlated to the Nap enzymes added (Figures 4, S8). Multiple 1 mL replicates were set up following identical reaction conditions and times, as larger volume reactions generated an increased number and intensity of byproducts. After the threestep addition of the first four Nap biosynthetic enzymes, inorganic metal cofactors and organic pyrophosphate and hydrogen peroxide cosubstrates to 1 (9.6 mg, 50 μmol, divided over 10 × 1 mL) in one pot, 2 (5.4 mg, 22%) was isolated after a 24 h reaction time; analogous reaction conditions with the inclusion of NapH4 produced 3 (4.6 mg, 18%), which has yet to be chemically synthesized in an enantioselective or racemic strategy. Moreover, the chemoenzymatic application of the napyradiomycin biosynthetic pathway established all five stereocenters of 3, three of which are chlorinated, using 1.1 mol equiv of organic pyrophosphates and 0.2−1 mol % of biological catalysts over a reaction time of 24 h.

Many impressive syntheses of complex natural products have been recently reported using enzymes from their biosynthetic pathways, highlighting the synthetic utility of this methodology.[28] While challenges still exist in the application of chemoenzymatic synthesis, it represents a complementary approach to overcome some of the biological challenges in microbial secondary natural product production (long culturing times, low production yields, reliance on naturally occurring biosynthetic precursors), while offering exquisite catalysis of regio-and stereospecific chemical reactions that would be difficult to mirror in a round-bottomed flask. We are particularly encouraged by the application of NapH1 and NapH4 enzymology in the chiral installation of halogenated stereocenters and aim to further extend the utility and scalability of VHPO catalysis within the challenging field of catalytic asymmetric halogenation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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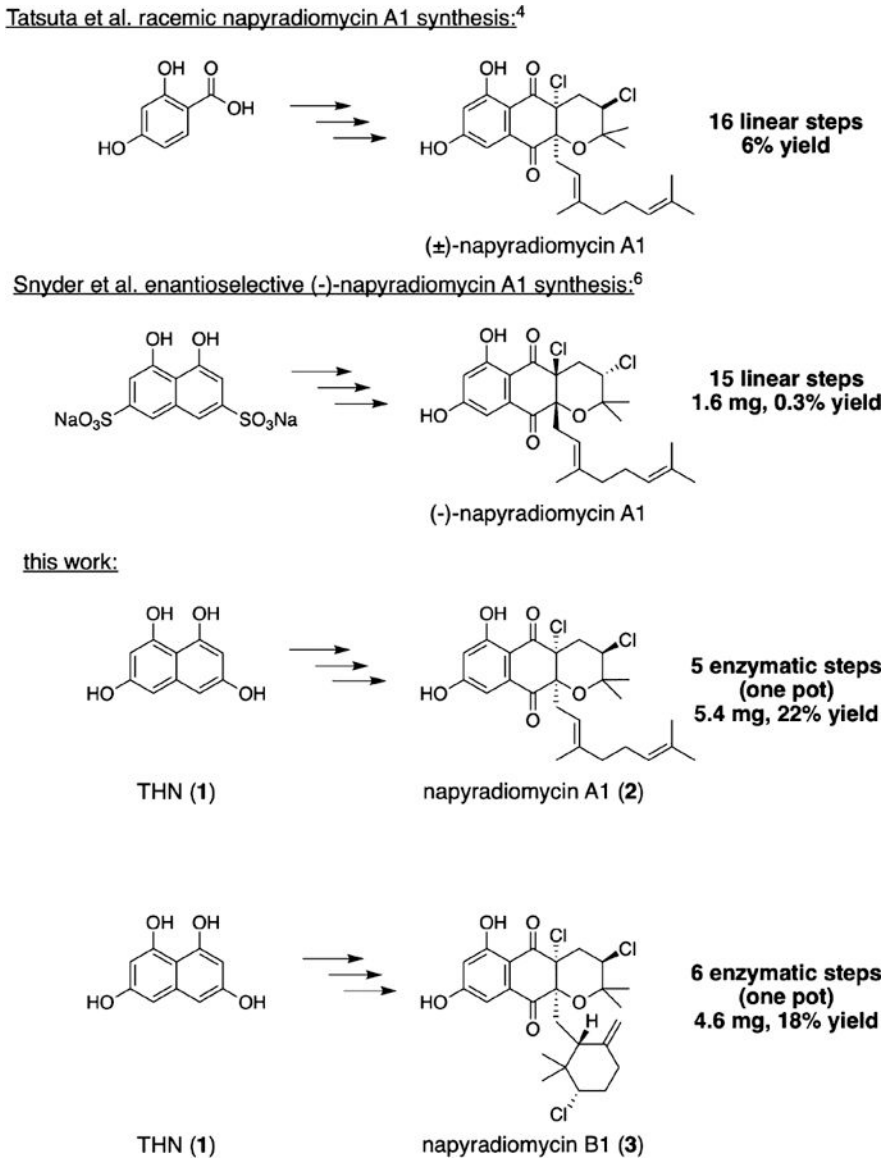


Figure 1.

Total chemical syntheses of napyradiomycin A1 and comparison with chemoenzymatic approaches described herein.

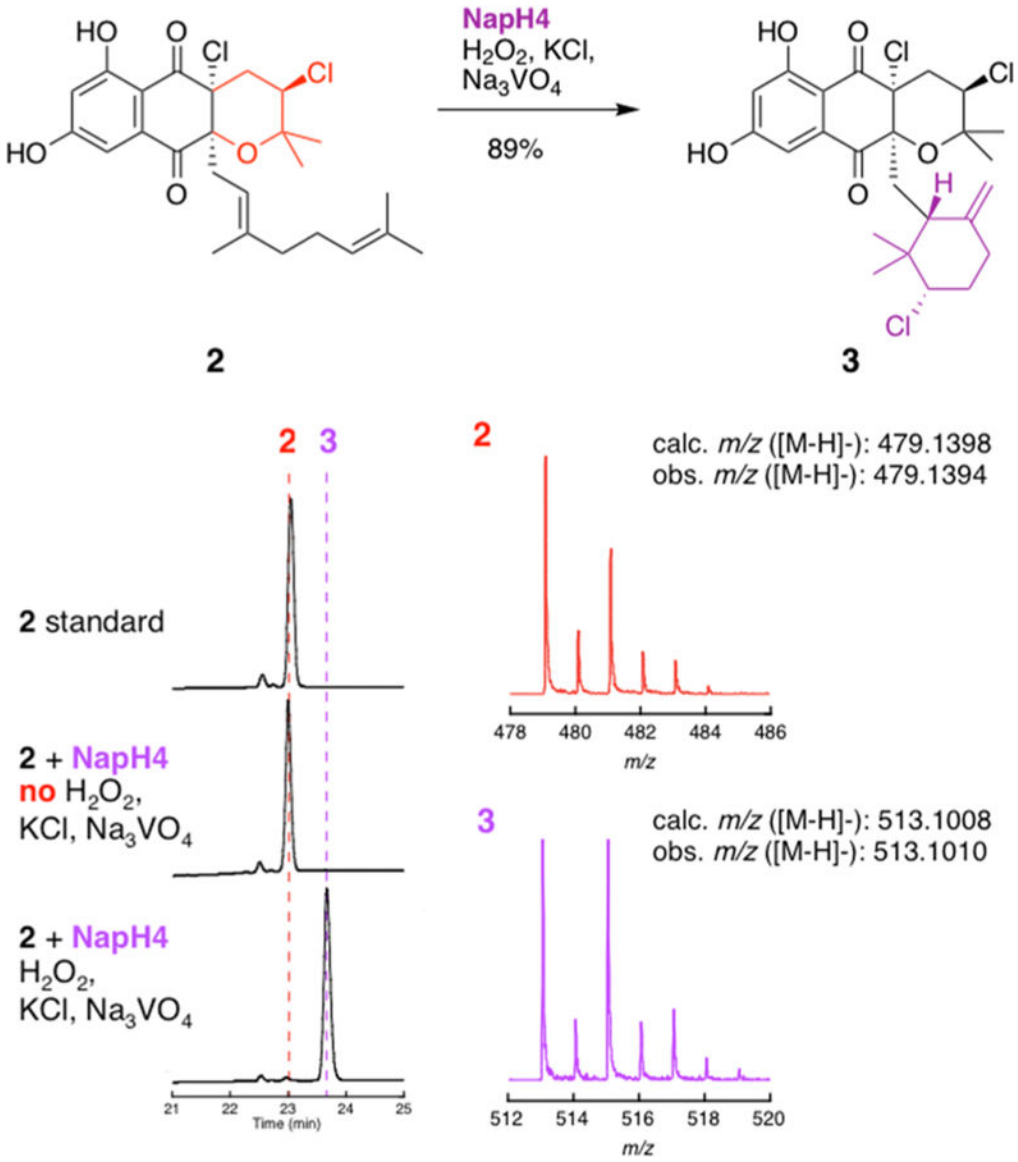


Figure 2.

NapH4-catalyzed chloronium-mediated cyclization of the geranyl moiety of 2 to form 3.

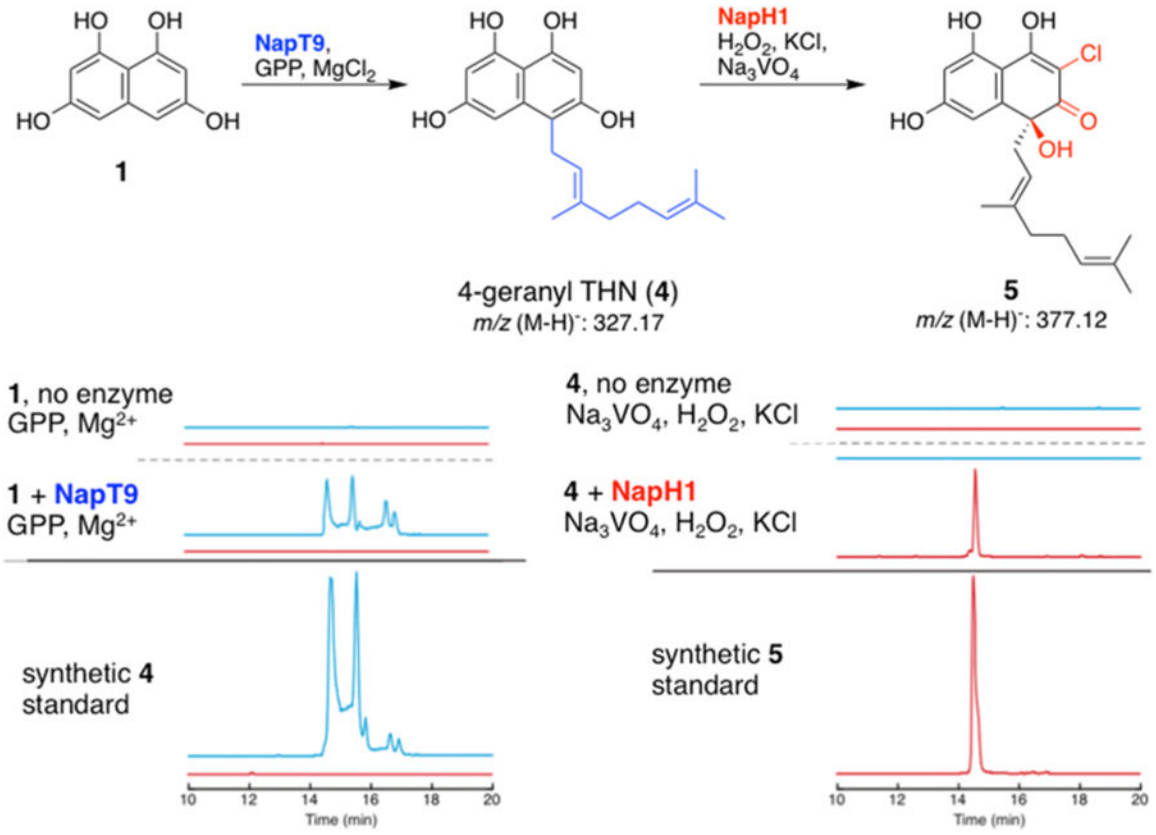


Figure 3.

Negative mode extracted ion chromatograms showing conversion of 1 to 4 (blue trace, 327.1 *m*/*z*) and 5 (red trace, 377.1 *m*/*z*) by NapT9 and NapH1 activities, respectively.

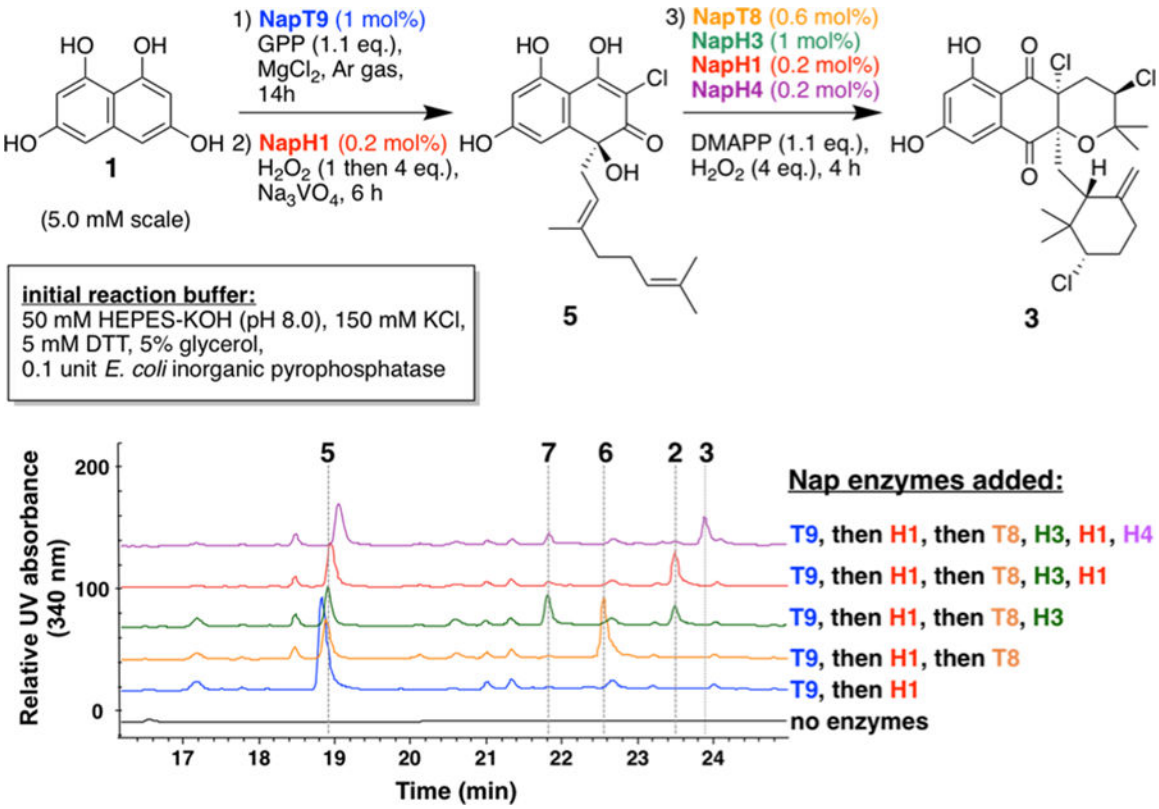
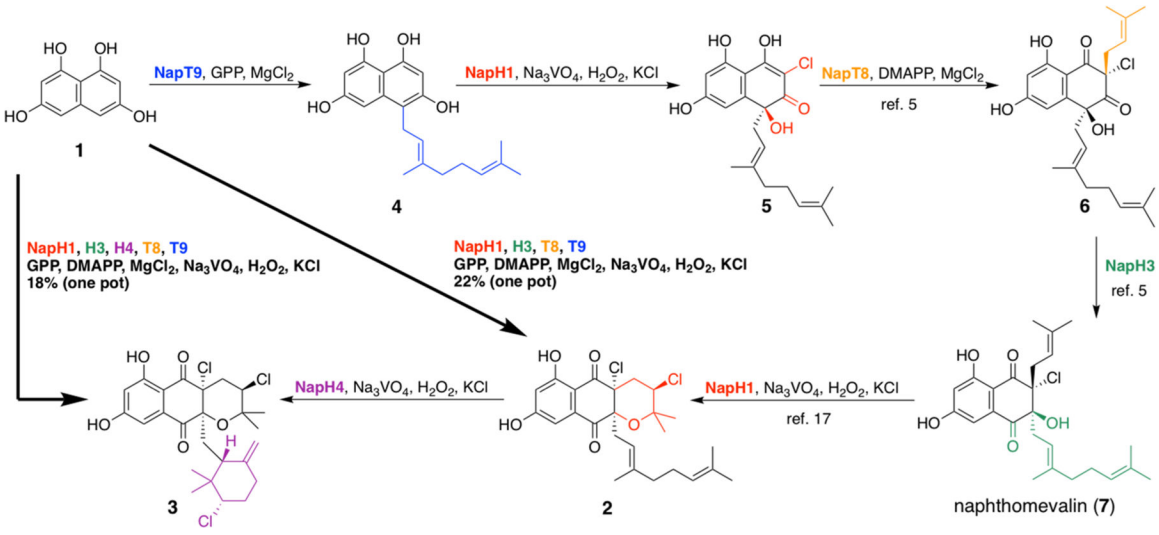


Figure 4.

Chemoenzymatic strategy to the one pot, three-step formation of napyradiomycin biosynthetic intermediates from metabolic precursors.



Scheme 1.

Biosynthetic Pathway from 1,3,6,8-Tetrahydroxynaphthalene (1) to Napyradiomycin B1 (3)