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Articles

Heterologous Catalysis of the Final Steps of Tetracycline Biosynthesis by *Saccharomyces cerevisiae*

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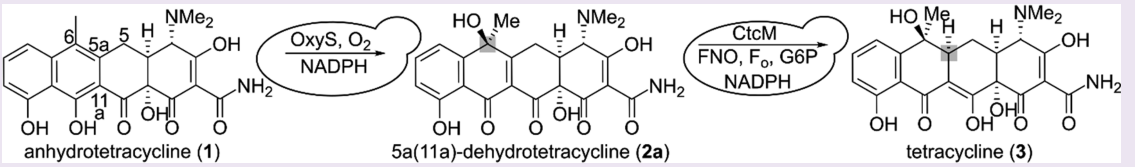

ABSTRACT: Developing treatments for antibiotic resistant bacterial infections is among the highest priority public health challenges worldwide. Tetracyclines, one of the most important classes of antibiotics, have fallen prey to antibiotic resistance, necessitating the generation of new analogs. Many tetracycline analogs have been accessed through both total synthesis and semisynthesis, but key C-ring tetracycline analogs remain inaccessible. New methods are needed to unlock access to these analogs, and heterologous biosynthesis in a tractable host such as *Saccharomyces cerevisiae* is a candidate method. C-ring analog biosynthesis can mimic nature's biosynthesis of tetracyclines from anhydrotetracyclines, but challenges exist, including the absence of the unique cofactor F_{420} in common heterologous hosts. Toward this goal, this paper describes the biosynthesis of tetracycline from anhydrotetracycline in *S. cerevisiae* heterologously expressing three enzymes from three bacterial hosts: the anhydrotetracycline hydroxylase OxyS, the dehydrotetracycline reductase CtcM, and the F_{420} reductase FNO. This biosynthesis of tetracycline is enabled by OxyS performing just one hydroxylation step in *S. cerevisiae* despite its previous characterization as a double hydroxylase. This single hydroxylation enabled us to purify and structurally characterize a hypothetical intermediate in oxytetracycline biosynthesis that can explain structural differences between oxytetracycline and chlortetracycline. We show that F_o, a synthetically accessible derivative of cofactor F_{420}, can replace F_{420} in tetracycline biosynthesis. Critically, the use of *S. cerevisiae* for the final steps of tetracycline biosynthesis described herein sets the stage to achieve a total biosynthesis of tetracycline as well as novel tetracycline analogs in *S. cerevisiae* with the potential to combat antibiotic-resistant bacteria.

INTRODUCTION

The development of treatments for antibiotic resistant bacterial infections is an urgent public health challenge.[1] Indeed, the U.S. Center for Disease Control reports over 2 million illnesses and 20 000 deaths annually in the United States as a result of antibiotic resistance,[1] and the World Health Organization reports over 700 000 deaths annually worldwide from drug-resistant diseases.[2] Novel antibiotic drug candidates have traditionally been accessed through natural product screening, total synthesis, semisynthesis, and combinatorial chemistry. However, the number of new antibiotics approved by the FDA dropped significantly from previous decades, from 29 approvals in the 1980s to 23 in the 1990s, 9 in the 2000s and 15 in the 2010s.[3,4] To reverse this trend, transformative new technologies for the discovery of new antibiotics are needed.

Beginning in the 1940s, tetracyclines were used widely for respiratory, gastrointestinal, and genitourinary tract infections. Indeed, tetracyclines were among the first antibiotic agents with broad-spectrum activity against numerous Gram-positive and Gram-negative bacteria,[5] with their mode of action established as inhibiting bacterial protein synthesis by binding reversibly to the 30S ribosomal subunit and sterically hindering aminoacyl-tRNA binding to the ribosomal A-site.[6,7] However, as with any member of the antibiotic armamentarium, tetracycline use has also led to the spread of antibiotic resistance,[8] with bacteria having acquired a growing number of resistance mechanisms including efflux pumps,[9] ribosomal protection proteins,[10] rRNA mutations,[11] and enzymatic degradation.[12]

The main methods for the production of tetracycline antibiotics are biosynthesis and semisynthesis using the original microbial hosts, as well as total synthesis.[13−15] Key subclasses of tetracycline antibiotic analogs remain inaccessible through these methods, and new methods are needed to access these analogs. A candidate new method is heterologous biosynthesis and semisynthesis. The use of the original tetracyclines host strains, *Streptomyces aureofaciens* and *Streptomyces rimosus*, limits the possible range of tetracycline

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scaffolds for semisynthetic modifications to chlortetracycline, oxytetracycline, tetracycline, and demeclocycline.[13] The use of alternative original hosts such as the dactylocycline producer *Dactylosporangium* sp. SC 14051 is theoretically possible but practically limited, because of a very slow growth rate and an inaccessibility of genetic modifications.[14] The Myers convergent tetracycline synthesis has been incredibly effective in generating D-ring tetracycline analogs, including the FDA-approved eravacycline.[15] However, this powerful synthetic strategy for forming the AB-ring precursor and then coupling it with the D-ring precursor across the C-ring limits the overall number of achievable modifications at the AB-ring junction and the C-ring.[16,17]

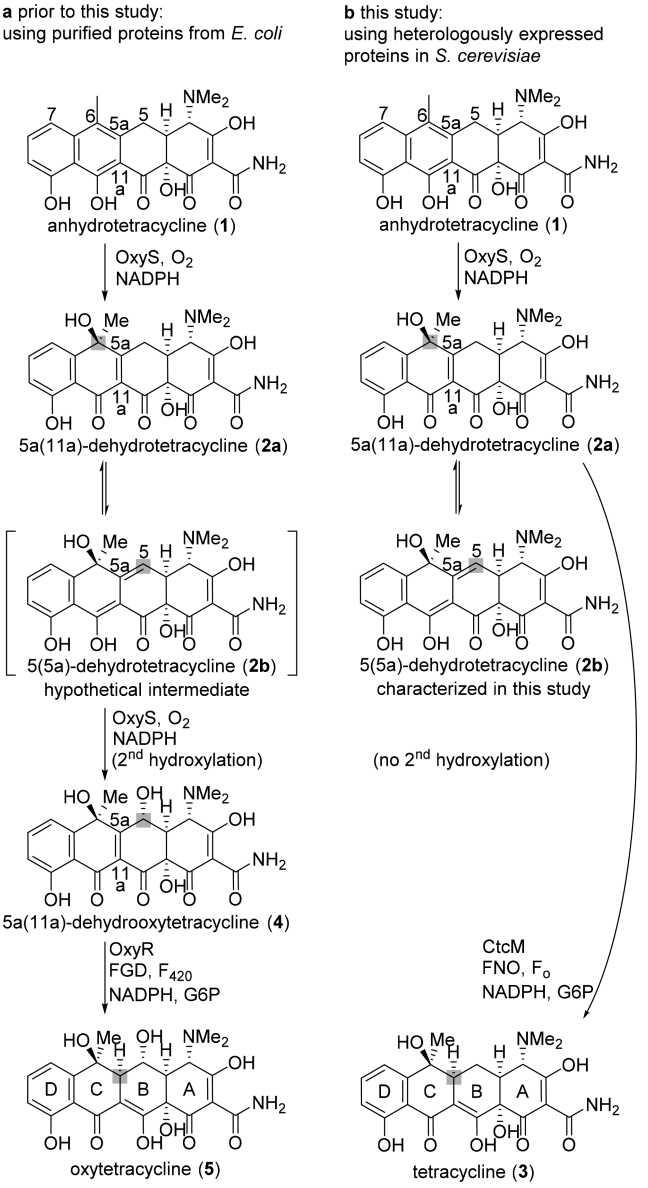
A major heterologous host candidate for the production of tetracycline analogs is *Saccharomyces cerevisiae*, which has proven itself by heterologously producing a variety of natural products of importance to human health. A key example is the production of artemisinic acid, a precursor to artemisinin.[18] More recent examples include the production of hydrocodone, Δ9-tetrahydrocannabinolic acid (2-carboxy-THC), and penicillin.[19−21] One of the promises in natural product biosynthesis using *S. cerevisiae* is the access to unnatural analogs of natural products.[20] Specifically, heterologous production of tetracyclines in *S. cerevisiae* has the potential to yield currently inaccessible analogs of tetracyclines to combat the resistance to clinically available tetracycline antibiotics. We envisioned engineering *S. cerevisiae* to heterologously express tetracycline biosynthetic enzymes from original hosts that are genetically intractable and/or slow growing followed by semisynthetic modifications. Heterologous biosynthesis of tetracyclines in *S. cerevisiae* would also be instructive for the study of tetracycline biosynthesis.

The last steps in the biosynthesis of the tetracyclines are hydroxylation and reduction, starting from an anhydrotetracycline, catalyzed by an FAD-dependent anhydrotetracycline hydroxylase and an F_{420}-dependent dehydrotetracycline reductase (Scheme 1). In the biosynthesis of oxytetracycline (5-hydroxytetracycline), these steps are catalyzed by OxyS and OxyR, respectively, and in the biosynthesis of chlortetracycline (7-chlorotetracycline), these steps are catalyzed by CtcN and CtcM, respectively. Two key differences between the two pathways with regard to these steps are the starting anhydrotetracycline structure and the number of hydroxylation steps on that anhydrotetracycline. While OxyS catalyzes two hydroxylation steps on anhydrotetracycline in the oxytetracycline pathway, CtcN catalyzes only one hydroxylation step on anhydrochlortetracycline (7-chloroanhydrotetracycline) in the chlortetracycline pathway.[22,23] It was hypothesized that structural differences between OxyS and CtcN lead to a difference in the number of hydroxylation steps between the two enzymes.[22]

A unique cofactor to the last steps of the tetracyclines' biosynthesis that is not native to *S. cerevisiae* is cofactor F_{420}, a lactyl oligoglutamate phosphodiester derivative of 7,8-didemethyl-8-hydroxy-5-deazariboflavin (F_o). F_o is much more synthetically accessible than F_{420}. As such, F_o can act as a substitute for F_{420} in some F_{420}-dependent reactions in vitro, but it does not appear to have a redox role in living cells.[24] F_{420}-reducing NADPH dehydrogenase enzymes such as F_{420} NADPH oxidoreductase (FNO) from *Archaeoglobus fulgidus* can reduce F_{420} to its reductively active form, $F_{420}H_2$.[24,25]

Here, we describe the use of *S. cerevisiae* for the final steps of tetracycline biosynthesis, specifically the conversion of anhydrotetracycline to tetracycline, by the heterologous expression of OxyS, CtcM, and FNO. Importantly, we report that synthetic F_o, exogenously supplied to the engineered *S. cerevisiae* strain, can successfully replace F_{420} in this biosynthetic pathway. Additionally, we report the character-

Scheme 1. Conversion of Anhydrotetracycline to Tetracycline by *Saccharomyces cerevisiae*[a]



[a](a) The conversion of anhydrotetracycline (**1**) to oxytetracycline (**5**) using purified proteins OxyS, OxyR, and FGD along with NADPH, F_{420}, and G6P prior to this study.[22] (b) The conversion of anhydrotetracycline (**1**) to tetracycline (**3**) using +OxyS +CtcM +FNO *Saccharomyces cerevisiae* whole cells or cell lysates along with NADPH, F_o, and G6P in this study; 5(5a)-dehydrotetracycline (**2b**) and tetracycline (**3**) were isolated in this study following incubation of a +OxyS and a +OxyS +CtcM +FNO *S. cerevisiae* cell lysate with **1**, respectively; the conversion of 5(5a)-dehydrotetracycline (**2b**) to oxytetracycline (**5**) was expected when a +OxyS + OxyR + FNO cell lysate was used in this study but was not observed. Gray squares emphasize the carbons at which key chemical transformations occur. FGD = F_{420}-dependent glucose-6-phosphate dehydrogenase; F_o = 7,8-didemethyl-8-hydroxy-5-deazariboflavin; FNO = F_{420}-NADP oxidoreductase from *A. fulgidus*; G6P = glucose-6-phosphate

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ization of a previously hypothetical intermediate in oxytetracycline biosynthesis[22] that can explain structural differences between oxytetracycline and chlortetracycline. Tetracycline biosynthesis is enabled in our study because OxyS performs in *S. cerevisiae* cells and cell lysates just a single hydroxylation step on anhydrotetracycline and not two, as previously reported when purified enzymes were used.[22] Thus, our study enhances the understanding of tetracycline biosynthesis and paves the road for total heterologous biosynthesis of tetracyclines in *S. cerevisiae*.

RESULTS

To carry out the final steps of a tetracycline biosynthesis in *S. cerevisiae*, namely the conversion of anhydrotetracycline to tetracycline, we heterologously expressed three enzyme types. The first, an anhydrotetracycline hydroxylase, converts anhydrotetracycline to dehydrotetracycline. Here, we employed OxyS from the oxytetracycline pathway as the anhydrotetracycline hydroxylase.[22] The second, a dehydrotetracycline reductase, converts dehydrotetracycline into tetracycline. In this case, we tested three dehydrotetracycline reductase candidates, OxyR, DacO4, and CtcM from the oxytetracycline, dactylocycline, and chlortetracycline pathways, respectively. The third, an F_{420} reductase, reduces the cofactor used by the dehydrotetracycline reductase. Here, three F_{420} reductase candidates from *Mycobacterium tuberculosis*, *Archaeoglobus fulgidus*, and *Streptomyces griseus* were explored. We used commercially available anhydrotetracycline as the substrate in this biosynthesis and synthetic F_o as a dehydrotetracycline reductase-cofactor instead of its much more complex derivative cofactor F_{420}.

6-Hydroxylation of Anhydrotetracycline in *Saccharomyces cerevisiae*. The first step required to convert anhydrotetracyclines to tetracyclines is 6-hydroxylation. In the biosynthesis of oxytetracycline, this step is catalyzed by OxyS.[22] To test the capacity of *S. cerevisiae* to hydroxylate anhydrotetracycline, we chose OxyS and anhydrotetracycline as the enzyme−substrate pair (Scheme 1). We made this choice because OxyS functionally expresses in *Escherichia coli* and because anhydrotetracycline is commercially available.[22] The expression plasmid pSP-G1 was chosen to facilitate the coexpression of a dehydrotetracycline reductase enzyme, to append antibody tags to both the hydroxylase and the reductase and to constitutively express both enzymes.[26] OxyS was cloned into pSP-G1 under the transcriptional control of the strong constitutive promoter *TEF1* with a FLAG antibody tag at its C-terminus.[26]

First, we tested for the catalysis of anhydrotetracycline hydroxylation by OxyS in *S. cerevisiae* cell lysate by mass spectrometry. For the cell lysate experiment, we supplied +OxyS cells or the control −OxyS cells with the anhydrotetracycline starting material as well as with NADPH, a cofactor of OxyS.[22,27] Indeed, when a lysate of *S. cerevisiae* cells expressing OxyS is incubated with anhydrotetracycline, the molecular ion corresponding to dehydrotetracycline (**2**, $[M + H]^+$ = 443.3) has over 20 times higher counts compared to when a lysate of *S. cerevisiae* cells not expressing OxyS is incubated with anhydrotetracycline. In addition, the ion counts for the molecular ions corresponding to anhydrotetracycline (**1**, $[M + H]^+$ = 427.3) are decreased in the case of lysates of cells expressing OxyS, compared to lysates of cells not expressing OxyS, supporting the biosynthesis of dehydrotetracycline from anhydrotetracycline (**2**, Figure 1).

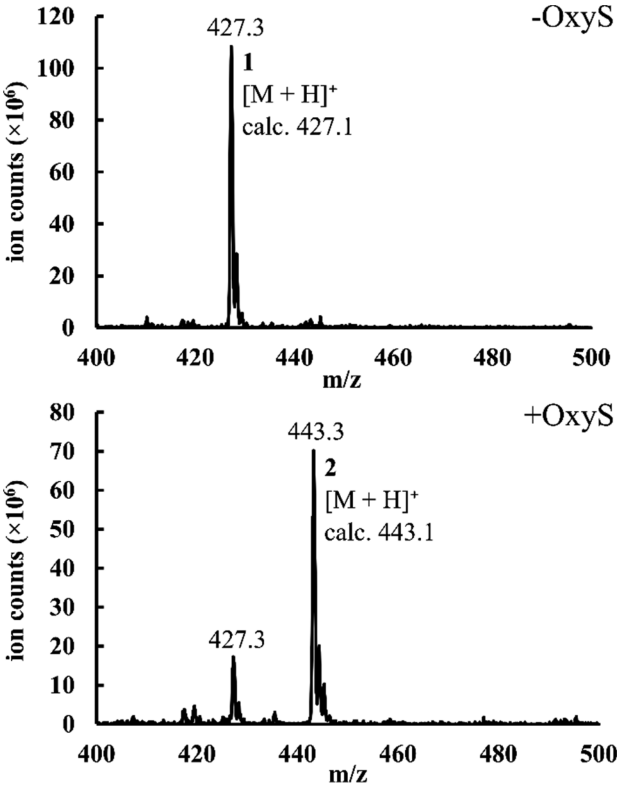

Figure 1. Mass spectrometry analysis of anhydrotetracycline hydroxylation in lysate of *S. cerevisiae* cells expressing OxyS. Cell lysate of EH-3-248-1 expressing OxyS (+OxyS) or the control strain EH-3-248-8 expressing no hydroxylase (−OxyS) was placed overnight in Tris buffer (100.0 mM, pH 7.45) containing anhydrotetracycline (**1**, 5.4 mM), glucose (27.8 mM), NADPH (3.0 mM), and mercaptoethanol (18.5 mM). After overnight incubation, methanol was added, the contents were mixed, and the reaction was filtered prior to MS analysis. *m/z* calcd for protonated anhydrotetracycline ($C_{22}H_{23}N_2O_7^+$): 427.15. Found: 427.3 $[M + H]^+$. *m/z* calcd for protonated 5a(11a)-dehydrotetracycline and for protonated 5(5a)-dehydrotetracycline (**2a** and **2b**, respectively, $C_{22}H_{23}N_2O_8^+$): 443.15. Found: 443.3 $[M + H]^+$. The expected $[M + H]^+$ value for **2** holds for either **2a** or **2b** as the two are isomers (Scheme 1).

Following this cell lysate result, the hydroxylation of anhydrotetracycline by *S. cerevisiae* cells expressing OxyS was tested by incubation of whole cells with anhydrotetracycline. Cultures of OxyS expressing cells and control cells were pelleted, and the pellets were resuspended and incubated overnight with anhydrotetracycline in Tris buffer (pH 7.45) prior to spectroscopic measurements. Indeed, the molecular ion corresponding to dehydrotetracycline had over 10 times higher ion counts in the OxyS expressing strain relative to the control strain (Figure S1).

Next, the hypothesis that anhydrotetracycline is converted to the hydroxylation intermediate, 5a(11a)-dehydrotetracycline (**2a**), was tested by a larger scale reaction, purification, and NMR analysis of the isolates. A cell lysate of the +OxyS strain was incubated overnight with anhydrotetracycline, and the hydroxylation product was isolated by liquid−liquid extraction using ethyl acetate and water followed by reverse phase HPLC purification. To prevent acidic and thermal degradation of the hydroxylation intermediate, reverse phase HPLC was performed with a mobile phase gradient of acetonitrile in Tris buffer (pH 7.45), and NMR analysis was performed at 273 ± 5 K in methanol-d_4. Gradients such as acetonitrile in NH_4OAc,

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H_2O:TFA, and H_2O, as well as ambient temperature NMR after the use of these aqueous phases led to degradation of the intermediate (data not shown).

Notably, after the reaction of the +OxyS cell lysate with anhydrotetracycline, 5(5a)-dehydrotetracycline (**2b**) was obtained instead of the dehydrotetracycline isomer that was anticipated, 5a(11a)-dehydrotetracycline (**2a**, Figure 2,

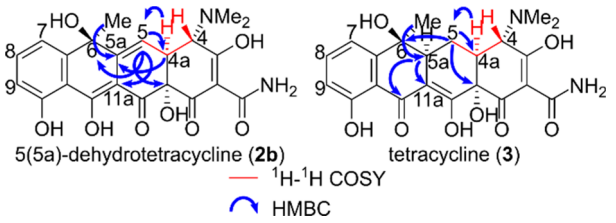
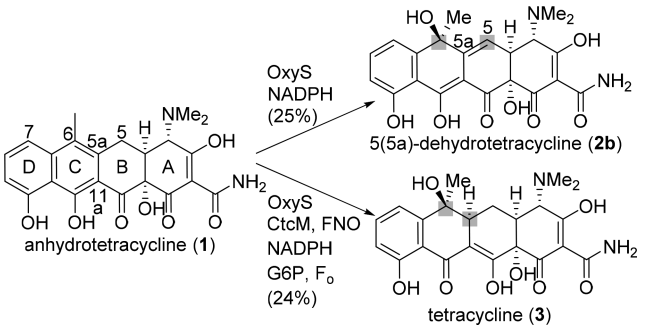


Figure 2. Key interactions of ^{1}H−^{1}H COSY and HMBC in the NMR of 5(5a)-dehydrotetracycline (**2b**) and tetracycline purified from cell lysate reaction of +OxyS and +OxyS +CtcM +FNO strains, respectively (methanol-d_4, 500 MHz).

Scheme 2. Biosynthesis of 5(5a)-Dehydrotetracycline (2b) and Tetracycline (3) in *S. cerevisiae* Cell Lysates[a]



[a]5(5a)-dehydrotetracycline (**2b**) and tetracycline (**3**) were isolated in this study in 25% and 24% yield, following incubation of anhydrotetracycline (**1**) with lysates of +OxyS and +OxyS +CtcM +FNO *S. cerevisiae* cells, respectively. NADPH, G6P, and F_o were supplemented to the lysates as indicated. **2b** and **3** were purified by liquid−liquid extraction and reverse phase HPLC purification and characterized by NMR and HRMS (Figures S5−S13). Gray squares emphasize the carbons at which key chemical transformations occur. F_o = 7,8-didemethyl-8-hydroxy-5-deazariboflavin; FNO = F_{420}-NADP oxidoreductase from *A. fulgidus*; G6P = glucose-6-phosphate

Scheme 2). All protons of 5(5a)-dehydrotetracycline (**2b**) have chemical shifts within 0.3 ppm of the corresponding protons in tetracycline except for the protons attached to the C_5 and C_{5a} positions (Table 1). As expected, a proton on C_{5a} exists in tetracycline but not in 5(5a)-dehydrotetracycline (**2b**). In addition, while tetracycline has two aliphatic protons on C_5 at δ 1.93 and 2.22, 5(5a)-dehydrotetracycline (**2b**) has only one C_5 proton at δ 5.66, typical of olefinic protons. By contrast, 5a(11a)-dehydrotetracycline (**2a**), the expected product of OxyS hydroxylation of anhydrotetracycline, should have two aliphatic protons on C_5 and no additional olefinic proton (Scheme 1). The interconversion between 5(5a)-dehydrotetracycline (**2b**) and 5a(11a)-dehydrotetracycline (**2a**) is supported by a D−H exchange experiment, where the 5-H peak is eliminated over time at 300 K (Figure S2) in methanol-d_4. Despite this interconversion, only the 5(5a)-

Table 1. ^{1}H-NMR Data for 5(5a)-Dehydrotetracycline (2b) and for Tetracycline (Methanol-d_4, 500 MHz)

no.	5(5a)-dehydrotetracycline (**2b**) δ_H (J in Hz)	tetracycline (**3**) δ_H (J in Hz)
4	3.95, d (11.3)	4.08, d (3.0)
4a	3.03, dd (11.5, 6.2)	2.95, ddd (12.7, 3.0, 2.5)
4-NMe2	2.77, s	3.03, s
5	5.66, d (6.2)	2.22 dddd (13.4, 3.0, 3.0, 2.5); 1.93 ddd (13.4, 13.3, 11.2)
5a		3.05, m
6-Me	1.51, s	1.63, s
7	7.13, d (7.8)	7.17, dd (7.8, 0.7)
8	7.28, dd (8.0, 7.9)	7.54, dd (8.1, 8.0)
9	6.63, d (8.1)	6.94, dd (8.4, 0.7)

dehydrotetracycline isomer (**2b**), and not the 5a(11a)-dehydrotetracycline form (**2a**), was observed in the ^{1}H and ^{13}C NMR spectra, implying that 5(5a)-dehydrotetracycline (**2b**) is the prevalent isomer (Scheme 1). The ^{1}H and ^{13}C assignments of 5(5a)-dehydrotetracycline (**2b**) are supported by one- and two-dimensional NMR experiments (Figure 2 and Figures S5−S11).

Reduction of the Hydroxylation Intermediate Using *Saccharomyces cerevisiae*. The second step in the biosynthesis of tetracycline from anhydrotetracycline is the reduction of the 5a(11a) α,β-unsaturated double bond (Scheme 1). The reduction at the 5a(11a) bond is known to be essential to the antibiotic activity of the tetracyclines. For example, 7-chlorotetracycline is over 20 times more potent than 7-chloro-5a(11a)-dehydrotetracycline against *Staphylococcus aureus*.[28] To execute the reduction step, we placed OxyR, the reductase of 5a(11a)-dehydrooxytetracycline (**2a**) from the oxytetracycline pathway,[22] under the control of the *PGK1* promoter in the pSP-G1-OxyS plasmid.

The catalytic activity of OxyR is known to be dependent on cofactor F_{420}, a unique cofactor not native to *S. cerevisiae*. F_o is an intermediate in cofactor F_{420} biosynthesis and is known to successfully replace cofactor F_{420} as a substrate of F_{420} reductase enzymes with similar K_m and k_{cat} values.[24,29] For example, a cofactor F_{420} reductase from *Methanobacterium thermoautotrophicum* had a K_m value of 19 μM with F_{420} and 34 μM with F_o.[30] In another example, a cofactor F_{420} reductase from *Methanococcus vannielii* catalyzed the reduction of F_{420} and F_o with k_{cat}/K_m values of 158 and 56 min^{-1} μM^{-1}, respectively.[31] To test if OxyR could be functional in *S. cerevisiae* with F_o as a cofactor, we exogenously added synthetic F_o to a lysate of *S. cerevisiae* cells expressing OxyS, OxyR, and an F_o reductase.[25] We employed F_o in a concentration of 400 μM, about 10× the experimental K_m values of F_o with F_{420} reductases. We tested if F_{420} reductases can function as F_o reductases in our system. Toward this end, we used F_{420} reductases from three hosts, *M. tuberculosis*, *A. fulgidus*, and *S. griseus*, by expression under the control of *pGPD* (*pTDH3*) on pRS413.[32−35] The reaction mixture containing the *S. cerevisiae* cell lysate in Tris buffer (pH 7.45) also contained anhydrotetracycline, glucose, and NADPH. In the reactions with F_{420} reductase from *M. tuberculosis*, glucose-6-phosphate (G6P), the reducing agent used by this enzyme, was also included for F_o reduction. Since the other two F_{420} reductases use NADPH as the reducing agent of F_{420}, and given that NADPH was already included in the reaction setup as a cofactor for OxyS, no additional reducing agent was added to

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the reactions of F_{420} reductases from *A. fulgidus* and *S. griseus*. We found that the levels of the molecular ion peak corresponding to tetracycline ($[M + H]^+$ = 445.2), the reduction product of 5a(11a)-dehydrotetracycline (**2a**, $[M + H]^+$ = 443.2), increased when G6P was added (Figure 3a vs c, dashed line, Figure S3). Contrary to the expectation that G6P serves as a substrate for the F_o reductase, we found that neither F_o, the F_o reductase, nor OxyR contributed to the increase in the levels of the molecular ion peak corresponding to tetracycline in the presence of G6P (data not shown). However, the low conversion rates of this setup did not allow the purification of tetracycline in levels permitting NMR analysis, and we therefore turned to test alternative reductase enzymes.

Given that OxyS performs one hydroxylation in *S. cerevisiae* (Figure 1) as opposed to two in vitro and in *S. rimosus*,[22] it was logical to test an alternative dehydrotetracycline reductase with OxyR. The native substrate for OxyR is hypothesized to be the doubly hydroxylated 5a(11a)-dehydrooxytetracycline (**4**) and not the singly hydroxylated 5a(11a)-dehydrotetracycline (**2a**, Scheme 1). Furthermore, using the alternative enzyme CtcM from the chlortetracycline pathway instead of OxyR yielded in vitro an increased ratio of tetracycline to oxytetracycline.[22] Another reasonable alternative candidate to OxyR was DacO4 from the dactylocycline pathway, an additional OxyR homologue, whose hypothetical native substrate, 5a(11a)-dehydrodactylocyclinone, is also not hydroxylated at C_5.[14] We therefore tested two candidate dehydrotetracycline reductases, CtcM and DacO4, along with synthetic F_o and three F_{420} reductase candidates, from *M. tuberculosis*, *A. fulgidus*, or *S. griseus*, leading to six combinations.[14,23,25,32,34,35] Gratifyingly, incubating the cell lysate of the strain encoding OxyS, CtcM, and FNO from *A. fulgidus* with anhydrotetracycline resulted in an F_o-dependent peak corresponding to tetracycline (Figure 3b vs a, solid line). As expected, such an F_o-dependent increase in the peak corresponding to tetracycline was not observed in the control strain lacking CtcM and FNO (Figure 3b vs a, dotted line).

In light of the G6P-dependent increase of the molecular ion counts corresponding to tetracycline even in the absence of CtcM (Figure S3), a potential synergy between F_o and G6P was tested. The cell lysate of the +OxyS +CtcM +FNO strain was incubated with anhydrotetracycline and NADPH in the presence of both F_o and G6P in Tris buffer (pH 7.45). Indeed, a major decrease in the molecular ion counts corresponding to dehydrotetracycline (**2**) and a major increase in the molecular ion counts corresponding to tetracycline (**3**) were observed (Figure 3d vs a−c, solid line). As expected, this result was not observed in the +OxyS −CtcM −FNO cell lysate (Figure 3d vs a−c, dotted line).

The conversion of anhydrotetracycline to tetracycline by the +OxyS +CtcM +FNO cell lysate in the presence of F_o and G6P was confirmed by purification and NMR characterization (Scheme 2). Tetracycline was isolated in 24% yield using liquid−liquid extraction with ethyl acetate and water followed by reverse-phase HPLC separation using a mobile phase gradient of acetonitrile in H_2O/TFA (99.9:0.1). Tetracycline was obtained from the cell lysates at a yield of 36 mg/L of the original culture of the cells. The identity of the tetracycline thus obtained to a tetracycline standard was confirmed by ^1H NMR and HRMS (Figures S12 and S13). In addition, the conversion of anhydrotetracycline to tetracycline upon incubation of anhydrotetracycline and F_o with unlyzed yeast

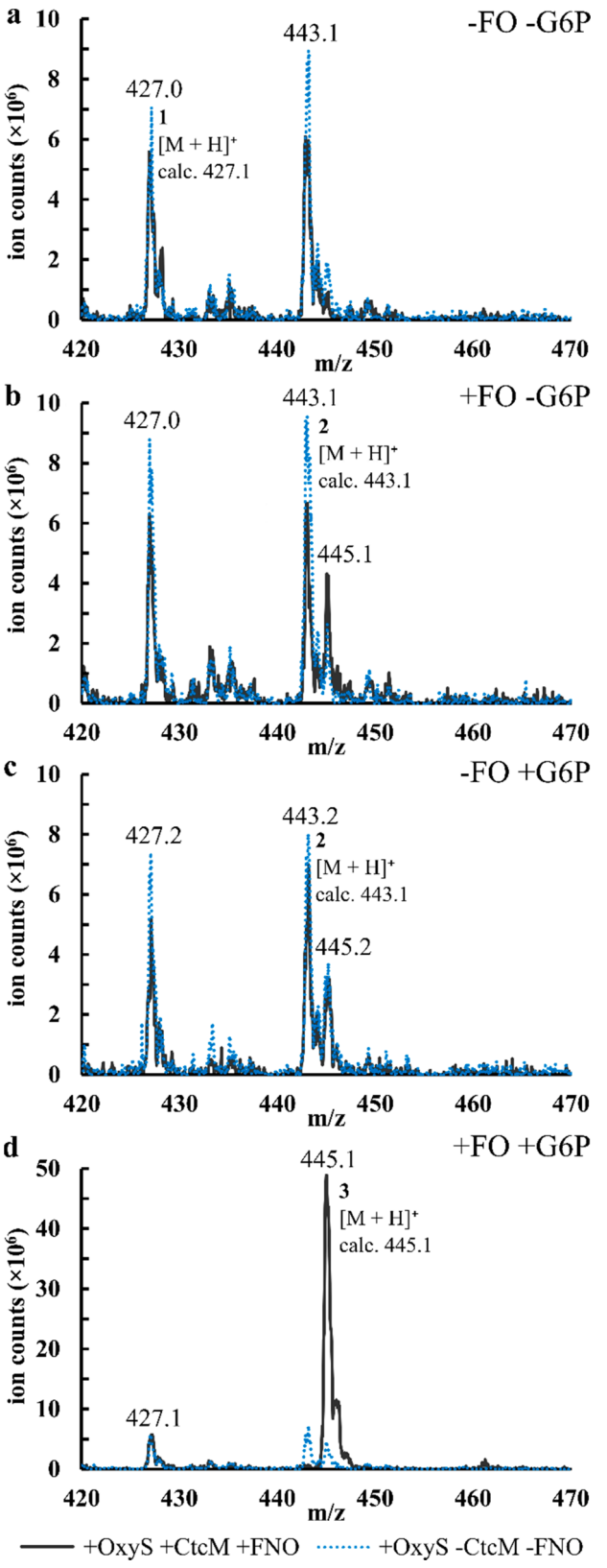

Figure 3. Mass spectrometry analysis of anhydrotetracycline hydroxylation and reduction in lysates of *S. cerevisiae* cells expressing OxyS, CtcM, and FNO in the presence of G6P and F_o. Cell lysates of +OxyS +CtcM +FNO strain EH-6-77-3 (—) or the +OxyS −CtcM −FNO control strain EH-3-204-9 (···) were placed overnight in Tris buffer (100.0 mM, pH 7.45) containing anhydrotetracycline (5.4 mM), glucose (27.8 mM), NADPH (3.0 mM), F_o (0.4 mM (+FO) or

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Figure 3. continued

0 mM (−FO)), glucose-6-phosphate (10.0 mM (+G6P) or 0 mM (−G6P)), and mercaptoethanol (18.5 mM). *m/z* calcd for protonated anhydrotetracycline (**1**, $C_{22}H_{23}N_2O_7^+$): 427.15. Found, 427.0−427.2 $[M + H]^+$. *m/z* calcd for protonated 5a(11a)-dehydrotetracycline or 5(5a)-dehydrotetracycline (**2a** and **2b**, respectively, $C_{22}H_{23}N_2O_8^+$): 443.15. Found: 443.1−443.2 $[M + H]^+$. *m/z* calcd for protonated tetracycline (**3**, $C_{22}H_{25}N_2O_8^+$): 445.16. Found: 445.1−445.2 $[M + H]^+$. The expected $[M + H]^+$ value for **2** holds for either **2a** or **2b** as the two are isomers (Scheme 1). F_o = 7,8-didemethyl-8-hydroxy-5-deazariboflavin; FNO = F_{420}-NADP oxidoreductase from *A. fulgidus*; G6P = glucose-6-phosphate.

cells expressing OxyS, CtcM, and FNO in Tris buffer is supported by mass spectrometry (Figure S4).

DISCUSSION

This study demonstrates the use of *S. cerevisiae* for the final steps of tetracycline biosynthesis, the hydroxylation of anhydrotetracycline, and the reduction of dehydrotetracycline (Scheme 1). These steps are key toward the total biosynthesis of tetracyclines in *S. cerevisiae* in an effort to biosynthesize new tetracycline analogs to combat antibiotic resistance. Heterologous biosynthesis of oxytetracycline was shown in *Streptomyces lividans* K4−11, a much closer relative of the original oxytetracycline biosynthesis host, *Streptomyces rimosus*.[14] The choice of *S. cerevisiae* as a heterologous host in our study enabled the biosynthesis of tetracycline, instead of oxytetracycline. This difference resulted from two reasons: first, a single hydroxylation catalyzed by OxyS from the oxytetracycline pathway in *S. cerevisiae*, as opposed to a double hydroxylation in *Streptomyces* and in vitro (Figure 1), and second, our use of CtcM from the chlortetracycline pathway, instead of OxyR from the oxytetracycline pathway, as the dehydrotetracycline reductase (Figure 3). Possible reasons for the single hydroxylation of OxyS in *S. cerevisiae* instead of the double hydroxylation observed in purified enzymes could be differences in the post-translational modifications of OxyS or differences in the chemical environment of OxyS, such as pH differences. Future research could determine the relevant factor for this difference.

Production of tetracycline in our work requires supplementation of anhydrotetracycline to *S. cerevisiae* cells or cell lysates. For a total biosynthesis of tetracycline and its analogs in *S. cerevisiae*, the additional enzymes coding for tetracycline biosynthesis would have to be heterologously expressed as well. Our work solves important challenges toward the goal of total biosynthesis of tetracyclines in *S. cerevisiae*, such as the challenge of reducing dehydrotetracycline to tetracycline in a heterologous host that does not biosynthesize the native cofactor, coenzyme F_{420}. Importantly, for industrial production of tetracyclines by *S. cerevisiae*, the yield of tetracycline would need to be improved from our current yield of 36 mg/L to yields that are closer to yields of tetracyclines from industrial strains, such as the industrial strains of *Streptomyes rimosus* producing oxytetracycline at 100 g/L.[36]

Our study is the first to isolate and analyze a dehydrotetracycline intermediate when OxyS is expressed in the absence of a dehydrotetracycline reductase, such as OxyR or CtcM (Figures 1 and 2). The reaction product of OxyS rapidly degraded both in vitro, when OxyR was not concurrently used, and in vivo, in Δ*oxyR S. lividans*.[22] Two explanations are noted for our ability to purify and analyze 5(5a)-dehydrotetracycline (**2b**) from the +OxyS *S. cerevsiae* cell lysate. First, the additional degradation pathways that become possible in the presence of the additional 5-hydroxy installed by OxyS in vitro and in *Streptomyces* but not in our study. For example, anhydrooxytetracycline (5-hydroxyanhydrotetracycline) is known to undergo B-ring scission degradation, which is not possible in anhydrotetracycline.[37] Second, in our study, the hydroxylated product of anhydrotetracycline was protected from acid, while in the in vitro study of OxyS, acidic organic extraction was employed.[22] Anhydrooxytetracycline is known to undergo B-ring scission specifically in the presence of dilute acid.[37]

Our characterization of 5(5a)-dehydrotetracycline (**2b**) enhances the current understanding of the last steps of chlortetracycline and oxytetracycline biosynthesis and the differences between these pathways. First, prior to our study, 5(5a)-dehydrotetracycline (**2b**) was an uncharacterized hypothetical intermediate in the OxyS hydroxylation of anhydrotetracycline.[22] By characterizing 5(5a)-dehydrotetracycline (**2b**), our study supports the hypothesized mechanism of OxyS hydroxylation in the biosynthetic pathway of oxytetracycline (**5**, Scheme 1, Figure 2). Second, prior to our study, it was hypothesized that the double hydroxylation performed by OxyS as opposed to the single hydroxylation performed by CtcN results from structural differences between OxyS and CtcN.[22] An increased stability of 5(5a)-dehydrotetracycline (**2b**) over 5a(11a)-dehydrotetracycline (**2a**), supported by our study (Scheme 1, Figure 2), can promote an additional hypothesis. Namely, that structural differences between the dehydrotetracycline intermediates of the two pathways can also contribute to the difference in the number of hydroxylations between the two pathways. Specifically, 5(5a)-dehydrotetracycline (**2b**) is the substrate for a second hydroxylation step, and hence its increased stability over 5a(11a)-dehydrotetracycline (**2a**), the substrate for reduction, can promote a second hydroxylation (Scheme 1). Third, among the dehydrotetracycline intermediates in the biosynthesis of chlortetracycline, oxytetracycline, and tetracycline, the only dehydrotetracycline previously characterized is 5a(11a)-dehydrochlortetracycline (**2a**, Figure S14).[28,38] Future studies can determine whether the lack of the 7-chloro substituent can be a contributing factor to an increased stability of 5(5a)-dehydrotetracycline (**2b**) over 5a(11a)-dehydrotetracycline (**2a**).

Furthermore, we have shown that F_o, a synthetically accessible precursor in the biosynthesis of cofactor F_{420}, can successfully replace F_{420} in the biosynthesis of tetracycline from anhydrotetracycline (Figure 3). To function effectively in our setup, F_o needed to be accepted as a substrate both by the F_o reductase FNO and by the dehydrotetracycline reductase CtcM. The ability of F_o to replace F_{420} in enzymatic reactions of F_{420} reductases was previously known in the literature.[24,29−31,39] However, our study critically shows that this ability further extends to F_{420}-dependent biosynthetic enzymes such as CtcM from the chlortetracycline pathway. This result is important since F_{420} is a unique cofactor that is not native to common heterologous hosts such as *S. cerevisiae* and *E. coli*.[24] Previous attempts at biosynthesis of oxytetracycline using purified enzymes with exogenously added F_{420} or using the more closely related heterologous host *Streptomyces lividans* that natively biosynthesizes F_{420} have been employing cofactor F_{420} and were thus limited in the diversity of possible heterologous hosts that can be used or in the scale of

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tetracycline production, respectively. Moreover, future studies can now incorporate the biosynthesis of F_o into a tetracycline biosynthesis system in *S. cerevisiae* since F_o is only one biosynthetic step removed from common *S. cerevisiae* metabolites, tyrosine and 5-amino-6-ribitylamino-2,4-(1H,3H)-pyrimidinedione.[40−43] Our approach of using F_o to replace F_{420} in the heterologous biosynthesis of tetracyclines could be further extended to other F_{420}-dependent pathways, such as the pathways leading to lincosamides and aminoglycosides.[24]

The synergistic effect of F_o and G6P could be explained by a G6P-dependent increase of NADPH pools from $NADP^+$, catalyzed by G6P dehydrogenase, leading to the reduction of F_o to F_oH_2 (Scheme S1).[44] Interestingly, G6P increases the ion counts corresponding to protonated tetracycline also in the absence of F_o, albeit to a lesser degree than in the presence of F_o (Figure 3). Future research can determine whether a native *S. cerevisiae* enzyme is catalyzing the reduction of 5a(11a)-dehydrotetracycline (**2a**) in the absence of F_o in a G6P-dependent manner. Such G6P dependence could be direct or through an increase in another cellular reducing agent, such as NADPH.

Beyond the use of *S. cerevisiae* toward tetracycline biosynthesis, *S. cerevisiae* can offer access to novel tetracycline analogs. This opportunity exists because of the widely available tools for genetically modifying *S. cerevisiae* as well as enhanced accessibility of biosynthetic enzymes such as P450s.[45−48] Specifically, *S. cerevisiae* can be used to express alternative hydroxylases such as DacO1 and CtcN that proved previously to be insoluble when expressed in other heterologous hosts such as *E. coli* and *Streptomyces*.[22] Such enzymes can hydroxylate alternative anhydrotetracycline substrates, as well as lead to hydroxylated products of the opposite stereochemistry in the 6-position, thereby covering additional chemical space. Importantly, such chemical space is not covered by existing methods of synthesizing tetracyclines, despite the promise of 6-position tetracycline analogs for potent antibiotic activity.[49] Moreover, the use of *S. cerevisiae* for the conversion of anhydrotetracyclines to tetracyclines can readily utilize fungal anhydrotetracycline analogs for further diversity generation.[17,50,51]

CONCLUSION

This study shows that with expression of three heterologous genes and the exogenous supply of a cofactor intermediate, *S. cerevisiae* could be used for the final steps of tetracycline biosynthesis. This study further confirms the structure of a hypothetical intermediate in the biosynthesis of oxytetracycline, providing a potential explanation for the structural difference between the chlortetracycline and oxytetracycline natural products. Additionally, of significant practical importance, the simple cofactor F_o is shown to substitute efficiently for cofactor F_{420}.

While at an early stage of development, the results presented here are the first step toward the total biosynthesis of tetracycline and its analogs in *S. cerevisiae*. These tetracycline analogs could have the potential to combat existing and future mechanisms of bacterial antibiotic resistance. Furthermore, they could introduce unique chemical handles for further derivatization by semisynthesis. Finally, in the future, yeast secreting tetracyclines could have myriad applications not yet envisioned significantly impacting the field of synthetic biology.

MATERIALS AND METHODS

General Methods. Absorption and fluorescence spectra were recorded on an Infinite-M200 fluorescent spectrometer. DNA sequences were purchased from IDT. Polymerases, restriction enzymes, and Gibson Assembly mix were purchased from New England Biolabs. Sanger sequencing was performed by Genewiz. Yeast strains were grown at 30 °C, and shaker settings were 200 rpm, unless otherwise indicated. Yeast transformations were done using the lithium acetate method.[52] Plasmids were cloned and amplified using Gibson Assembly and cloning strain C3040 (New England Biolabs).[53] Unless otherwise indicated, yeast strains were grown on synthetic minimal media lacking histidine and/or uracil and/or tryptophan and/or leucine, as indicated by the abbreviation HUTL.[54] Yeast strain patches were obtained from glycerol stocks by streaking on an agar plate of synthetic medium lacking the appropriate amino acid markers, incubating at 30 °C for 3 days, patching single colonies onto a fresh agar plate, and incubating at 30 °C overnight. Protein homology was calculated by BLAST (https://blast.ncbi.nlm.nih.gov) using the standard sequence alignment parameters. DataExpress was used to analyze Advion CMS data, and MassLynx was used to analyze Waters XEVO QTOF data.

Codon optimization by COOL (http://cool.syncti.org/index.php) was used for all hydroxylases and reductases unless noted explicitly that JCAT or no optimization was used (http://www.jcat.de/). Optimization parameters chosen were individual codon use, a codon context GC content of 39.3%, and the *S. cerevisiae* organism. The following restriction sites were generally excluded: GAGACC, GGTCTC, GGATCC, GAGCTC, CTCGAG, GAAGAC, GTCTTC, CGTCTC, GAGACG, GAATTC, TTAATTAA, TCTAGA, ACTAGT, CCCGGG, CTGCAG, AAGCTT, GTCGAC, ACGCGT, GGTACC, GCGGCCGC, AGATCT, GGCCGGCC, CCGCGG, GCTAGC, and CCATGG.

Preparative HPLC was carried out with a C-18 5 μ column, 250 × 10 mm, eluent given in parentheses. NMR spectra were obtained using Bruker 400 or 500 MHz instruments, as indicated. Unless stated otherwise, mass spectroscopy measurements were performed on an Advion CMS mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. HRMS spectra and MS analyses of whole cell supernatants were taken on a Waters ACQUITY UPLC XEVO QTOF equipped with a BEH C18 column (2.1 × 50 mm) at 30 °C with a flow rate of 0.8 mL/min. Unless stated otherwise, all reagents, salts, and solvents were purchased from commercial sources and used without further purification.

General Protocol for Hydroxylation/Reduction Assay with Cell Lysate. Fresh patches of strains harboring the plasmid for the hydroxylation with/without the reduction enzyme and with/without the plasmid for the F_{420} reductase enzyme and control strains were inoculated in 5 mL of selective media (U^- or HU^-) in 15 mL culture tubes (Corning 352059) and placed in a shaker overnight to OD_{600} 2−3. Overnight cultures were used to inoculate 100 mL of selective media (U^- or HU^-) cultures in 500 mL conical flasks with a starting OD_{600} of 0.01−0.05. Cells were grown to final OD_{600} of 0.6−0.8 before pelleting in two 50 mL tubes (Corning 352098) at 4 °C and 4000 rpm for 20 min. Each pellet was redissolved in 0.5 mL of H_2O, and the suspension was distributed into two presterilized 1.5 mL Eppendorf tubes and pelleted at 14 000 rpm for 10 min at 4 °C. Pellets were stored at −20 °C prior to further use.

Pellets were weighed and thawed on ice. A 99:1 mixture of Y-PER yeast protein extraction reagent (ThermoFisher Scientific 78991) and HALT protease inhibitor cocktail (ThermoFisher Scientific PI87786) was added in a ratio of 3 μL of mixture per milligram pellet and placed on an orbital shaker for 20 min at 22 °C, followed by 10 min of centrifugation at 14 000 rpm at 4 °C, and the cell lysate was transferred to a new 1.5 mL Eppendorf tube, kept on ice, and used within 1 h.

The cell lysate (0.080 mL) was added as the last component to a 4 mL vial (Chemglass CG-4900−01) containing 0.280 mL of 143.0 mM Tris (pH 7.45), 7.7 mM anhydrotetracycline·HCl (AdipoGen CDX-A0197-M500), 4.3 mM NADPH tetrasodium hydrate (Sigma-

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Aldrich N7505), 26.4 mM mercaptoehtanol, 0.5 mM of F_o (in experiments labeled + FO, 0 mM in all other experiments), 14.3 mM glucose-6-phosphate (in experiments labeled + G6P, 0 mM glucose-6-phosphate in all other experiments), and 0.040 mL of glucose (278.0 mM) for final concentrations of 100.0 mM Tris, 5.4 mM anhydrotetracycline·HCl, 3 mM NADPH, 18.5 mM mercaptoethanol, 0 or 0.4 mM F_o as indicated, 0 or 10.0 mM G6P as indicated, and 27.8 mM glucose. A septum was placed on top of the vial through which a needle was inserted to allow air exchange and the reaction was left at 22 °C overnight. After the night, 1 mL of MeOH was added. The contents were mixed, and the reaction was filtered through a PTFE 0.2 μm filter (Acrodisc 4423) prior to analysis by mass and UV/vis spectrometry.

Protocol for Hydroxylation/Reduction Assay with Whole Cells. Fresh patches of strains harboring the plasmid for the hydroxylation and/or reduction enzyme and control strains were inoculated in 5 mL of selective media (U^- or HU^-) in 15 mL culture tubes (Corning 352059) and placed in shaker overnight to OD_{600} 2−3. Overnight cultures were used to inoculate 100 mL of selective media (U^- or HU^-) cultures in 500 mL conical flasks with a starting OD_{600} of 0.05−0.1. Cells were grown for 22 h before being placed at 15 °C for an additional 10−12 h. Cells were then pelleted in two 50 mL tubes (Corning 352098) at 10 °C and 3500 rpm for 5 min. Each pellet was redissolved in 0.5 mL of H_2O, and the suspension was distributed into a presterilized 1.5 mL Eppendorf tube and pelleted at 11 000 rpm for 3 min at 10 °C. Pellets were placed on ice and used within 1 h.

When strains EH-3-98-6 and EH-3-80-3 were used, pellets from 50 mL of culture were redissolved in H_2O (1.025 mL) and added as the last component to 15 mL culture tubes (Corning 352059) containing 1.100 mL of 8 mg mL^{-1} anhydrotetracycline·HCl, 0.125 mL of glucose solution in H_2O (40%), and 0.250 mL of 1 M Tris buffer at pH 7.45 and were placed in a shaker at 350 rpm at 21 °C for 27 h. Cultures were then pelleted, and the supernatant was diluted into H_2O before being used for mass and UV/vis spectroscopy.

When strains EH-6-77-3 and EH-3-204-9 were used, pelleted unlyzed cells were redissolved in H_2O (0.4 mL) and added as the last component to 15 mL culture tubes (Corning 352059) containing 1.100 mL of 8 mg mL^{-1} anhydrotetracycline·HCl, 0.125 mL of glucose solution in H_2O (40%), 0.250 mL of 1 M Tris buffer at pH 7.45, and 0.625 mL of 1.5 mM F_o (+FO) or 0.625 mL of H_2O (−FO) and were placed in a shaker at 350 rpm at 21 °C for 27 h. Cultures were then pelleted, and the supernatant was diluted into H_2O before being used for mass and UV/vis spectroscopy.

Biosynthesis of (5,5a)-Dehydrotetracycline (2b) Using *S. cerevisiae*. Fresh patches of EH-3-248-1 harboring the plasmid encoding OxyS were inoculated in 2 × 5 mL selective media (U^-) in 15 mL culture tubes (Corning 352059) and placed in a shaker overnight to OD_{600} 2−3. Overnight cultures were used to inoculate 500 mL of selective media (U^-) cultures in 2 L conical flasks with a starting OD_{600} of 0.01−0.05. Cells were grown to a final OD_{600} of 0.75 before pelleting in 500 mL tubes at 4 °C and 6000 rpm. The pellet was redissolved in 25 mL of H_2O, and the suspension was distributed into 50 mL falcon tubes and pelleted at 4 °C and 4000 rpm. The pellet was then transferred into four presterilized 1.5 mL Eppendorf tubes and pelleted at 14 000 rpm for 10 min at 4 °C. Pellets were stored at −20 °C prior to further use.

Pellets were weighed and thawed on ice. A 99:1 mixture of Y-PER yeast protein extraction reagent (ThermoFisher Scientific 78991) and HALT protease inhibitor cocktail (ThermoFisher Scientific PI87786) was added in a ratio of 3 μL of mixture per milligram pellet and placed on an orbital shaker for 20 min at 22 °C, followed by 10 min of centrifugation at 14 000 rpm at 4 °C, and the cell lysate was transferred to a new 1.5 mL Eppendorf tube, kept on ice, and used within 1 h.

The cell lysate (3.2 mL) was added as the last component to a 50 mL round-bottom flask with a stir bar containing 12.8 mL of 125.1 mM Tris (pH 7.45), 6.7 mM anhydrotetracycline·HCl (AdipoGen CDX-A0197-M500), 3.8 mM NADPH tetrasodium hydrate (Sigma-Aldrich N7505), 23.1 mM mercaptoethanol, and 34.7 mM glucose for final concentrations of 100.0 mM Tris, 5.4 mM anhydrotetracycline·HCl, 3.0 mM NADPH, 18.5 mM mercaptoethanol, and 27.8 mM glucose. A septum was placed on top of the flask through which a needle was inserted to allow air exchange, and the reaction was left at 22 °C overnight. After 14 h, the aqueous mixture was extracted two times with EtOAc. The combined organic fraction was extracted with water. MeCN was then added to the combined aqueous phase, and it was then dried at 20 °C. The contents were then dissolved in a mixture of water, MeCN, and MeOH and purified by preparative RP-HPLC (1−20% MeCN in Tris (pH 7.45, 20.0 mM, 60 min)) to afford 5(5a)-dehydrotetracycline (**2b**) after lyophilization (11.1 mg, 25% yield) as a yellow solid. **2b**: ^{1}H NMR (500 MHz, methanol-d_4): δ 7.28 (dd, J = 8.0, 7.9 Hz, 1 H), 7.13 (d, J = 7.8 Hz, 1 H), 6.63 (d, J = 8.1 Hz, 1 H), 5.66 (d, J = 6.2 Hz, 1 H), 3.95 (d, J = 11.3 Hz, 1 H), 3.03 (dd, J = 11.5, 6.2 Hz, 1 H), 2.77 (s, 6 H), 1.51 (s, 3 H). ^{13}C NMR (500 MHz, D_2O): δ 192.6, 188.3, 182.9, 180.4, 172.1, 160.7, 148.1, 146.3, 134.3, 116.3, 116.1,115.0, 106.1, 103.5, 102.7, 77.8, 73.1, 69.1, 42.4, 39.3, 34.4. HRMS (ES+), m/z calcd for $C_{22}H_{23}N_2O_8^+$: 443.1449. Found: 443.1415 $[M + H]^+$. HRMS (ES^-), m/z calcd for $C_{22}H_{21}N_2O_8^-$: 441.1303. Found: 441.1318 $[M - H]^-$.

Biosynthesis of Tetracycline Using *S. cerevisiae*. Fresh patches of EH-6-77-3 harboring the plasmids encoding OxyS, CtcM, and FNO were inoculated in selective media (HU^-) in 15 mL culture tubes (Corning 352059) and placed in a shaker overnight to OD_{600} 2−3. Overnight cultures were used to inoculate 100 mL of selective media (HU^-) cultures in 500 mL conical flasks with a starting OD_{600} of 0.01−0.05. Cells were grown to a final OD_{600} of 0.75 before pelleting in 500 mL tubes at 4 °C and 6000 rpm. The pellet was redissolved in 25 mL of H_2O, and the suspension was distributed into 50 mL falcon tubes and pelleted at 4 °C and 4000 rpm. The pellet was then transferred into four presterilized 1.5 mL Eppendorf tubes and pelleted at 14 000 rpm for 10 min at 4 °C. Pellets were stored at −20 °C prior to further use.

Pellets from 50 mL of culture were weighed and thawed on ice. A 99:1 mixture of Y-PER yeast protein extraction reagent (ThermoFisher Scientific 78991) and HALT protease inhibitor cocktail (ThermoFisher Scientific PI87786) was added in a ratio of 3 μL of mixture per milligram of pellet and placed on an orbital shaker for 20 min at 22 °C, followed by 10 min of centrifugation at 14 000 rpm at 4 °C, and the cell lysate was transferred to a new 1.5 mL Eppendorf tube, kept on ice, and used within 1 h.

The cell lysate (0.440 mL) was added as the last component to four borosilicate vials of 4 mL each (4 × 0.110 mL) containing 1.760 mL (0.440 mL each) of 125.1 mM Tris (pH 7.45), 6.7 mM anhydrotetracycline·HCl (AdipoGen CDX-A0197-M500), 3.8 mM NADPH tetrasodium hydrate (Sigma-Aldrich N7505), 23.1 mM mercaptoethanol, 0.5 mM F_o, 125.1 mM G6P, and 34.7 mM glucose for final concentrations of 100.0 mM Tris, 5.4 mM anhydrotetracycline·HCl, 3.0 mM NADPH, 18.5 mM mercaptoethanol, 0.4 mM F_o, 100.0 mM G6P, and 27.8 mM glucose. A septum was placed on top of each vial through which a needle was inserted to allow air exchange, and the reaction was left at 22 °C overnight. After 16 h, the aqueous mixture was extracted two times with EtOAc (7.5 mL in each round). The combined organic fraction was extracted with water (7.5 mL). MeOH was then added to the combined aqueous phase, and it was then dried at 25−30 °C. The contents were then dissolved in a mixture of water, MeCN, and MeOH and purified by preparative RP-HPLC (1−50% MeCN in 99.9:0.1% H_2O/TFA, 90 min) to afford, after drying, tetracycline (1.8 mg, 24% yield) as a yellow solid with HRMS and ^{1}H NMR spectra identical to the tetracycline standard (Figures S12 and S13).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00259.

Supplementary figures, tables, and schemes (PDF)

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Notes

The authors declare no competing financial interest.

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