Novosphingobium aromaticivorans uses a Nu-class glutathione-S-transferase as a glutathione lyase in breaking the β-aryl ether bond of lignin

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ABSTRACT

As a major component of plant cells walls, lignin is a potential renewable source of valuable chemicals. Several sphingomonad bacteria have been identified that can break the β-aryl ether bond connecting most phenylpropanoid units of the lignin heteropolymer. Here, we tested three sphingomonads predicted to be capable of breaking the β-aryl ether bond of the dimeric aromatic compound guaiacylglycerol-β-guaiacyl ether (GGE) and found that Novosphingobium aromaticivorans metabolizes GGE at one of the fastest rates thus far reported. After the ether bond of racemic GGE is broken by replacement with a thioether bond involving glutathione, the glutathione moiety must be removed from the resulting two stereoisomers of the phenylpropanoid conjugate β-glutathionyl-γ-hydroxypropiovanillone (GS-HPV). We found that the Nu-class glutathione-S-transferase NaGSTNu is the only enzyme needed to remove glutathione from both (R)- and (S)-GS-HPV in N. aromaticivorans. We solved the crystal structure of NaGSTNu and used molecular modeling to propose a mechanism for the glutathione lyase (deglutathionylation) reaction in which an enzyme-stabilized glutathione thiolate attacks the thioether bond of GS-HPV, and the reaction proceeds through an enzyme-stabilized enolate intermediate. Three residues implicated in the proposed mechanism (Thr51, Tyr166, and Tyr224) were found to be critical for the lyase reaction. We also found that Nu-class GSTs from Sphingobium sp. SYK-6 (which can also break the β-aryl ether bond) and Escherichia coli (which cannot break the β-aryl ether bond) can also cleave (R)- and (S)-GS-HPV, suggesting that glutathione lyase activity may be common throughout this widespread but largely uncharacterized class of glutathione-S-transferases.

INTRODUCTION

As society looks to diversify its sources of fuels and chemicals, there are reasons to produce them from renewable resources, such as lignocellulosic plant biomass, the most abundant organic material on Earth. Lignin, which can compose ~25% of lignocellulosic plant biomass (1), is a heteropolymer of phenylpropanoid units linked together via several classes of covalent bonds (2). Because of its abundance, its recalcitrance to
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degradation, and the potential value of its aromatic substituents, there is interest in developing economical and environmentally sustainable methods to depolymerize lignin (3).

The β-aryl ether (β-O-4) bond typically constitutes >50% of all the linkages between aromatic units in lignin (2). Thus, methods for breaking this bond will be important for developing systems for lignin depolymerization. The β-etherase pathway, found in some sphingomonad bacteria, is a promising biological route for cleaving the β-aryl ether bond (Fig. 1). While several sphingomonads, such as *Sphingobium* sp. SYK-6, *Erythrobacter* sp. SG61-1L, and *Novosphingobium* sp. MBES04, are known to contain this pathway (4–6), identifying additional species with it could advance development of biological systems for depolymerizing lignin. Ohta et al. (6) identified several additional sphingomonads whose genomes are predicted to contain genes for the enzymes known to be necessary for the pathway. In this work, we test three of these species- *Novosphingobium aromaticivorans* DSM 12444 (7, 8), *Novosphingobium* sp. PPIY (9), and *Sphingobium xenophagum* NBRC 107872 (10, 11)- for the ability to break the β-aryl ether bond of the dimeric aromatic compound guaiacylglycerol-β-guaiacyl ether (GGE; Fig. 1) and find that *N. aromaticivorans* most rapidly and completely metabolizes GGE.

Breaking the β-aryl ether bond of GGE via the β-etherase pathway involves three steps (Fig. 1). First, the α-hydroxyl of GGE is oxidized by stereospecific NAD⁺-dependent dehydrogenases (LigL, LigN, LigD, LigO) to generate the α-ketone, β-(2-methoxyphenoxy)-γ-hydroxypropiovanillone (MPHPV) (12). Next, stereospecific β-etherases (LigF, LigE, LigP) replace the β-ether bond of MPHPV with a thioether bond involving glutathione (GSH), producing guaiacol and the glutathione conjugate, β-glutathionyl-γ-hydroxypropiovanillone (GS-HPV) (13–15). Finally, the glutathione moiety is removed from GS-HPV and combined with another GSH, producing hydroxypropiovanillone (HPV) and glutathione disulfide (GSSG). The Omega-class glutathione-S-transferase (GST) LigG from *Sphingobium* sp. SYK-6 has been shown to remove the glutathione moiety from β(R)-GS-HPV *in vitro* (13), but no enzyme has yet been characterized from this organism that can react with the β(S)-stereoisomer. The Nu-class GST3 from *Novosphingobium* sp. MBES04 has been reported to react with both β(R)- and β(S)-GS-HPV *in vitro* (6), although the physiological role of this enzyme has not been established. Here, we find that Nu-class GSTs from *N. aromaticivorans* (NaGSTₐ) and *Sphingobium* sp. SYK-6 (SYK6GSTₐ) can react with both β(R)- and β(S)-GS-HPV *in vitro*, and that NaGSTₐ is the sole enzyme required for these reactions in *N. aromaticivorans*. We also use kinetic and structural data to propose mechanisms for the glutathione lyase (deglutathionylation) reactions of NaGSTₐ with both GS-HPV stereoisomers.

The most distinguishing characteristic of Nu-class (Main.2 (16)) GSTs is their apparent ability to bind two molecules of GSH and/or a single molecule of GSSG (16–20). Nu-class GSTs are found in many organisms (16, 18), but their physiological roles are largely unknown. Several Nu-class GSTs have been tested for activity *in vitro*; most, including EcYfcG and EcYghU from *Escherichia coli*, showed disulfide bond reductase activity toward small molecules such as 2-hydroxyethyl disulfide (16–18, 21), which led to the proposal that this is their main physiological activity. A strain of *E. coli* in which the *yfg* gene was deleted was impaired in its response to oxidative stress, leading to the proposal that EcYfcG may naturally function as a peroxidase, although this enzyme exhibited low *in vitro* peroxidase activity with model peroxides (22). At least one other Nu-class GST besides GST3 has been found to have glutathione lyase activity *in vitro*: PcuRe2pB1 from the white-rot fungus *Phanerochaete chrysosporium* can cleave GS-phenacylacetophenone, which led to the suggestion that this enzyme may naturally function as a glutathione lyase (19). To investigate the prevalence of glutathione lyase activity throughout the Nu-class, we assayed EcYfcG and EcYghU and found that each cleaves both β(R)- and β(S)-GS-HPV *in vitro*, though less efficiently than NaGSTₐ. Furthermore, EcYghU complements growth of an *N. aromaticivorans* ΔNaGSTₐ mutant, showing that it can perform these reactions *in vivo*. Our results thus suggest that glutathione lyase activity may be common
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throughout the Nu-class, and the lower catalytic efficiencies we find with EcYghU and EcYfcG toward GS-HPV may reflect the fact that this compound is not a natural substrate for E. coli.

RESULTS

GGE metabolism by sphingomonads. To test for the β-etherase pathway in the sphingomonad bacteria N. aromaticivorans DSM 12444, Novosphingobium sp. PP1Y, and S. xenophagum NBRC 107872, we fed them erythro-GGE (Fig. 1), both alone and in the presence of another organic molecule, in case one or more of the strains can break the β-aryl ether bond of GGE but cannot use it to support growth, as is the case for Novosphingobium sp. MBES04 (6).

For these studies, we created a strain of N. aromaticivorans in which the Saro_1879 (putative sacB)-containing cloning vector. When fed erythro-GGE alone, N. aromaticivorans 12444A1879 completely removed the compound from the medium (Fig. 2A,B and Supplemental Fig. S1) and incorporated ~22% of its organic material (measured as chemical oxygen demand, COD) into biomass (Supplemental Table S1). The majority of the electrons from the erythro-GGE (~53%) were likely combined with oxygen to support respiration during growth with the compound (Supplemental Table S1). When fed erythro-GGE plus vanillate, 12444A1879 completely removed both substrates from the medium (Fig. 2G,H) and incorporated ~41% of the COD from the vanillate (based on the results from a culture fed vanillate alone) and ~22% of the COD from the GGE into biomass (Supplemental Table S1). The known β-etherase pathway intermediates threeo-GGE, MPHPV, and HPV transiently appeared in the media of both erythro-GGE-fed 12444A1879 cultures (Fig. 2B,H and Supplemental Fig. S1), while the pathway intermediate guaiacol was only detected at a low level in the medium of the culture fed erythro-GGE plus vanillate (Fig. 2H).

Transcripts of predicted N. aromaticivorans β-etherase pathway genes increase in abundance in the presence of GGE. To test the transcriptional response of N. aromaticivorans to GGE, we investigated expression levels of several of its genes predicted to code for enzymes of the β-etherase pathway, including Saro_2595, which encodes a Nu-class glutathione-S-transferase (named here NaGST_Nu). With the exception of ligL, transcript levels from these genes were higher (by >6-fold) in cells grown in the presence of GGE versus its absence (Table 1).

NaGST_Nu cleaves β(R)- and β(S)-GS-HPV. NaGST_Nu is 38% identical in amino acid sequence to GST3 from Novosphingobium sp. MBES04 (Supplemental Fig. S3), which can convert β(R)- and β(S)-GS-HPV into HPV in
vitro (Fig. 1; (6)). Since N. aromaticivorans lacks any homologues of LigG (the enzyme from Sphingobium sp. SYK-6 that cleaves the β(R)-stereoisomer of GS-HPV (Fig. 1; (13))), we tested whether NaGST\textsubscript{Nu} could cleave both β(R)- and β(S)-GS-HPV. We found that recombinant NaGST\textsubscript{Nu} cleaved both stereoisomers of GS-HPV in vitro (Supplemental Figs. S4 and S5), with slightly higher $k_{\text{cat}}$ and ~5-fold higher $K_M$ with β(R)-GS-HPV than with β(S)-GS-HPV, resulting in a ~4-fold higher $k_{\text{cat}}/K_M$ with the β(S)-isomer (Table 2).

NaGST\textsubscript{Nu} is necessary for GGE metabolism by N. aromaticivorans. To test for an in vivo role for NaGST\textsubscript{Nu}, we generated an N. aromaticivorans strain (12444Δ2595) lacking Saro\textsubscript{2595}, the gene that encodes NaGST\textsubscript{Nu}. When 12444Δ2595 was provided only erythro-GGE, a small amount of MPHPV and threo-GGE appeared in the medium (Fig. 2D), but no cell density was detected (Fig. 2C) and no detectable COD was converted into biomass (Supplemental Table S1). When 12444Δ2595 was fed both vanillate and erythro-GGE, all of the vanillate was consumed, and almost all of the GGE was converted into MPHPV (Fig. 2J). The maximum cell density and amount of COD incorporated into biomass were the same for this culture as for a culture fed vanillate only (Fig. 2I; Supplemental Table S1), suggesting that 12444Δ2595 cannot convert GGE into cell material. A small amount of guaiacol appeared in the medium of the 12444Δ2595 culture fed vanillate and erythro-GGE (Fig. 2J), showing that this strain cleaved some MPHPV. However, unlike for its parent strain (12444Δ1879), no extracellular HPV was detected in any erythro-GGE-fed 12444Δ2595 culture (Fig. 2D). These results show that NaGST\textsubscript{Nu} is necessary for complete GGE metabolism by N. aromaticivorans.

NaGST\textsubscript{Nu} is sufficient and necessary for conversion of GS-HPV into HPV in N. aromaticivorans. To determine which step in the β-etherase pathway requires NaGST\textsubscript{Nu}, we incubated cell extracts of 12444Δ2595 and its parent strain (12444Δ1879) with racemic MPHPV and GSH. With the 12444Δ1879 extract, MPHPV was completely converted to roughly equimolar amounts of guaiacol and HPV, along with a small amount of GS-HPV (Fig. 3A). In contrast, the 12444Δ2595 extract incompletely cleaved the MPHPV, producing roughly equimolar amounts of guaiacol and GS-HPV (along with a low level of HPV, ~2% of the level of GS-HPV formed; Fig. 3B). Thus, the 12444Δ2595 extract was defective in converting GS-HPV into HPV. When recombinant NaGST\textsubscript{Nu} was added to the 12444Δ2595 extract after one day of incubation with MPHPV (Fig. 3B), the initially accumulated GS-HPV rapidly disappeared, with a concomitant increase in HPV, showing that the defect in GS-HPV cleavage by the 12444Δ2595 extract was caused by the lack of NaGST\textsubscript{Nu}.

Structural characterization of NaGST\textsubscript{Nu}. To gain insight into the mechanism of GS-HPV cleavage by NaGST\textsubscript{Nu}, we solved two structures of the enzyme with resolutions of 1.25 (pdb 5uuo) and 1.45 (pdb 5uun) \(\text{Å}\) (Table 3). The structures align with each other with an RMS distance of 0.108 \(\text{Å}\) over 7381 atoms. NaGST\textsubscript{Nu} is a homodimer; each subunit contains a characteristic N-terminal GST1 (thioredoxin-like) domain (Val39 to Gly129), a C-terminal GST2 domain (Ser135 to Leu257), and N-terminal (Met1 to Pro38) and C-terminal (Val258 to Phe288) extensions not present in most other characterized classes of GSTs (Fig. 4A).

Structures of Nu-class GSTs solved in the presence of GSH typically contain either two GSH molecules (e.g., pdb 3c8e, 4ikh) or a single GSGG molecule (e.g., pdb 4mzw, 3gx0) in each active site. Consistent with this, each NaGST\textsubscript{Nu} subunit contained electron density data that was best modeled as a mixed population of GSH1 and GSH2 thiols with HS–SH distance of 2.4 \(\text{Å}\) (~60% occupancy) and a GSGG disulfide with S–S distance of 2.0 \(\text{Å}\) (~40% occupancy) (Fig. 4B; Supplemental Fig. S6), suggesting a heterogeneous population of protein molecules in the crystals in which each subunit contained

* The residue numbers used here for NaGST\textsubscript{Nu} are for the native protein; residue numbers in the pdb entries differ by +5 since the protein used for crystallization contained an N-terminal extension left after proteolytic removal of the His\textsubscript{tag}
either two GSHs or a single GSSG (see legend for Supplemental Fig. S6).

In NaGST_Nu, seven residues make close contacts with GSH1 (Thr51, Asn53, Gln86, Lys99, Ile100, Glu116, and Ser117) and three residues make close contacts with GSH2 (Asn25, Asn53, and Arg177 from the opposite chain in the dimer) (Fig. 4B). These residues and contacts with the GSHs are conserved throughout much of the Nu-class (Supplemental Fig. S3; (16, 19, 20)), although members of this family with truncated N-termini lack an analogue of Asn25 (Supplemental Fig. S3; Supplemental Materials of (19)).

In NaGST_Nu, a short channel connects the active site to the solvent (Fig. 5 and Supplemental Fig. S7). This channel is also present in Nu-class GSTs from Escherichia coli (EcYghU; pdb 3c8e (18)) and Streptococcus sanguinis SK36 (SsYghU; pdb 4mzw (23)). In most other structurally characterized Nu-class GSTs, the active site is more solvent exposed, since these proteins lack N-terminal residues that contribute to one of the channel walls (Supplemental Fig. S3; Supplemental Materials of (19)).

In each NaGST_Nu homodimer we solved (5uun and 5uuo), the individual monomers differ in the positioning of residues Gln282 to Phe288 (Fig. 5A,B,C). The C-terminal Phe288 resides near the active site channel in one subunit (NaGST_Nu closed, Fig. 5B), and ~18 Å away from the channel entrance in the other (NaGST_Nu open, Fig. 5C), resulting in a difference between the monomers in the opening to the active site pocket. The closed NaGST_Nu configuration is stabilized by hydrogen-bonds between Lys286 and both Lys262 and Arg220. The open configuration lacks these interactions and its Arg220 sidechain has two rotamer positions. In other structurally characterized Nu-class GST homodimers, the individual subunits are symmetric, with C-termini that generally extend away from the active site (alignments for EcYghU and SsYghU subunits are shown in Fig. 5A,D,E).

Modeling of substrate binding and proposed reaction mechanism. We separately modeled the GS-moiety of β(R)- and β(S)-GS-HPV into the GSH2 active site position (Fig. 4B) of the closed C-terminal configuration subunit of NaGST_Nu, and found that the HPV moieties extend into the active site channel in different orientations without generating unfavorable steric clashes (Fig. 6A,B; Supplemental Fig. S7A,B). For both GS-HPV stereoisomers, there are predicted hydrogen-bonds between the γ-hydroxyl and the hydroxyl of Tyr224, and between the HPV phenolic group and the carboxyl group of the C-terminal Phe288 (Fig. 6A,B). For β(R)-GS-HPV, the γ-hydroxyl is also predicted to hydrogen-bond with the α-ketone, which in turn is predicted to hydrogen-bond with the hydroxyl of Tyr166 (Fig. 6A). For β(S)-GS-HPV, the hydroxyl of Tyr166 is predicted to form a hydrogen-bond with the HPV aromatic ring (24) and a long-range interaction with the HPV α-ketone (Fig. 6B).

We propose that the thiol of a GSH molecule in the GSH1 active site position of NaGST_Nu is activated by hydrogen-bonding with the side-chain hydroxyl of Thr51 (3.0 Å) and the side-chain amide of Asn53 (3.2 Å) (Figs. 4B, 7A,B). These residues are part of a Thr-Pro-Asn motif that is highly conserved throughout the Nu-class, the Asn residue of which was found to be critical for the glutathione lyase activity of PcUre2pB1 (19). Interactions with active site residues have been shown to lower the pKs of a GSH thiol and stabilize the reactive thiolate anion in members of other GST classes (for example, the GSH thiol pKs is lowered to ~6.6 by hydrogen-bonds with Tyr and Arg side chains in human GSTA3-3 (25)). In addition, the Thr51 hydroxyl closely contacts the backbone amides of Asn53 and Gly54, and the side-chain amide of Asn53 closely contacts the side-chain amine of Lys56 and the Gly carboxylate of the GS-moiety of GS-HPV (Supplemental Fig. S8). Extended hydrogen-bonding networks (“second-sphere interactions”) such as this are proposed to contribute to GSH thiolate stabilization in other GST classes (26).

In our proposed reaction mechanism for NaGST_Nu, the GS1 thiolate attacks the thioether of GS-HPV to form a disulfide GS–SG. Since the C-S bond lyase reaction proceeds in the absence of a redox cofactor such as flavin or pyridine nucleotide, we propose that a transient enolate (Fig. 7C,D) stores the 2e− reducing equivalents released by disulfide bond formation. Formation of this enolate is proposed to be facilitated by polarization of the GS-HPV α-ketone, which is stabilized by the predicted interactions between...
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GS-HPV and Y166 and Y224 (Figs. 6, 7C,D). Due to active site steric constraints, our modeling places the reactive portion (S-Cp-(Cn =O)-aryl) of both β(R)- and β(S)-GS-HPV into roughly planar configurations in the NaGSTNu channel (Fig. 6A,B and Supplemental Fig. S7A,B), which should also promote enolate formation. Collapse of the proposed enolate intermediate proceeds with carbanion trapping of a solvent-derived proton, corresponding to reduction of the carbon atom originally containing the thioether bond (27). In contrast to NaGST, LigG is not known or predicted to be able to bind two glutathione molecules in a single active site (27, 28). NaGSTNu contains a single Cys residue (Supplemental Fig. S3), whose thiol is >20 Å away from either GSH thiol, and so is unlikely to be involved in the lyase reaction.

This proposed mechanism for NaGSTNu, in which the activated thiol of a glutathione molecule is used to break the thioether bond of GS-HPV, is different from that proposed for the Omega-class GST LigG, in which the activated thiol of a Cys residue in the enzyme breaks the thioether bond (27). In contrast to NaGSTNu, LigG is not known or predicted to be able to bind two glutathione molecules in a single active site (27, 28). NaGSTNu contains a single Cys residue (Supplemental Fig. S3), whose thiol is >20 Å away from either GSH thiol, and so is unlikely to be involved in the lyase reaction.

Single amino acid variants of NaGSTNu are catalytically impaired. To test the plausibility of our proposed reaction mechanism, three residues in NaGSTNu predicted to be important for catalysis were separately mutated: Thr51 into Ala (T51A), Tyr166 into Phe (Y166F), and Tyr224 into Phe (Y224F). All three mutated enzymes had decreased reactivity with GS-HPV compared to wild-type NaGSTNu (Supplemental Fig. SSB-D; Table 2). The T51A variant had similar Km values to wild-type NaGSTNu, but kcat values that were ~1,000-fold lower, consistent with our proposal that Thr51 is primarily involved in stabilizing the reactive GSH1 thiolate and not in binding GS-HPV. Compared to wild-type NaGSTNu, the Y166F variant had kcat values that were ~1,000-fold and ~400-fold lower, and Km values that were ~2.5-fold and ~15-fold higher, for the β(R)- and β(S)- stereoisomers of GS-HPV, respectively. These results suggest that the Tyr166 hydroxyl is critical for catalysis with both GS-HPV stereoisomers and is important for binding of β(S)-GS-HPV. Compared to wild-type NaGSTNu, the Y224F variant had kcat values that were ~40-fold and ~60-fold lower, and Km values

that were ~7.5-fold and ~6-fold higher, for the β(R)- and β(S)- stereoisomers of GS-HPV, respectively. These results implicate the Tyr224 hydroxyl in both binding and catalysis, though it is less important to catalysis than that of Tyr166.

Modeling GS-conjugated syringyl phenylpropanoids into the NaGSTNu active site. Besides GS-HPV, depolymerization of natural lignin oligomers via the β-etherase pathway is expected to produce syringyl phenylpropanoids (3). Modeling of the β(R)- and β(S)-isomers of a GS-conjugated syringyl phenylpropanoid into the NaGSTNu active site predicts that they should bind in essentially the same orientations as the corresponding GS-HPV isomers, since the active site channel can accommodate each syringyl conjugate’s additional methoxy group (Supplemental Fig. S7C,D). Thus, Nu-class GSTs may be the only enzymes necessary for the glutathione lyase step in deconstructing natural lignin oligomers via the β-etherase pathway.

A Nu-class GST from Sphingobium sp. SYK-6 can cleave β(R)- and β(S)-GS-HPV. Although the sphingomonad Sphingobium sp. SYK-6 can metabolize GGE (5, 12), and its LigG enzyme can cleave β(R)-GS-HPV (13), no enzyme capable of cleaving β(S)-GS-HPV has been identified in this organism (Fig. 1). To identify an enzyme capable of cleaving β(S)-GS-HPV, we tested a Nu-class GST from Sphingobium sp. SYK-6 (with 63% sequence identity to NaGSTNu; Supplemental Fig. S3) coded for by SLG_04120 and named here SYK6GSTNu and found that recombinant SYK6GSTNu cleaved both β(S)- and β(R)-GS-HPV in vitro (Supplemental Fig. SSE). SYK6GSTNu had higher kcat and lower Km with β(S)-GS-HPV than with β(R)-GS-HPV, leading to a ~10-fold greater kcat/Km with the β(S)-isomer (Table 2). Thus, SYK6GSTNu could cleave β(S)-GS-HPV and potentially contribute, along with LigG, to β(R)-GS-HPV cleavage in Sphingobium sp. SYK-6 (13).

Nu-class GSTs from Escherichia coli can cleave β(R)- and β(S)-GS-HPV. To investigate the prevalence of glutathione lyase activity throughout the GST Nu-class, we tested two Nu-class GSTs from E. coli (EcYghU and EcYfcG),

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an organism not known to metabolize lignin-derived glutathione conjugates. We found that recombinant EcYghU cleaves β(R)- and β(S)-GS-HPV in vitro (Supplemental Fig. S5F); while its $K_M$ values were comparable to those of NaGST$_{Nu}$, its $k_{cat}$ values were much lower, resulting in $k_{cat}/K_M$ values for EcYghU ~100-fold lower than those of NaGST$_{Nu}$ (Table 2). To investigate the cause of these differences in catalytic efficiency, we modeled β(R)- and β(S)-GS-HPV into the active site of EcYghU (pdb 3e8e). While EcYghU is similar to NaGST$_{Nu}$ in sequence (61% identical, including analogues of catalytic residues T51, Y166, and Y224; Supplemental Fig. S3) and structure (RMSD of 0.49 Å over 3116 atoms; Fig. 5), there are steric differences in the interior of the active site channel that lead to different predicted orientations of the bound substrates between the enzymes (Fig. 6; Supplemental Fig. S7). The hydroxyls of Tyr167 and Tyr225 in EcYghU (analogues of Tyr166 and Tyr224 in NaGST$_{Nu}$) are predicted to interact with the HPV moiety of both β(R)- and β(S)-GS-HPV (Fig. 6C,D). For β(R)-GS-HPV, Tyr225 is predicted to form hydrogen-bonds with both the γ-hydroxyl and α-ketone, and Tyr167 is predicted to form hydrogen-bonds with the α-ketone and the aromatic ring (Fig. 6C). For β(S)-GS-HPV, Tyr225 is predicted to form a hydrogen-bond with the γ-hydroxyl, and Tyr167 is predicted to form a hydrogen-bond with the aromatic ring and a long-range interaction with the γ-hydroxyl (Fig. 6D). These interactions could help stabilize an enolate intermediate in the reactions between EcYghU and GS-HPV, and so EcYghU could follow a reaction mechanism similar to that proposed for NaGST$_{Nu}$ (Fig. 7). However, the reactive atoms (S-C$_{β}$-(C$_α$ =O)-aryl) of both β(R)- and β(S)-GS-HPV bound to EcYghU are predicted to be ~45° out of alignment (Fig. 6C,D; Supplemental Fig. S7E,F), which could hinder formation of the enolate and lead to the lower $k_{cat}$ values for EcYghU than for NaGST$_{Nu}$ (for which these reactive atoms were in a roughly planar configuration (Fig. 6A,B; Supplemental Fig. S7A,B)).

Despite these lower in vitro $k_{cat}$ values, we found that EcYghU was able to substitute for NaGST$_{Nu}$ in the β-etherase pathway in vivo: a strain of N. aromaticivorans (12444EcYghU) in which Saro_2595 was replaced in the genome by the E. coli yghU gene removed all of the GGE from its culture media and assimilated it into biomass (Fig. 2E,F,K,L; Supplemental Table S1), whereas 12444Δ2595 (which lacks NaGST$_{Nu}$) could not (Fig. 2C,D,I,J). 12444EcYghU metabolized GGE slower than 12444Δ1879 (which contains NaGST$_{Nu}$) (Fig. 2), which we propose reflects the slower conversion of GS-HPV into HPV in 12444EcYghU than in 12444Δ1879, due to the lower catalytic efficiencies of EcYghU than NaGST$_{Nu}$.

The other E. coli Nu-class GST we tested, EcYfcG, is less similar to NaGST$_{Nu}$ in sequence (42% identical; Supplemental Figs. S3 and S9) and structure (pdb 3gx0; RMSD of 0.775 Å over 2016 atoms, and lacking an enclosed active site channel (17)). While we found that recombinant EcYfcG cleaved both β(R)- and β(S)-GS-HPV in vitro (Supplemental Fig. S5G), $K_M$ values were higher and $k_{cat}$ values were much lower than those of NaGST$_{Nu}$, leading to $k_{cat}/K_M$ values with GS-HPV ~10,000-fold lower for EcYfcG than for NaGST$_{Nu}$ (Table 2). The lower catalytic efficiencies of EcYfcG are likely due, at least in part, to the absence of analogues of NaGST$_{Nu}$ residues Tyr166 and Tyr224 (Supplemental Fig. S3), which were found to be important for catalysis and substrate binding in NaGST$_{Nu}$. Given the dramatically lower catalytic efficiencies of EcYfcG, it is unlikely that EcYfcG could substitute for NaGST$_{Nu}$ in N. aromaticivorans.

DISCUSSION

In developing bio-based systems to generate products from renewable materials such as lignin, optimized cellular and enzyme catalysts are needed. Toward this end, we tested sphingomonads for the ability to break the β-aryl ether bond commonly found in lignin (Fig. 1), and identified and characterized a Nu-class glutathione-S-transferase from N. aromaticivorans that acts as a glutathione lyase in the process (NaGST$_{Nu}$).

Differences in β-aryl ether bond breaking by sphingomonads. We found that N. aromaticivorans was the most effective species studied here at breaking the β-aryl ether bond of the dimeric aromatic compound GGE and assimilating GGE into cellular biomass. The rate
of GGE β-aryl ether bond breaking by \textit{N. aromaticivorans} (~165 μM in ~25 h; Supplemental Fig. S1) is comparable to rates for \textit{Erythrobacter} sp. SG61-1L (~180 μM in ~74 h (5)) and \textit{Novosphingobium} sp. MBES04 (~700 μM in ~40 h (6)). \textit{Sphingobium} sp. SYK-6 (~180 μM in ~160 h (5)), \textit{Novosphingobium} sp. PP1Y (Supplemental Fig. S2), and \textit{S. xenophagum} (Supplemental Fig. S2) are slower and/or less efficient at breaking the β-aryl ether bond of GGE under laboratory conditions, even though they each contain enzymes implicated in the β-etherase pathway. Understanding the bases for these differences can aid future efforts to develop microbial systems for converting lignocellulosic biomass into commodities.

\textbf{GGE metabolism by \textit{N. aromaticivorans}.} The transient appearance of extracellular MPHPV, \textit{threo}-GGE, and HPV in \textit{N. aromaticivorans} cultures fed \textit{erythro}-GGE suggests that the bacterium excreted these metabolites, then subsequently transported them back into the cell (Fig. 2). The production of \textit{threo}-GGE in cultures fed \textit{erythro}-GGE suggests that MPHPV reduction occurs \textit{in vivo}, as was previously found for \textit{Pseudomonas acidovorans} D3 (29), since the two forms of GGE are not directly interconvertible. The low level of extracellular guaiacol and the absence of extracellular GS-HPV suggest that MPHPV cleavage occurred intracellularly, as was proposed for \textit{Novosphingobium} sp. MBES04 (6), and that the products of this cleavage were largely retained within the cells. Since we did not observe any additional aromatic compounds in our culture media, any aromatic metabolites downstream of HPV and guaiacol were also likely largely retained within the cells.

Since NaGST\textsubscript{Nu} catalyzes the conversion of GS-HPV into HPV, the accumulation of extracellular MPHPV by \textit{N. aromaticivorans} 12444Δ2595 fed \textit{erythro}-GGE and vanillate (Fig. 2J) was unexpected. Cleavage of MPHPV into guaiacol and GS-HPV is catalyzed by LigF and LigE (Fig. 1), enzymes that are likely expressed in 12444Δ2595, since crude extract from this strain can cleave MPHPV (Fig. 3B). We hypothesize that without NaGST\textsubscript{Nu}, GS-HPV accumulates in 12444Δ2595, and cells become limited for the free GSH that is needed to cleave MPHPV.

It is unclear whether the trace amount of HPV formed in assays using 12444Δ2595 extract (Fig. 3B) resulted from activity of an unknown enzyme or spontaneous cleavage of GS-HPV. Even if there were another enzyme in \textit{N. aromaticivorans} with low glutathione lyase activity, our results with the 12444Δ2595 strain and its extract show that NaGST\textsubscript{Nu} is sufficient for cleavage of GS-HPV and necessary for complete metabolism of GGE by \textit{N. aromaticivorans}.

\textbf{The role of Nu-class GSTs in the β-etherase pathway.} This work demonstrates that a Nu-class GST is likely the only enzyme necessary for metabolizing the β\textsubscript{(R)}- and β\textsubscript{(S)}-isomers of lignin-derived GS-phenylpropanoids (both guaiacyl (HPV) and syringyl) in nature. This raises the question of why some sphingomonads (such as \textit{Sphingobium} sp. SYK-6) contain both a Nu-class GST and LigG, an Omega-class GST that is reported to be specific for the β\textsubscript{(R)}-isomer (Fig. 1; (13)). Indeed, cell extract from a \textit{Sphingobium} sp. SYK-6 \textit{ΔLigG} mutant was found to completely cleave racemic GS-HPV, presumably because the lysate contained active SYK6GST\textsubscript{Nu} (13). Notably, we found that the \(k_{\text{cat}}/K_M\) value for SYK6GST\textsubscript{Nu} with β\textsubscript{(R)}-GS-HPV (~240 mM\textsuperscript{-1}\textsuperscript{s}\textsuperscript{-1}; Table 2) is ~7-fold lower than that reported for LigG with β\textsubscript{(R)}-GS-HPV (~1700 mM\textsuperscript{-1}\textsuperscript{s}\textsuperscript{-1}) (28)). Thus, while SYK6GST\textsubscript{Nu} likely cleaves β\textsubscript{(S)}-GS-HPV in \textit{Sphingobium} sp. SYK-6, LigG may play an important role in cleaving β\textsubscript{(R)}-GS-HPV in that organism.

\textit{Novosphingobium} sp. MBES04 contains not only a LigG homologue (GST6, which preferentially reacts with β\textsubscript{(R)}-GS-HPV (6)), but also two Nu-class GSTs (GST3, and a protein encoded by MBENS4.4395). While GST3 cleaves β\textsubscript{(R)}- and β\textsubscript{(S)}-GS-HPV \textit{in vitro} (6), its catalytic efficiencies and physiological role have not been reported. No investigation of MBENS4.4395 has been reported, but it is 66% identical to NaGST\textsubscript{Nu} in amino acid sequence and contains analogues of catalytic residues Tyr166 and Tyr224 (Supplemental Fig. S3), and so can likely react with β\textsubscript{(R)}- and β\textsubscript{(S)}-GS-HPV. While the relative roles of these enzymes in \textit{Novosphingobium} sp. MBES04 is unknown,
given the effect of deleting NaGST_Nu on N. aromaticivorans (its only known glutathione lyase) (Fig. 2C,D,I,J), perhaps they represent redundant enzymes, or are specialized for different GS-conjugates.

The potential roles of Nu-class GSTs as glutathione lyases in organisms that do not contain the β-etherase pathway. All five of the Nu-class GSTs that have been tested for GS-HPV cleavage (NaGST_Nu, SYK6GST_Nu, EcYghU, and EcYfG (tested here), and GST3 (6)) show in vitro glutathione lyase (deglutathionylation) activity with the β(R)- and β(S)-stereoisomers of this substrate, though with a wide range of catalytic efficiencies. Phylogenetic analysis of Nu-class GSTs shows that these five enzymes lie in separate sub-clades (Supplemental Fig. S9), although the residues predicted to be involved in the binding of two GSH molecules (or one GSH molecule and the glutathione moiety of a GS-conjugate) (Fig. 4B) and inactivating the thiol of the GSH1 molecule (Thr51 and Asn53) are conserved in many members of the class (Supplemental Fig. S3; (16, 19, 20)). Another Nu-class GST, PcUre2pB1 from P. chrysosporium, has also been shown to exhibit in vitro glutathione lyase activity (19). Glutathione lyase activity may thus be widespread throughout this large class of GSTs, most of which are found in organisms (like E. coli) not known or predicted to break the β-aryl ether bond of lignin. While EcYghU and EcYfG cleave β(R)- and β(S)-GS-HPV in vitro with lower catalytic efficiencies than NaGST_Nu and SYK6GST_Nu (Table 2), this may reflect the fact that GS-HPV is not a natural substrate for the E. coli enzymes. While the overall structures of Nu-class GSTs are similar, differences between them, particularly in the region surrounding the active site where the GS-conjugate would be located, could make individual enzymes optimally suited for binding and cleaving GS-conjugates that their respective organisms encounter more frequently. For example, we propose that steric differences in the active site channels between NaGST_Nu and EcYghU contribute to the observed differences in catalytic efficiencies between these enzymes with GS-HPV as substrate (Fig. 6).

Conclusions

The rate of GGE metabolism by N. aromaticivorans makes it an attractive organism for studying various aspects of the β-etherase pathway, and may allow it to be developed into a biological system for converting lignin oligomers into useful compounds. Our finding that NaGST_Nu is the only enzyme needed for the glutathione lyase step of the β-etherase pathway is notable, since the other pathway steps require multiple stereospecific enzymes (Fig. 1). In addition, this is the first demonstration of a Nu-class GST having a defined physiological role as a glutathione lyase in an organism. Our analyses of wild-type NaGST_Nu and its variants define roles for residues conserved throughout the Nu-class in GS-HPV cleavage. Our identification of SYK6GST_Nu likely solves the enigma of how both β(R)- and β(S)-GS-HPV are cleaved in Sphingobium sp. SYK-6: based on kinetic analyses, we propose that SYK6GST_Nu cleaves β(S)-GS-HPV, whereas LigG and SYK6GST_Nu both contribute to cleaving β(R)-GS-HPV in that organism. Finally, the ability of EcYghU and EcYfG to cleave GS-HPV shows that Nu-class GSTs from organisms lacking the β-etherase pathway can also act as glutathione lyases, offering new possibilities for the physiological roles of members of this large enzyme class.

Experimental procedures

Bacterial strains and growth media. Strains used are listed in Supplemental Table S3. We deleted Saro_1879 (putative sacB; SARO_RS09410 in the recently reannotated genome in NCBI) from the Novosphingobium aromaticivorans DSM 12444 genome to create a strain (12444Δ1879) amenable to markerless genomic modifications using a variant of pK18mobSacB (30), which contains sacB and a kanamycin resistance gene. We used 12444Δ1879 as parent strain to generate strains in which Saro_2595 (SARO_RS13080 in the recently reannotated genome in NCBI) was deleted from the genome (12444Δ2595), and in which Saro_2595 was replaced in the genome by the E. coli DH5α.yghU gene (12444EcYghU). See Supporting Information for details of genomic modifications.

Unless otherwise noted, E. coli cultures were grown in Lysogeny Broth (LB), and shaken at ~200 rpm at 37 °C. For routine storage and
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manipulation, sphingomonad cultures were grown in LB or GluSis at 30 °C. GluSis is a modification of Sistrom’s minimal medium (31) in which the succinate has been replaced by 22.6 mM glucose. Standard Mineral Base (SMB) (32) used for growth experiments contains 20 mM Na₂HPO₄, 20 mM KH₂PO₄, 1 g/L (NH₄)₂SO₄, and 20 mL Hutner’s vitamin-free concentrated base (adapted from (33), but lacking nicotinic acid, thiamin, and biotin; see Supporting Information) per liter, final pH 6.8. SMB was supplemented with carbon sources as described below. Where needed to select for plasmids, media were supplemented with 50 μg/mL kanamycin and/or 20 μg/mL chloramphenicol.

**Sphingomonad growth experiments.** Cell densities were measured using a Klett-Summerson photoelectric colorimeter with a red filter. For *N. aromaticivorans*, 1 Klett Unit (KU) is equal to ~8 × 10⁶ CFU/mL (Supplemental Table S5). Experimental cultures of *N. aromaticivorans* and *Novosphingobium* sp. PP1Y were grown in SMB containing either vanillate or GGE alone (4 mM and 3 mM, respectively), or a combination of vanillate and GGE (4 mM and 1.5 mM, respectively). For *S. xenophagum* cultures, vanillate was replaced by glucose, since we found this strain to be unable to metabolize vanillate. *N. aromaticivorans* was also grown in SMB containing 165 μM GGE. Starter cultures were grown in SMB with 4 mM vanillate or glucose, and cells were pelleted and washed with PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl; pH = 7.4). Pellets were resuspended into culture medium and used to inoculate experimental cultures to initial cell densities of < 5 KU.

Cultures were grown aerobically at 30 °C, in 125 mL conical flasks containing 20-40 mL of medium and shaken at ~200 rpm. Aliquots (400-600 μL) were removed at specified time points and filtered through 0.22 μm syringe tip filters (e.g. Whatman Puradisc filters, GE Healthcare) before HPLC analysis of extracellular aromatics. Every culture was grown at least three times; data shown are from representative cultures.

**Enzyme purifications.** Saro_2595 from *N. aromaticivorans*, yghU and yfcG from *E. coli* DH5α, and SLG_04120 (SLG_RS02030 in the recently reannotated genome in NCBI) from *Sphingobium* sp. SYK-6 (codon-optimized for expression in *E. coli*) were individually cloned into plasmid pVP302K (34) so that transcripts from the plasmids would be translated into proteins containing a His⁶-tag connected to the N-terminus via a tobacco etch virus (TEV) protease recognition site (construction of plasmids described in Supporting Information). The plasmid containing Saro_2595 was modified via PCR to generate plasmids for expressing variants of NaGSTₙu containing the single-residue mutations T51A, Y166F, and Y224F. Recombinant proteins were expressed in *E. coli* B834 (35, 36) containing plasmid pRARE2 (Novagen, Madison, WI) grown for ~25 hours at 25 °C in ZYM-5052 Autoinduction Medium (37) containing kanamycin and chloramphenicol. Recombinant proteins were purified as described previously (15) (see Supporting Information for modifications to the procedure). After removal of His⁶-tags using TEV protease, recombinant proteins retained a Ser-Ala-Ile-Ala-Gly- peptide on their N-termini, derived from the linker between the protein and the TEV protease recognition site. Recombinant LigE and LigF1 from *N. aromaticivorans* were purified as previously described (34).

**Kinetics of converting GS-HPV into HPV.** The reaction buffer (RB) consisted of 25 mM Tris-HCl (pH 8.5) and 25 mM NaCl. The β(R)- and β(S)- stereoisomers of GS-HPV were separately generated by incubating racemic β(S)- and β(R)-MPHPV (0.46 mM) in RB with 5 mM GSH and either 38 μg/mL LigF1 or 36 μg/mL LigE for several hours (Supplemental Fig. S4). This sample, containing a single GS-HPV stereoisomer, guaiacol, and the unreacted MPHPV stereoisomer (as well as LigE or F1), was diluted with RB to achieve the desired concentration of GS-HPV for the time course reaction (0.005, 0.01, 0.02, 0.1, or 0.2 mM). An additional 5 mM GSH (dissolved in RB) was added prior to initiation of each time course. At time zero, 100 μL of the indicated enzyme (resuspended in RB) was combined with 1800 μL of the diluted GS-HPV reaction mixture to achieve final concentrations of 8 nM NaGSTₙu, 20 nM NaGSTₙu (T51A), 100 nM NaGSTₙu
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(Y166F), 10 nM NaGST_Na (Y224F), 195 nM EcYghU, 195 nM EcYfcG, or 47 nM or 18 nM SYK6GST_Na (for the \( \beta(R) \)- and \( \beta(S) \)-GS-HPV reactions, respectively). Reactions were performed at 25 °C. At specified time points, 300 µL of the reaction was removed and combined with 100 µL 1 M HCl (Acros Organics; Geel, Belgium) to stop the reaction before HPLC analysis to quantify HPV formed.

**N. aromaticivorans cell extract assays.** *N. aromaticivorans* cells were grown in 500 mL conical flasks containing 267 mL SMB medium with 4 mM vanillate and 1 mM GGE. When cell densities reached ~1 x 10^8 cells/mL, cells were lysed by the sonication procedure used to generate *E. coli* lysates for protein purification (Supporting Information). Samples were centrifuged at 7,000 x g for 15 min, and the supernatants were used as crude cell extracts.

Assays containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM GSH, 0.407 mM racemic \( \beta(R) \)- and \( \beta(S) \)-MPHPV and cellular extract from 12444Δ1879 or 12444Δ2595 (final concentrations of 269 and 186 µg protein/mL, respectively) were performed at 30 °C. At defined time points, 300 µL aliquots were combined with 100 µL 1 M HCl to stop the reaction before HPLC analysis. At the indicated time, recombinant NaGST_Na (30 µg protein/mL) was added to the 12444Δ2595 cell extract reaction, along with an additional 10 mM GSH.

**HPLC analysis.** After extracellular aromatics were identified using LC-MS with a PFP column (Supporting Information), routine analysis and quantification of aromatics were performed using an Ultra AQC18 5µm column (Restek) attached to a System Gold HPLC (Beckman Coulter) with running buffers described in Supplemental Fig. S11A. The eluent was analyzed for light absorbance between 191 and 600 nm, and absorbances at 280 nm were used for quantification of aromatic metabolites by comparing peak areas to those of standards (retention times of measured metabolites are shown in Supplemental Figs. S4 and S11B).

**Production of cDNA libraries from *N. aromaticivorans* cultures and real-time quantitative PCR.** *N. aromaticivorans* cultures were grown in 120 mL of SMB containing either 4 mM vanillate or 4 mM vanillate and 1 mM GGE. Cells were harvested when the vanillate concentration of a culture’s medium was ~20% of its initial value (cell densities of ~8 x 10^8 cells/mL); at this point, ~65% of the GGE initially present in the GGE-fed culture was converted into downstream intermediates. Since both cultures were actively metabolizing vanillate, and only one culture was actively metabolizing GGE, the differences in transcript levels between the cultures should largely be related to GGE metabolism.

40 mL of harvested culture was removed and combined with 5.71 mL ice-cold Stop Solution (95% ethanol, 5% acid phenol: chloroform (5:1 solution, pH 4.5)). These mixtures were centrifuged at 4 °C for 12 min at 6,000 x g. Cell pellets were resuspended into 2 mL Lysis Solution (2% SDS, 16 mM EDTA in RNase-free water), then incubated at 65 °C for 5 min. RNA purification and cDNA synthesis were performed as previously described (38), using SuperScript III reverse transcriptase (Thermo Fisher Scientific) to construct the cDNA library. Genes selected for transcript analysis were the top BLASTp hits in the *N. aromaticivorans* genome of gene products from *Sphingobium* sp. SYK-6 or *Novosphingobium* sp. MBES04 previously shown to catalyze reactions of the \( \beta \)-etherase pathway (Table 1).

Real-time qPCR was performed on a 7500 Real Time PCR System (Applied Biosystems, Forest City, CA) using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). Primers used to detect transcripts are contained in Supplemental Table S6. Transcript levels were normalized to those of Saro_0141 (rpoZ, coding for the RNA polymerase omega subunit).

**Determination of chemical oxygen demand (COD).** Initial COD values for cultures were obtained either from uninoculated medium, or from inoculated medium that was immediately passed through a 0.22 µm filter. Final COD samples were collected when cultures reached their maximum cell densities, both from unfiltered culture (cells and medium) and filtered culture (medium). The difference in COD between the unfiltered and filtered final samples is defined as the COD of cellular biomass.
Samples were diluted as needed and combined with High Range COD Digestion Solution (Hach, Loveland, CO). The mixtures were heated to 150 °C for 120 min to oxidize the materials before absorbances were measured at 600 nm. Standards with known COD values were analyzed in parallel.

**Chemicals.** Vanillate, guaiacol, reduced L-glutathione (GSH), and 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) were purchased from Sigma-Aldrich. Erythro-guaiacylglycerol-β-guaiacyl ether (erythro-GGE) was purchased from TCI America (Portland, OR).

A racemic mixture of β-(2-methoxyphenoxy)-γ-hydroxypropiovanillone (MPHPV) was synthesized by dissolving erythro-GGE into ethyl acetate (Fisher Scientific), then slowly adding 1.25 molar equivalents of 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ) and stirring for 30 min. The reaction was washed three times with saturated NaHCO₃ to remove DDQH₂ formed during the reaction. The MPHPV was purified via flash chromatography using hexane/ethyl acetate (0.33/0.67 v/v), as previously described (15), then crystallized from the eluent via solvent evaporation.

Hydroxypropiovanillone (HPV) was synthesized as previously described for synthesis of β-deoxy-α-veratrylglycerone, except using 4-O-benzyl-acetovanillone as starting material, rather than acetoveratrone (15). Synthesis of HPV required an additional debenzylation step that was unnecessary in the synthesis of β-deoxy-α-veratrylglycerone.

**Structure determination.** NaGST₅u was screened for crystal formation against several commercial screens at 277 and 293K using a TTP Labtech Mosquito® crystallization robot. The best diffracting ammonium acetate precipitated crystal was obtained at 293K, by mixing 0.2 μL of protein solution (277 μM protein preincubated for 50 min with 10 mM GSH (neutralized with NaOH)) with 0.2 μL of reservoir solution (4 M ammonium acetate buffered with 100 mM sodium acetate, pH 4.6). This crystal was mounted directly from the growth solution by drawing it through a layer of fomblin oil, thinning the surrounding liquid with a paper wick, and plunging into liquid nitrogen. The best diffracting ammonium sulfate precipitated crystal was obtained at 293K using 0.13 μL of protein solution and 85 nL of reservoir solution (1.35 M ammonium sulfate, 0.1 M lithium sulfate, and 0.1 M bis-trispropane, pH 7.5). This crystal was cryopreserved by adding 0.5 μL of a solution composed of 2 parts reservoir solution and 1 part neat glycerol to the droplet containing the crystal, and equilibrating for 11 min prior to looping and plunging into liquid nitrogen.

Diffraction data were obtained at the GM/CA beam-line at Argonne National Laboratory with an Eiger 16M detector (39). Data were collected on the ammonium acetate (pdb 5uuu) and ammonium sulfate (pdb 5uuo) crystal forms using 1.033 Å (for 1.45 Å resolution) or 0.7749 Å (for 1.25 Å resolution) X-rays, respectively. Diffraction data were reduced using XDS (40). Both crystals belonged to space group P2₁2₁2₁ with a predicted solvent content of 60%. The structure was solved by molecular replacement with Phaser (41) in the Phenix suite (42), using a search model based on PDB ID 3c8e:A (EcYghU (18)) modified with phenix.sculptor (43), based on primary sequence alignment. Phenix.refine (44) and COOT (45) were alternatively used to refine the structure and fit the model to electron density maps. Structure solution revealed two copies of the protein per asymmetric unit, with strong electron density present for the paired active site glutathione molecules. The composite simulated annealing omit map for the active site glutathiones was calculated in Phenix using default parameters, and contoured at 2 sigma. The active sites were modeled containing either two GSH molecules or a single GSSG molecule, and difference density maps were calculated by setting the occupancy of either conformation to one or zero, then running one round of standard refinement, contoured at 4 sigma. The omit map data were best fit to a model in which each active site contained electron density from both a pair of GSHs (locked to be of equal occupancy, Q(GSHA)=Q(GSHB)) and a single GSSG (occupancy 1-Q(GSHA,B)), which was interpreted as a heterogeneous population of protein molecules containing either two GSHs or one GSSG throughout the crystal (Supplemental Fig. S6).
Molecular modeling of substrate binding. For modeling $\beta(R)$- and $\beta(S)$-GS-HPV and their syringyl phenylpropanoid analogues into the active sites of NaGST$_{Nu}$ and EcYghU, the PyMOL Builder function (46) was used to create molecules of GS-HPV or GS-syringyl by adding atoms onto the GSH2 molecule bound in each active site. Atoms were added so as to visually minimize steric clash with the proteins. The potential energies of the protein-GS-phenylpropanoid complexes were minimized using the Minimize Structure function of UCSF Chimera (47). For the energy minimization, all of the atoms of the protein-GS-phenylpropanoid complex were held rigid except for those of the phenylpropanoid moiety and the Cys sidechain of GSH2. One-hundred steepest descent steps were run, followed by twenty conjugate gradient steps, and all step sizes were 0.05 Å.
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CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article

AUTHOR CONTRIBUTIONS
WSK, TJD, and DRN designed the study. WSK, BGF, and TJD wrote the paper. WSK, DRW, CNO, and LMY created mutant *N. aromaticivorans* strains and performed qPCR experiments. WSK, CNO, and LMY performed growth experiments, expressed and purified the enzymes investigated, and performed *in vitro* enzyme assays. AU and JJC identified metabolites via LC-MS. DLG purified LigE and LigF and synthesized MPHPV and HPV. CAB and RWS crystallized NaGST\textsubscript{Nu} and solved its structure. WSK performed molecular modeling. BGF developed the NaGST\textsubscript{Nu} mechanism proposal. All authors reviewed the results and approved the final version of the manuscript.
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Deglutathionylation of a GS-phenylpropanoid by Nu-class GSTs

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FOOTNOTES

**Abbreviations used:** GSH, reduced glutathione; GSSG glutathione disulfide; GST, glutathione-S-transferase; GGE, guaiacylglycerol-β-guaiacyl ether; MPHPV, β-(2-methoxyphenoxy)-γ-hydroxypropiovanillone; GS-HPV, β-glutathionyl-γ-hydroxypropiovanillone; HPV, hydroxypropiovanillone; COD, chemical oxygen demand; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; TEV, tobacco etch virus
Table 1: Differences in transcript levels between *N. aromaticivorans* cultures grown in vanillate or vanillate plus GGE

| Gene    | BLASTp query                  | Fold-change |
|---------|-------------------------------|-------------|
| Saro_0205 | SLG_08640, LigD (442; 78%)  | 11 ± 3      |
| Saro_0793 | SLG_35880, LigO (224; 41%)   | 6 ± 2       |
| Saro_0794 | SLG_35900, LigN (224; 45%)   | 9 ± 1       |
| Saro_1875 | SLG_33660, LigL (261; 49%)   | 1 ± 1       |
| Saro_2091 | SLG_08650, LigF (310; 59%)   | 8 ± 1       |
| Saro_2405  | SLG_08660, LigE (344; 61%)  | 17 ± 9      |
|           | SLG_32600, LigP (391; 66%)   |             |
| Saro_2595  | MBENS4_2527, GST3 (135; 38%) | 8 ± 2       |

*a* Transcript levels for each culture were normalized to those of Saro_0141 (*rpoZ*).

*b* Gene product from *Sphingobium* sp. SYK-6 (SLG) or *Novosphingobium* sp. MBES04 (MBENS4) that has been shown to catalyze a reaction in the β-etherase pathway, and that was used in a BLASTp search to identify the indicated *N. aromaticivorans* (Saro) gene. Bit score and percentage identity from the BLASTp searches are shown in parentheses.

*c* Fold-change is the ratio of the normalized transcript level in cells grown in the presence of GGE to that in cells grown in the absence of GGE for the *N. aromaticivorans* genes.

*d* Only Saro_2405 showed high homology (Bit score >100) to both SLG_08660 (LigE) and SLG_32600 (LigP).
Table 2: Kinetic parameters for the enzymatic conversion of GS-HPV into HPV\(^a\)

| Protein       | GS- HPV\(^b\) | \(k_{cat}\) (s\(^{-1}\)) | \(K_M\) (\(\mu\)M) | \(k_{cat}/K_M\) (mM\(^{-1}\)s\(^{-1}\)) |
|---------------|---------------|--------------------------|-----------------|-----------------------------------|
| NaGST\(_R\)   | \(\beta(R)\) | 80 ± 10                  | 40 ± 6          | 1900                              |
|               | \(\beta(S)\) | 57 ± 9                   | 8 ± 3           | 8000                              |
| NaGST\(_R\) (T51A) | \(\beta(R)\) | 0.036 ± 0.005            | 16 ± 5          | 2.3                               |
|               | \(\beta(S)\) | 0.057 ± 0.008            | 4 ± 2           | 13                                |
| NaGST\(_R\) (Y166F) | \(\beta(R)\) | 0.07 ± 0.01              | 110 ± 30        | 0.7                               |
|               | \(\beta(S)\) | 0.16 ± 0.03              | 120 ± 50        | 1.3                               |
| NaGST\(_R\) (Y224F) | \(\beta(R)\) | 2 ± 1                    | 300 ± 200       | 8                                 |
|               | \(\beta(S)\) | 1.0 ± 0.1                | 50 ± 9          | 19                                |
| SYK6GST\(_R\) | \(\beta(R)\) | 13 ± 1                   | 55 ± 7          | 240                               |
|               | \(\beta(S)\) | 30 ± 5                   | 11 ± 2          | 2700                              |
| EcYghU        | \(\beta(R)\) | 0.43 ± 0.03              | 28 ± 4          | 16                                |
|               | \(\beta(S)\) | 0.29 ± 0.03              | 12 ± 3          | 24                                |
| EcYfcG        | \(\beta(R)\) | 0.04 ± 0.01              | 160 ± 60        | 0.2                               |
|               | \(\beta(S)\) | 0.017 ± 0.004            | 130 ± 40        | 0.14                              |

\(^a\)Kinetic parameters are from non-linear least squares best fits to plots of initial rate ([HPV] formed per second) versus GS-HPV concentration using the Michaelis-Menten equation (Supplemental Fig. S5).

\(^b\)Stereoisomer of GS-HPV used in reaction (see Fig. 1)
Table 3: Statistics for the crystal structure determinations of NaGST$_{\text{Nu}}$

| PDB entry | Suuo | Suun |
|-----------|------|------|
| Precipitant | Ammonium sulfate | Ammonium acetate |
| Wavelength | 0.7749 | 1.033 |
| Resolution range | 29.81 - 1.25 (1.295 - 1.25) | 43.97 - 1.45 (1.502 - 1.45) |
| Space group | P 21 21 21 | P 21 21 21 |
| Unit cell | 68.81 70.39 168.23 90 90 90 | 68.59 70.64 168.57 90 90 90 |
| Total reflections | 3049254 (309691) | 1854065 (113574) |
| Unique reflections | 225346 (22262) | 140421 (11067) |
| Multiplicity | 13.5 (13.9) | 13.2 (10.3) |
| Completeness (%) | 99.91 (99.70) | 96.48 (76.88) |
| Mean I/σ(I) | 23.67 (1.99) | 36.36 (8.82) |
| Wilson B-factor | 16.03 | 13.23 |
| R-merge | 0.05235 (1.297) | 0.04367 (0.2047) |
| R-meas | 0.05445 (1.346) | 0.04544 (0.2159) |
| R-pim | 0.01485 (0.3565) | 0.01239 (0.06599) |
| CC1/2 | 1 (0.841) | 1 (0.986) |
| CC* | 1 (0.956) | 1 (0.997) |
| Reflections used in refinement | 225262 (22242) | 140408 (11066) |
| Reflections used for R-free | 1963 (197) | 1852 (154) |
| R-work | 0.1303 (0.2607) | 0.1357 (0.206) |
| R-free | 0.1324 (0.2979) | 0.1365 (0.1595) |
| CC(work) | 0.978 (0.913) | 0.978 (0.971) |
| CC(free) | 0.988 (0.851) | 0.975 (0.9363) |
| Non-H atoms | 5658 | 5823 |
| macromolecules | 4626 | 4567 |
| Ligands | 252 | 248 |
| Solvent | 780 | 1008 |
| Protein residues | 569 | 1036 |
| RMS(bonds) | 0.007 | 0.007 |
| RMS(angles) | 1.02 | 1.05 |
| Ramachandran favored/allowed/ outliers (%) | 93.75/2.30/0.35 | 97.16/2.48/0.35 |
| Rotamer outliers (%) | 0.43 | 0.43 |
| Clashscore | 1.89 | 2.13 |
| Average B-factor | 22.39 | 19.61 |
| B-factor macromolecules/ligands/solvent | 20.02/26.98/34.97 | 16.55/19.017/33.64 |
| No. TLS groups | 9 | 9 |

Statistics for the highest-resolution shell are shown in parentheses.
Figure 1. Breaking of the β-aryl ether (β-O-4) bond of guaiacylglycerol-β-guaiacyl ether (GGE) via the sphingomonad β-etherase pathway. Enzymes shown were identified in Sphingobium sp. SYK-6 (“Lig” enzymes (12–14)), Novosphingobium sp. MBES04 (GST3 (6)), or Novosphingobium aromaticivorans DSM 12444 (NaGSTNu; this work). The sphingomonads investigated in this work for GGE metabolism that are predicted to contain a given enzyme (6) are listed under the enzyme name. The α, β, and γ carbons are labeled in the topmost GGE molecule. Erythro-GGE consists of the β(S)β(R) and β(R)β(S) stereoisomers; threo-GGE consists of the β(R)β(R) and β(S)β(S) stereoisomers. All chiral molecules are labeled with their chiralities. MPHPV: β-(2-methoxyphenoxy)-γ-hydroxypropiovanillone. GS-HPV: β-glutathionyl-γ-hydroxypropiovanillone. HPV: hydroxypropiovanillone. GSH: reduced glutathione. GSSG: glutathione disulfide. –SG: conjugated glutathione moiety.
Figure 2. Cell densities and extracellular metabolite concentrations of representative *N. aromaticivorans* cultures grown in SMB containing 3 mM GGE (panels A-F), or 4 mM vanillate and 1.5 mM GGE (panels G-L). Data are shown for strains 12444Δ1879 (effective wild-type; panels A,B,G,H), 12444Δ2595 (Saro_2595, which codes for NaGST\textsubscript{Nu}, deleted from the genome of 12444Δ1879; panels C,D,I,J), and 12444EcyghU (with *E. coli* yghU replacing Saro_2595 in the genome of 12444Δ1879; panels E,F,K,L). The y-axes of panels H, J, and L use multiple scales. For comparison, cell densities for cultures grown in SMB containing only 4 mM vanillate are included in panels G, I, and K. Cell densities are in Klett Units (KU); for *N. aromaticivorans*, 1 KU $\approx 8 \times 10^6$ CFU/mL (Supplemental Table S5).
Figure 3. Time courses for the reactions of *N. aromaticivorans* cell extracts with racemic β(R)- and β(S)-MPHPV. (A) Strain 12444Δ1879. (B) Strain 12444Δ2595. The red dotted line in panel B indicates the time at which recombinant NaGST$_{Nu}$ and additional GSH were added to the reaction.
Figure 4. Structure of NaGST$_{Nu}$ (pdb 5uuo). (A) Domain structure of one subunit of the homodimer (with the open C-terminal configuration). The monomer contains a GST1 N-terminal (thioredoxin) domain (Val39 to Gly129; green), a GST2 C-terminal domain (Ser135 to Leu257; maroon), and N-terminus (Met1 to Pro38; white) and C-terminus (Val258 to Phe288; gold) extensions. Atoms in Phe82, Tyr224, Lys262, and Phe288 are shown as spheres. (B) Active site residue contacts to the GSH1 and GSH2 dithiol (60% occupancy; orange and cyan carbon atoms, respectively) and GS–SG disulfide (40%; gray carbon atoms). NaGST$_{Nu}$ residues are colored according to domain origin in (A). Interactions involving protein residues are shown in black; those between GSH1 and GSH2 are in silver. Selected distances between interacting atoms are shown.
Figure 5. Comparison of the region surrounding the active site in closely related Nu-class GSTs. (A) Alignment of subunits of NaGST$_{\text{Nu}}$ (pdb 5uu0) (closed C-terminal configuration: blue; open C-terminal configuration: white), EcYghU (pdb 3c8e; orange), and SsYghU (pdb 4mzw; green). Each subunit is labeled at its C-terminus. Residues labeled in panels B-E with atoms shown as spheres are spatially conserved between the subunits and define a triangle used to approximate the area of each active site channel opening. (B) Closed configuration of NaGST$_{\text{Nu}}$ (channel opening ~11 Å$^2$). (C) Open configuration of NaGST$_{\text{Nu}}$ (channel opening ~18 Å$^2$). (D) EcYghU (channel opening ~25 Å$^2$). (E) SsYghU (channel opening ~25 Å$^2$).
Figure 6. Modeling of β(R)- and β(S)-GS-HPV into the NaGST\textsubscript{Nu} and EcYghU active sites. The glutathione moiety of GS-HPV is modeled into the position occupied by GSH2 in the structures. Carbon atoms of GS-HPV are cyan. Those of GSH1 are orange. Coloring of NaGST\textsubscript{Nu} residues is the same as in Fig. 4. EcYghU residues are colored the same as their NaGST\textsubscript{Nu} analogues in Fig. 4. Panels (A) and (B) show modeling into NaGST\textsubscript{Nu} (pdb 5uu0; closed C-terminal configuration). Panels (C) and (D) show modeling into EcYghU (pdb 3c8e). Panels (A) and (C) show predicted interactions involving β(R)-GS-HPV. Panels (B) and (D) show predicted interactions involving β(S)-GS-HPV. Residues Phe82 and Phe288 in NaGST\textsubscript{Nu} and Arg260 and Asn262 in EcYghU contribute to different internal dimensions of the active site channels of NaGST\textsubscript{Nu} and EcYghU, leading to different predicted orientations of the bound substrates between the enzymes. Supplemental Fig. S7 shows space-filling models of the enzyme-substrate complexes.
**Figure 7.** Proposed mechanism for NaGST$_{Nu}$-catalyzed cleavage of the thioether bond in $\beta$(R)- (left column) or $\beta$(S)- (right column) GS-HPV. (A and B) Thr51 and Asn53 provide hydrogen-bonds that stabilize a reactive GS1 thiolate anion, which attacks the GS-moiety of GS-HPV (occupying the active site GSH2 position) to form a G1S–SG2 disulfide. (C and D) Rupture of the thioether bond is facilitated by formation of a transient enolate intermediate, which is stabilized by interactions between GS-HPV and Y166 and Y224 of NaGST$_{Nu}$. (E and F) Capture of a solvent-derived proton by the carbanion collapses the enolate to form HPV. The hydroxyl group of the C-terminal Phe288 in the closed NaGST$_{Nu}$ configuration provides a hydrogen-bond that stabilizes the positioning of the substrate throughout the process.
Novosphingobium aromaticivorans uses a Nu-class glutathione-S-transferase as a glutathione lyase in breaking the $\beta$-aryl ether bond of lignin

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