

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

INARI AGRICULTURE, INC.,
Petitioner,

v.

CORTEVA AGRISCIENCE LLC,
Patent Owner.

PGR2023-00022
Patent 11,371,055

DECLARATION OF J. MARTIN BOLLINGER, JR., Ph.D.

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I, J. Martin Bollinger, Jr., hereby declare as follows:

I. Introduction

1. I have been retained by Finnegan, Henderson, Farabow, Garrett & Dunner, LLP on behalf of Patent Owner Corteva Agriscience LLC as an independent expert in the field of protein biochemistry, including enzymology and enzyme variants. I am being compensated for the time I spend on this matter, but no part of my compensation depends on the outcome of this proceeding.

II. Professional Background and Qualifications

2. I am an enzymologist and the endowed Russell and Mildred Marker Professor of Natural Products Chemistry in the Department of Chemistry and the Department of Biochemistry and Molecular Biology at The Pennsylvania State University. I received my Ph.D. from the Massachusetts Institute of Technology (MIT) in 1993 and was an N.I.H.-sponsored postdoctoral fellow at Harvard Medical School from January 1993 through May 1995. I am a Fellow of the American Association for the Advancement of Science.

3. I have taught undergraduate courses in chemistry and biochemistry as well as graduate courses in the mechanisms of enzyme action. I have also lectured at numerous professional conferences, universities, and in corporate R&D laboratories on those topics.

4. I have expertise in the science of enzymes. In my research laboratory, we study enzymes involved in the production of DNA, natural-product drugs, and biofuels. Other enzymes that we investigate degrade natural and anthropogenic compounds to enable bacteria to acquire essential elements such as phosphorus and sulfur. Some of these enzymes, including the enzymes at issue in this case, are used in agricultural applications. I have consulted for several companies, including the biotechnology companies KinTek, LS9, Joule Unlimited, Dow AgroSciences (now Corteva), and the pharmaceutical company GlaxoSmithKline.

5. I have received several prizes and awards, including the Dreyfus Foundation New Faculty Award (1995), the Searle Scholar Award (1996), the International Society of Biological Inorganic Chemistry's Early Career Award (2008), Penn State University's Faculty Scholar Medal for Outstanding Achievement in the Physical Sciences (2009), Penn State's Howard Palmer Award for Faculty Mentoring (2012), Penn State's Distinguished Faculty Mentoring Award (2021), the American Society for Biochemistry and Molecular Biology William C. Rose Award (2022), the American Chemical Society Division of Biological Chemistry Abeles and Jencks Award (2023), and the American Chemical Society Nobel Laureate Signature Award for Graduate Education in the Chemical Sciences (2023).

6. I have directed the research activities of 21 Ph.D. graduates, 7 M.S. graduates, and approximately 24 post-doctoral fellows; most of these trainees have gone on to successful careers in the chemical and biochemical industries or in academia, including positions at Stanford University, the University of Pennsylvania, Brigham Young University, Carnegie Mellon University, North Carolina State University, Brandeis University, and Penn State University.

7. I have delivered several named and plenary lectures at universities and scientific conferences, including the 2018 TY Shen Lecture in Biological Chemistry at M.I.T. and keynote lectures at the 2009 International Conference in Biological Inorganic Chemistry in Nagoya, Japan, the 2015 Southeast Enzymes Conference in Atlanta, GA, and the 2020 Midwest Enzyme Chemistry Conference (remote). In total, I have delivered more than 50 lectures at conferences, nearly 80 lectures at colleges and universities, and 6 lectures at companies.

8. I have co-chaired two international conferences (Gordon Research Conferences) and served as Program Chair of the American Chemical Society's Division of Biological Chemistry. I have also served as co-chair of a federally funded, biennial international training program in biological inorganic chemistry at Penn State.

9. I have served on the editorial advisory boards for the scientific journals *Journal of Biological Chemistry*, *Biochemistry*, and *Journal of Biological Inorganic*

Chemistry. I have also served as section editor of reference book series and special issues of journals, including *Comprehensive Natural Products* and *Current Opinion in Chemical Biology*.

10. I have published ~140 original scientific research articles as well as ~21 invited scientific review articles, book chapters, and perspectives. My research team specializes in elucidating the structures and mechanisms of enzymes that use transition metals to react with oxygen gas (O₂) in the atmosphere to transform carbon compounds. All but a few of the more than 160 publications noted above focus on such oxygen-utilizing metalloenzymes.

11. One of our most successful long-term projects (since 2002) concerns iron- and α -ketoglutarate-dependent oxygenases, which are at issue in this case. I have co-authored 39 publications focused exclusively on one or more of the enzymes in this large class. Three of these articles were published in the high-profile journals *Science* and *Nature*, and five others appeared in the top journals *Proceedings of the National Academy of Sciences of the United States of America* and *Nature Chemical Biology*. I have co-authored seven other articles that discuss these enzymes at least in part.

12. My *curriculum vitae* (CV) is attached as Exhibit **Appendix A** and lists the litigations and other matters on which I have served as an expert witness. Also

listed on my CV are books and articles I have co-authored, including peer-reviewed journal publications.

III. Materials Considered

13. My opinions are based on my education, training, background and experience, the documents I reviewed, and information available to me to date. I have reviewed and considered the documents and materials cited in this declaration, as well as those listed in **Appendix B**. To the extent I am provided with additional documents or information in this proceeding, I reserve the right to modify or expand upon my opinions based on such new information, and in response to any additional reports and testimony.

IV. Legal Standards

14. The opinions I express in this declaration involve the application of my technical knowledge and experience to evaluating certain prior art and other scientific and technical issues relating to the '055 patent. In preparing this declaration, certain patent law concepts have been explained to me by counsel, including the legal standard for assessing written description and enablement.

15. I understand that written description and enablement must be analyzed from the perspective of a “*skilled artisan*” (or “skilled person”) in the same field as the challenged patent as of the “effective filing date” of the claims. I understand that

a skilled artisan is presumed to know of the relevant art (e.g., technical literature) as of the “effective filing date” of the claims.

16. I understand that the “*effective filing date*” is evaluated on a claim-by-claim basis and that it is the filing date of the earliest priority application that provides “*written description*” and “*enables*” the subject matter of the claim. In this case, as explained below, the relevant time period is in 2005-2006, when Corteva filed its initial patent applications that led to the ’055 patent. Ex. 2017 (PCT/US2006/042133); Ex. 2013 (Provisional 60/731,044).

17. I understand that the basic inquiry for written description is whether the specification describes the claimed subject matter such that a skilled person would understand that the inventor possessed what is claimed. I understand that a number of factors may be considered in evaluating the written description requirement, including: (1) the existing knowledge in the particular field; (2) the extent and content of the prior art; (3) the maturity of the science or technology; and (4) the predictability of the technical feature at issue. I have been instructed that a sufficient description of a genus (such as a group of enzymes) requires the disclosure of either a representative number of species or structural features common to the genus so that one of skill in the art can visualize or recognize the members of the genus.

18. I understand that a patent claim also can benefit from the filing date of an application if the disclosure in the application provides *enablement*, that is, if the

patent specification enables a skilled person to make and use the full scope of the claimed subject matter without undue experimentation. I understand that enablement requires disclosure of only one or a few examples if there is some general quality, such as a structural feature, that runs through a genus of compounds that makes them particularly suited for their function.

19. I understand that each claim of a patent must be enabled such that, at the effective filing date of the patent, a skilled person could practice the invention without undue experimentation. I understand that the factors I may consider in determining whether a disclosure would require undue experimentation, known as the “*Wands* factors,” include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented in the specification, (3) the presence or absence of working examples in the specification, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. I understand that enablement is met where routine experimentation would be required to practice a claimed invention. I also understand that enablement is not measured against the cumulative time and effort it might take to make every embodiment within a claim.

V. Background and State of the Art

20. Set forth below is a brief summary of what was known in the prior art about enzyme structure-function correlations, including for α KG-dependent

dioxygenases such as SdpA (AAD-12) and TfdA. This information would have been known to persons skilled in the art and generally accepted in 2005-2006.

A. Enzymes

1. Function, Structure, and Classification of Enzymes

21. Enzymes function as catalysts for biochemical reactions. Some enzymes catalyze reactions that would otherwise be too slow to be useful to cells and organisms. Without enzymes, many biochemical reactions would take much longer and would not be fast enough to sustain life. Ex. 2018 (StatPearls, Enzymes), 1. An enzyme catalyzes a chemical reaction by binding its substrate(s) (i.e., the chemical compounds required for the reaction) at a location within an interior pocket known as the active site. Ex. 2018 (StatPearls, Enzymes), 1.

22. The vast majority of enzymes are proteins (also known as polypeptides), which are large molecules built from 20 different types of amino acids. Ex. 2018 (StatPearls, Enzymes), 1. To form enzymes, these amino-acid building blocks are connected in a linear chain in a specific sequence. The sequence of amino acids in a polypeptide chain is called its primary structure. Ex. 2018 (StatPearls, Enzymes), 1. This linear chain then folds up into a “globular” (meaning more or less spherical) three-dimensional structure in order to function as an enzyme catalyst. The secondary structure of a protein describes the localized polypeptide chain structures such as α -helices and β -sheets. Ex. 2018 (StatPearls, Enzymes), 1.

The complete three-dimensional fold of a polypeptide chain into a globular protein subunit is known as its tertiary structure. Proteins may be composed of one or more subunits (e.g., a monomer or dimer). The three-dimensional arrangement of subunits in an enzyme is known as its quaternary structure. Ex. 2018 (StatPearls, Enzymes) 1.

23. Enzymes are classified using a specific enzyme nomenclature system known as an Enzyme Commission or “EC” number. This system was “the best developed and most widely used functional classification scheme” in 2005-2006. Ex. 2019 (Tian), 864. An EC number is a unique four-digit code that categorizes the chemical reaction that an enzyme catalyzes. Ex. 2019 (Tian), 864. The first digit of an EC number indicates the main type of enzymatic activity and ranges from 1 to 6¹, including: 1, oxidoreductases, 2, transferases, 3, hydrolases, 4, lyases, 5, isomerases, and 6, ligases. Ex. 2020 (Tipton), 36. The last digit usually represents the substrate specificity of a reaction, while the first three digits of the EC number usually describe the overall type of enzymatic reaction. Ex. 2020 (Tipton), 36-37. For example, the EC number of (*S*)-(2,4-dichlorophenoxy)propanoate:α-ketoglutarate dioxygenase, also known as SdpA, is 1.14.11.43. The first digit (1) indicates that it is an

¹ A new class of enzymes, the translocases (EC 7), was added in 2018, but was not a recognized class in 2005-2006.

oxidoreductase, the second digit (14) indicates that it acts on paired donors with incorporation or reduction of oxygen, the third digit (11) indicates that α -ketoglutarate (also known as 2-oxoglutarate) is one of the electron donors, and the fourth digit (43) indicates that its substrate is (*S*)-(2,4-dichlorophenoxy)propanoate.²

24. An enzyme's structure and the reaction it catalyzes (its function) are usually determined by its sequence of amino acids or primary structure. Ex. 2018 (StatPearls), 1 ("Subunit structure is determined by the sequence and characteristics of amino acids in the polypeptide chain."); *see also* Ex. 2021 (Rost 1999), 85 ("proteins with similar sequences adopt similar structures"). Enzymes with similar sequences usually catalyze similar or identical reactions, as further described below.

2. Degrees of Amino Acid Sequence Relatedness

25. Skilled artisans have long analyzed the similarity of enzymes by comparing their amino acid sequences. Before 2005-2006, skilled artisans in the field had arrived at a general consensus for how to determine and describe the degree of relatedness of enzymes based on their amino acid sequences.

26. Skilled artisans perform sequence alignments to identify structural, functional, or evolutionary relationships between two or more DNA, RNA, or

² The Enzyme Commission has not yet assigned a separate EC number for the degradation of pyridyloxy auxin substrates, such as triclopyr, by the same enzyme.

protein sequences. Nucleotides or amino acids (often referred to as residues) in a sequence are aligned to give the greatest possible number of matches between or among the sequences, which are typically represented as rows within a matrix. Gaps are inserted between the residues as necessary so that identical or similar residues are aligned in successive columns. *See, e.g.,* Ex. 2022 (Elkins), Fig. 5.

27. By 2005-2006, researchers commonly performed sequence alignments to compare between proteins of similar or dissimilar structure. Ex. 2022 (Elkins), 5189 (TfdA and TauD are “closely related proteins” based on ~30% sequence identity); Ex. 2021 (Rost 1999), 85. Sequence identities (or similarities) between two or more enzymes may range from <10% (very few matching residues) to 100% (identical). A sequence identity between two enzymes of above 35% or 40% is considered a relatively high level of sequence identity and indicates that those enzymes likely have similar structures and functions. Ex. 2021 (Rost 1999), 85. Often, two enzymes with this level of sequence identity will catalyze the same reaction or similar reactions. Enzymes having less than 20% sequence identity generally have different folded secondary and tertiary structures and catalyze different types of reactions.

28. Decades of comparing and aligning protein sequences led researchers to recognize a “twilight zone” for sequence similarity. Ex. 2023 (Chung), 1123. This “twilight zone” represents a range of sequence identity (approximately 20-35%) in

which the relatedness of proteins—how similar their structures are, whether they have the same function, and whether they evolved from a common precursor—can be more difficult to judge. Ex. 2023 (Chung), 1123; Ex. 2021 (Rost 1999), 85. In other words, relationships between sequence and function are often more unpredictable when sequence identities fall within the twilight zone, making enzymes in this sequence identity range particularly interesting for research purposes, with several groups spending significant time attempting to discern sequence/function relationships at different thresholds of sequence identity, including at and around the twilight zone of 20-35% sequence identity. Ex. 2021 (Rost 1999), 85; Ex. 2024 (Wilson), 245.

3. Predicting Enzyme Function Based on Sequence Identity

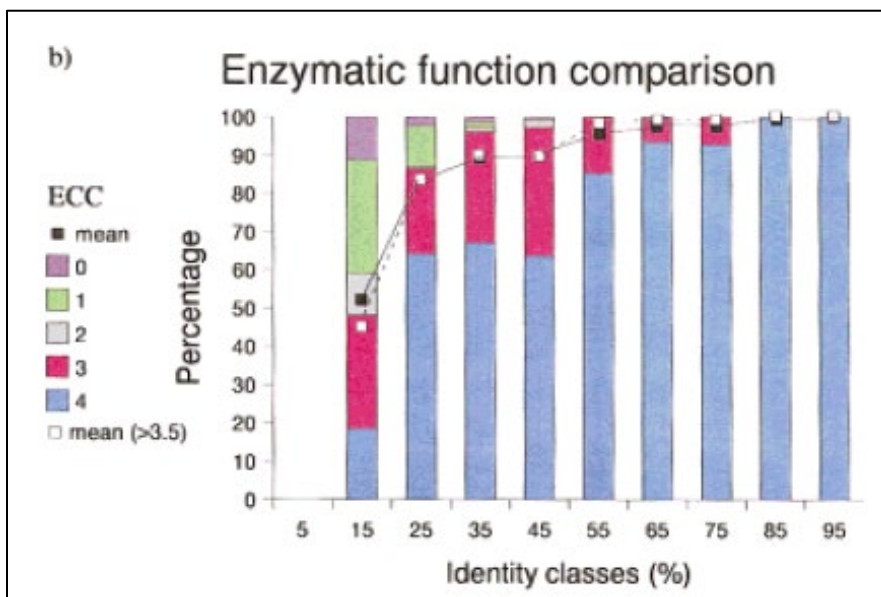
29. By the 2000s, genome sequencing was identifying new genes encoding proteins much faster than researchers could empirically determine the activities of those proteins. Researchers therefore sought to determine how reliably one could infer the function of a new protein by comparing its sequence to the sequence of another protein having a known function. Ex. 2025 (Minshull), 1 (two main reasons to predict an enzyme’s function from its sequence are “to identify the components and thus the functional capabilities of an organism” and “to create enzymes with specific properties”). Using pairwise sequence comparisons, numerous studies have shown that when the sequence identity between enzymes is above 60%, enzyme

function is well conserved. Ex. 2019 (Tian), 863; Ex. 2024 (Wilson), 233; Ex. 2026 (Devos), 98; Ex. 2027 (Todd), 1113; Ex. 2028 (Rost 2002), 595. This principle applied in 2005 and continued to apply through the '055 patent's March 24, 2017 filing date. Ex. 2030 (Price), 1. It remains true today.

30. A study by Wilson et al. in 2000, for example, evaluated pairwise sequence, structure, and function comparisons on ~30,000 pairs of protein domains. Ex. 2024 (Wilson), 233. Wilson et al. constructed their own functional classification system using EC numbers for enzymes. Ex. 2024 (Wilson), 242. They found that full conservation of enzyme function can occur between two proteins with as low as ~40% sequence identity. Ex. 2024 (Wilson), 233. They concluded that percent identity is a useful predictor of function for proteins that are close in structure and outside the twilight zone. Ex. 2024 (Wilson), 245.

31. A study in 2000 by Devos et al. analyzed a set of over 7,000 protein sequence alignments using the publicly available FSSP protein database. Ex. 2026 (Devos), 99. They found that, above an identity threshold of 50-80%, at least the first three digits of an EC number were identical, and above 80%, all four digits in the EC codes were “completely identical,” meaning that two enzymes having this level of identity can confidently be assumed to have the same activity. Ex. 2026 (Devos), 101-102 (Fig. 1b, replicated below). Further, Devos et al. found that binding site conservation follows a similar pattern that is parallel to the overall level

of sequence identity, such that “at 75% identity, most of the proteins conserve 75% of the binding site residues.” Ex. 2026 (Devos), 103.

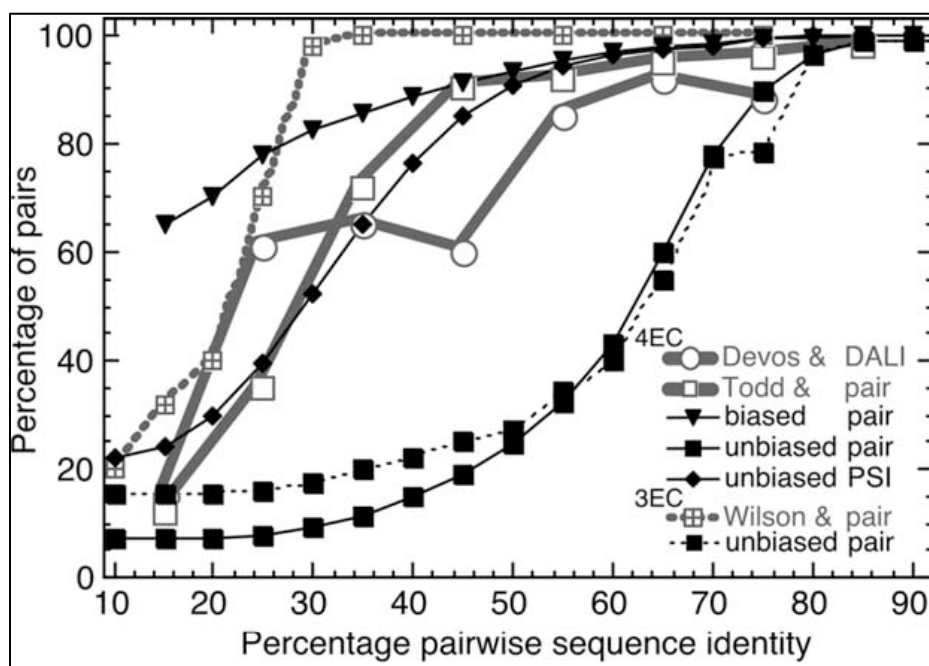


Ex. 2026 (Devos), 101 (Fig. 1b).

32. Similarly, a study by Todd et al. in 2001 assessed the functional variation of 31 diverse structural enzyme superfamilies using the EC classification scheme and found that functional variation is rare when the sequence identity is above 40%. Ex. 2027 (Todd), 1113. Todd et al. also found that “conservation of chemistry does appear to be the dominant theme,” further suggesting that high sequence identity between a pair of enzymes indicates that they will have the same biochemical function (i.e., catalyze the same reaction). Ex. 2027(Todd), 1138.

33. In 2002, Rost made a more conservative estimate for sequence-based conservation of enzyme function aimed at providing “important benchmarks for everyday sequence analysis,” suggesting that the prior analyses were either biased

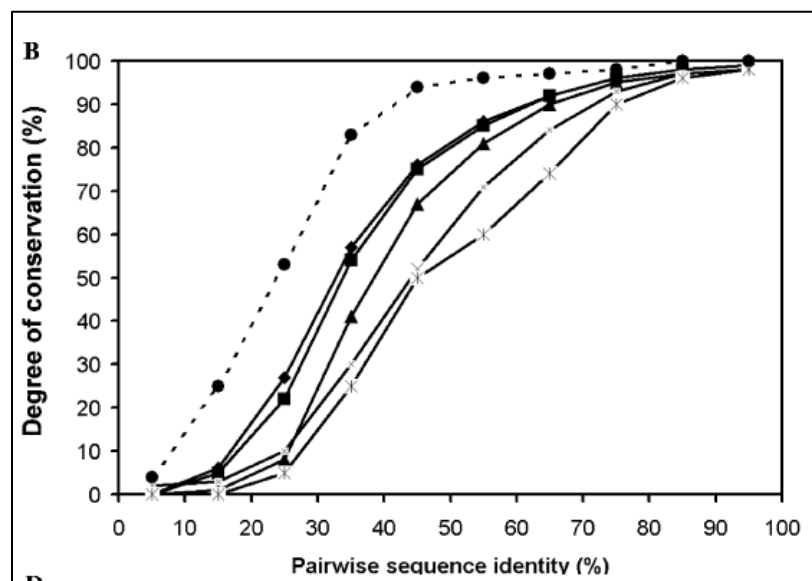
or too small. Ex. 2028 (Rost 2002), 595. Rost took a slightly different approach that considered sequence comparisons into the twilight zone and found that, when the sequence identity is below 70%, both the first digit and all four digits of EC numbers start to diverge more quickly than others had reported. Ex. 2028 (Rost 2002), 596; Ex. 2019 (Tian 2003), 863. Nevertheless, Rost confirmed previous reports that enzyme function is highly conserved above 70% sequence identity. Ex. 2028 (Rost 2002), 596. Indeed, Rost presented a graphical comparison between his study and those by Wilson, Devos, and Todd, which confirmed an even stronger conservation of function at higher levels of sequence identity, including at 85% or greater identity:



Ex. 2028 (Rost 2002), 604 (Fig. 5).

34. A study by Tian et al. in 2003 consolidated and built upon the prior studies by Devos, Wilson, Todd, and Rost, analyzing thousands of protein sequences

for establishing “the threshold of sequence identity necessary to transfer function from a protein of known function to an unknown protein.” Ex. 2019 (Tian), 863. Using a strategy similar to Rost’s to reduce database bias, Tian defined an enzyme family as a family of sequences that are all above a certain threshold of pairwise sequence identity and that also have the same function. Ex. 2019 (Tian), 865, 876-77. Tian found that “40% sequence identity can still be used as a confident threshold to transfer the first three digits of an EC number,” whereas “above 60% sequence identity is needed to have above 90% accuracy” for all four digits of an EC number. Ex. 2019 (Tian), 865.



Ex. 2019 (Tian), 866 (Fig. 1(B)). As shown in the figure, the x-axis represents percent sequence identity, and the y-axis represents percent function conservation.

35. Accordingly, by 2005-2006, persons skilled in the art routinely predicted enzyme function based on sequence similarity to proteins whose function

had already been experimentally determined. Extensive studies showed that this was a universal principle applied within the field of enzymology. *See, e.g.*, Ex. 2026 (Devos), 98; Ex. 2019 (Tian), 864 (“sequence identity is [] widely used as an indication of functional similarity”). It was established by then that enzyme function is well conserved and can be reliably predicted based on sequence identities above approximately 50-60%. These established function prediction values were well below the higher 85% minimum threshold of the ’055 patent claims. Ex. 2019 (Tian 2003), 863; Ex. 2026 (Devos), 102.

36. Indeed, sequence comparisons were the accepted “gold standard” for predicting protein function. Ex. 2029 (desJardins), 97; Ex. 2024 (Wilson), 233 (“percent identity is more effective at quantifying functional conservation” than other techniques); Ex. 2030 (Price), 1 (“Proteins with over 30% similarity are likely to have similar functions, but to be 90% confident of the substrate of an enzyme, over 60% similarity may be required.”).

37. The strong correlation between a high level of enzyme sequence identity and a conserved enzyme function was intuitive for skilled artisans in 2005-2006 for several reasons. Different enzymes that catalyze the same reaction were known generally to have significant sequence and structural similarity based on underlying sequence conservation. Ex. 2032 (Galperin), 779-80. Structural analyses of enzymes having relatively high sequence identity have revealed recurrent

structural patterns, such as common folds and conservation of specific sequence motifs. Ex. 2032 (Galperin), 780. Persons skilled in the art recognized the evolutionary structural patterns of enzymes and often used their structures as a basis for inferring function for enzymes having related sequences.

38. Even before the 2000s, skilled artisans had learned to recognize amino acid sequence patterns—known as motifs—in enzymes to identify elements of their structures that are essential for their functions. Such motifs consist of individual amino acids or stretches of amino acids that are always identical in a set of enzymes with the same or similar functions but that can be interspersed by individual or stretches of amino acids (known as spacers) that differ among enzymes of the set. The always identical amino acids (referred to as “conserved” amino acids) within such a motif often have important functions in the common activity of the set of enzymes, such as in binding of substrates or enzyme components (known as cofactors) that are required to promote the chemical reaction. Motifs are amino acid sequence patterns that are generally related to a specific function of the protein. By 2005-2006, researchers had recognized that, when a set of enzymes has an overall level of sequence identity of about at least 60-70%, such sequence motif patterns that are fully conserved within the set of enzymes can readily help identify residues that are important to the common structure and function and, importantly, be used to assign new members to the set as new protein sequences are reported. Ex. 2033

(Dym), 1712. A motif is often one of the most reliable structural features for inferring common function between enzymes. Ex. 2002 (Müller 2006), 1358; Ex. 2034 (Minarik), 261 (explaining how “[r]esidue 53 is conserved with an acidic side chain in all NAD-dependent MDHs”). For instance, malate dehydrogenases are a class of enzymes that promote one common chemical reaction and use a conserved catalytic mechanism. Malate dehydrogenases from different species share a high degree of 3D (tertiary) structural similarity. Ex. 2034 (Minarik), 257. The common functional part of malate dehydrogenases—their catalytic domain—is characterized by a particular motif that is conserved throughout the class and contributes to both the conserved structure and the shared enzymatic activity. Ex. 2034 (Minarik), 259.

39. In sum, by 2005-2006, high similarity of enzyme amino acid sequences was known to correlate well with conservation of both overall three-dimensional structure and function, which enzymologists recognized and referred to as sequence-function (or structure-activity) relationships. Ex. 2035 (Chaparro-Riggers), 187. Thus, when an amino acid sequence appearing to represent a new enzyme was determined as part of the genome-sequencing efforts of the 1990s and 2000s, skilled artisans routinely assigned a biochemical function to the new enzyme using sequence comparisons and motif recognition. These principles were well established and also routinely applied in protein engineering to make enzyme variants with

conserved or even improved functional traits. *See* Section V.B, below; Ex. 2025 (Minshull), 2-3 (“Using homologous sequences for protein engineering”).

4. Enzymes Are Unique Compared to Other Types of Proteins

40. Several categories of proteins occur in nature, including enzymes, antibodies, structural proteins, hormones, and many others. Skilled artisans were familiar with differences between the various types of proteins and what made enzymes unique. Among these categories of proteins, enzymes and antibodies have been extensively used in medicine and biotechnology and thus have been the subjects of considerable scientific research.

41. A major difference between enzymes and antibodies is their evolutionary background. Because enzymes catalyze biochemical reactions, many of which were likely important even to early life forms, they are generally products of evolution over long time periods. Ex. 2018 (StatPearls, Enzymes), 1. Even enzymes that catalyze the identical reaction can have quite different amino acid sequences as a result of the long time over which they evolved from their common ancestral enzyme. However, any amino acid changes (mutations) appearing over time that disturbed the enzyme’s overall structure or replaced amino acids that promoted the reaction would have led to a loss or reduction of the enzyme’s activity, likely with negative consequences for the organism that depended on the enzyme. Accordingly, pairs of enzymes catalyzing the same reaction (isofunctional enzymes)

have retained common, relatively optimized structures with important amino acids that are responsible for certain facets of the overall enzyme activity (e.g., structural stability, substrate binding, transition state stabilization). *See, e.g.*, Ex. 2019 (Tian), 877. Amino acids in a protein sequence that are less important to an optimized structure or catalytic function are replaced over time by different amino acids, with no deleterious effect on protein function. The retained portions are functional motifs, such as the AAD-12 motif at issue here. In other words, enzymes that have evolved over time to perform the same function (or similar functions) can span a relatively broad range of sequence space. *See, e.g.*, Ex. 2034 (Minarik), 258-59 (malate dehydrogenases have ~20% sequence similarity but have homologous active sites, coenzyme binding sites and quaternary structure).

42. The evolutionary background of antibodies is starkly different from that of enzymes. Antibodies develop rapidly over a matter of weeks as part of an immunological response to an antigen molecule, to which the antibody binds. Ex. 2037 (StatPearls, Antibodies), 1-2. Antibody structures and sequences are evolutionarily adapted to this rapid evolution, as large portions of antibodies have generally fixed sequences (known as constant regions) while sequence changes leading to diversity in antigen recognition are rapidly generated in smaller variable regions, particularly the hypervariable regions known as complementarity determining regions (CDRs). Ex. 2037 (StatPearls, Antibodies), 1-2. These variable

regions are, on average, much shorter in sequence than enzymes. Thus, the sequence of antibodies can rapidly evolve in relatively discrete regions as part of an immunological response, with small changes within the variable regions of an antibody often leading to enormous differences in function. In other words, the variable regions of antibodies, which generally determine their different functions, generally cannot be grouped into “buckets” of similar sequences as can be done in the case of enzymes.

43. In addition, modifications in enzyme structure tend to be located throughout the enzyme’s amino acid sequence, unlike the focused tailoring of the variable and hypervariable regions of an antibody to change its binding activity and confer recognition of a new antigen. Ex. 2037 (StatPearls, Antibodies), 2. Because antibodies serve a different biological role, which requires rapid evolution of their structures to confer the ability to recognize new antigens, even small changes in variable region sequence can lead to entirely different antigen targets and therapeutic properties. The same does not hold true for enzymes, which generally have conserved and slowly evolving structures among enzymes having identical or similar functions.

B. Enzyme Engineering and Variants

44. In his declaration, Dr. Silverstone appears to imply that a skilled artisan would need 3D structural information in choosing substitutions to make on a target

protein to retain or improve its activity. Ex. 1003, ¶¶ 45-46. He fails to acknowledge the full knowledge of skilled artisans as of 2005-2006, which went well beyond selection of substitutions based on 3D structure alone.

1. Sequence Alignments

45. One important available technique was use of sequence alignments. Ex. 2025 (Minshull), 2-3 (“The sequences of orthologous proteins are thus a potentially rich source of information regarding sequence changes that are acceptable without drastically disrupting protein function”); Ex. 2035 (Chaparro-Riggers), 180 (in protein engineering, “[b]esides crystal structures, a wide variety of tools can be applied, ranging from *sequence alignments* or phylogenic trees to computational models”).

46. By 2005-2006, alignment tools for comparing protein sequences were recognized and widely used by persons skilled in the art. These tools allowed researchers to compare two or more protein sequences and calculate percent identity or similarity scores using known algorithms. These algorithms had been incorporated into various programs, such as BLAST (Basic Local Alignment Search Tool) and FASTA (FAST-All), which were publicly available and widely used by skilled artisans in 2005-2006. Ex. 2040 (Altschul), 3389; Ex. 2041 (Müller), 761; Ex. 1001 (’055 Pat.), 21:61-22:10.

47. Aligned sequences of amino acid residues are typically represented as rows within a matrix. Matching residues in the aligned sequences are typically marked using asterisks or pipe symbols. When the aligned sequences differ in length, gaps are inserted between the residues so that identical or similar residues can be aligned in successive columns. An exemplary sequence alignment is shown in Figure 5 of Elkins et al. Ex. 2022 (Elkins), 5190 (Fig. 5).

48. The quality of a possible alignment of two or more sequences is frequently assessed using a substitution matrix, which have been known since the 1970s and were automated and computer-implemented (e.g., with the SIFT (Sorting Intolerant From Tolerant) program) by 2005-2006. These matrices assign probabilities of seeing one of the 20 common amino acids replaced by another. One class of scoring matrix, given the acronym PAM (for Point Accepted Mutation), explicitly accounts for evolutionary approximations regarding the rates and probabilities of the possible amino acid substitutions. Ex. 2041 (Müller), 761. Another common scoring matrix is known as BLOSUM (Blocks Substitution Matrix) and encodes empirically derived substitution probabilities. Ex. 2041 (Müller), 761. Results generated from either type of matrix allow skilled artisans to quantify the level of sequence relatedness or divergence. For example, in searching sequence databases to identify proteins related to an enzyme of interest (the query sequence), users of BLAST or FASTA can set threshold values either to restrict the

identified sequence set (the “hits”) to more closely related matches or to expand the set to include less similar (more divergent) sequences.

49. When interpreting aligned sequences, it was common for skilled artisans in 2005-2006 to examine the degree of conservation of the amino acids within a stretch of the sequence as a rough measure of how conserved a particular region is among lineages. In a multiple sequence alignment, positions that have the identical amino acid in all the aligned sequences are considered highly conserved, and, if the side chain of the amino acid found there has an appropriate chemical functional group (such as the carboxylic acid group of Asp or Glu, the imidazole of His, the sulfhydryl of Cys, the amine of Lys, or the phenol of Tyr), then the amino acid always found in that sequence position likely has an important role in the enzyme’s mechanism(s) of catalysis or the overall structure. Positions that have only a few different amino acids harboring chemically similar side chains (such as Leu, Ile, or Val) in the aligned sequences are considered to be somewhat conserved and may be involved in important interactions with substrates, cofactors, or other parts of the enzyme. Sequence positions that vary more widely, meaning that representative sequences have different types of amino acids with chemically dissimilar side chains (such as Glu, Lys, and Leu) in that position, are not conserved. It can be concluded that the common overall structure of the set of aligned proteins is robust to even drastic changes to the amino acids in these unconserved positions.

Going beyond individual sites, stretches of sequence with a high density of highly or somewhat conserved positions are likely to have structural or functional importance. Often, conservation will be high in stretches of sequence that contribute to the active site. Sequence alignments can thus provide guidance as to regions of the enzyme that are likely to tolerate substitutions without loss of function and other regions in which any substitution, or particular classes of substitutions, will likely disrupt the enzyme's structure and cause a loss of activity. *See e.g.*, Ex. 2022 (Elkins), 5190 (Fig. 5).

2. Principles for Designing Enzyme Variants

50. By 2005-2006, it was common for skilled artisans to develop enzyme variants with desired functional properties. A fundamental paradigm in the art was this established link between protein sequence and function: an enzyme's amino acid sequence determines its three-dimensional fold (structure), and its structure determines its biochemical function (activity). Therefore, persons skilled in the art generally analyzed an enzyme's amino acid sequence by comparing it to other known, similar sequences. Ex. 2025 (Minshull), 2-3 ("sequences of orthologous proteins are thus a potentially rich source of information regarding sequence changes that are acceptable without drastically disrupting protein function"); *id.*, 9 ("tools may now be used to aid in enzyme redesign: identifying suitable proteins as catalytic

starting points, choosing changes to make, and assessing the effects of those changes in a powerfully predictive way”); Ex. 2035 (Chaparro-Riggers), 184.

51. When making enzyme variants, a person skilled in the art in 2005-2006 would not have randomly picked any of the 19 other possible amino acids for a given residue, as Dr. Silverstone suggests, but would have instead relied on known principles for generating enzyme variants. Ex. 1003, ¶ 80, n. 1

52. To guide these substitutions, known matrices such as PAM or BLOSUM, which were commonly used to score alignments of protein sequences, would have provided guidance as to which amino acid replacements have been accepted by the processes of natural selection. *See, e.g.*, Ex. 2083 (Dayhoff), 345; Ex. 2041 (Müller), 761. These matrices were developed from sets of high-confidence alignments of many homologous proteins by assessing the frequencies of all possible amino acid substitutions. Ex. 2025 (Minshull), 6. They show which changes are favored or disfavored, providing guidance to skilled researchers on how to modify enzymes without losing activity. Ex. 2025 (Minshull), 6-7.

53. As noted above, skilled artisans performed sequence alignments with related proteins to identify highly conserved residues and segments that likely delineate important structural or functional elements. *See* Ex. 2035 (Chaparro-Riggers), 185 (homology-based methods “can be used to identify residues that can tolerate mutation without radically changing function”). Comparing amino acid

sequences with low identity (~30%) is particularly helpful here because residues found to be conserved even at a relatively high level of divergence among aligned sequences are likely important to structure, function, or both, whereas sets of sequences with higher identity will have more coincidental identities at positions that may not have important roles. For example, in this case, Figure 2 of the '055 patent illustrates a sequence alignment among AAD-12 and other homologous proteins and provides valuable guidance to a skilled artisan by identifying conserved residues and the claimed AAD-12 motif by lining up the sequences with varying lengths of amino acids. Ex. 1001 ('055 Pat.), Fig. 2.

54. A person skilled in the art in 2005-2006 would have had access to computational tools that took into account information provided by the substitution scoring matrices and sequence alignments to predict which substitutions in a given enzyme would be tolerated and which would cause loss of function. For example, SIFT was one available program that predicted whether an amino acid substitution would affect protein function by considering the position at which the change occurred and the type of amino acid change. Ex. 2044 (Ng), 3812.

55. Skilled artisans also used directed evolution techniques (often combined with rational design) that mimic natural evolution to produce variants. Ex. 2038 (Chica), 378 (targeting “multiple, specific residues to mutate on the basis of prior structural or functional knowledge [to] create ‘smart’ libraries”). Such

approaches enhanced directed evolution by incorporating strategies based on known sequence-activity relationships, such as the protein sequence activity relationships (ProSAR) method that was able to quickly generate enzymes with desired characteristics for commercial use. Ex. 2039 (Fox), 338-339.

56. In summary, a skilled person would have followed several fundamental scientific principles of protein engineering in 2005-2006 when designing enzyme variants based on the claimed methods:

1. Maintain high sequence identities in view of the known sequence-function correlations for enzymes. Skilled artisans understood that high levels of sequence identity between enzymes are generally correlated with the same or similar function. By 2005-2006, studies consistently confirmed that the minimum threshold for confidently predicting enzyme function (e.g., typically 60-70%) was well below the 85% threshold recited in the '055 patent claims. Ex. 2019 (Tian); Ex. 2028 (Rost 2002); Ex. 2025 (Minshull).

2. Maintain the active site motif. The active site residues are those in the enzyme's interior pocket that allow the enzyme to push and pull on the substrates to catalyze a chemical reaction. A skilled person would be careful to preserve the active site motif residues. Ex. 2025 (Minshull), 6 ("changes at key active site residues or other highly conserved residues are generally deleterious").

3. Retain other non-motif residues that are highly conserved across related enzymes. This is particularly true for enzymes with low sequence identities (pairwise identities of ~30%) where “changes at well-conserved positions tend to be deleterious.” Ex. 2044 (Ng), 3812; Ex. 2025 (Minshull), 6.

4. Retain overall surface charge distribution and steric bulk. Skilled artisans understood not to introduce mutations that would change the enzyme’s overall surface charge or steric bulk. *See* Ex. 2035 (Chaparro-Riggers), 186 (suggesting restricting mutation diversity where small amino acids can create additional space in a binding pocket whereas large amino acids can narrow the binding pocket). For instance, Müller et al. tested the effect of various mutations, including one that resulted in a bulkier side chain and presented steric hindrance that interfered with the enzyme’s reactive conformation. Ex. 2002 (Müller 2006), 1360. Skilled artisans would therefore avoid mutations that change side chain bulk. Skilled artisans, recognizing that changing an amino acid’s charge or polarity to the opposite charge would likely affect enzyme function, would have also avoided these types of substitutions. Ex. 2044 (Ng), 3812.

57. Once enzyme variants had been obtained by following these principles, a skilled artisan would have understood that the genes encoding the enzymes could be passed on to an artisan skilled in plant transformation for expression in plants.

C. α Ketoglutarate-Dependent Dioxygenases

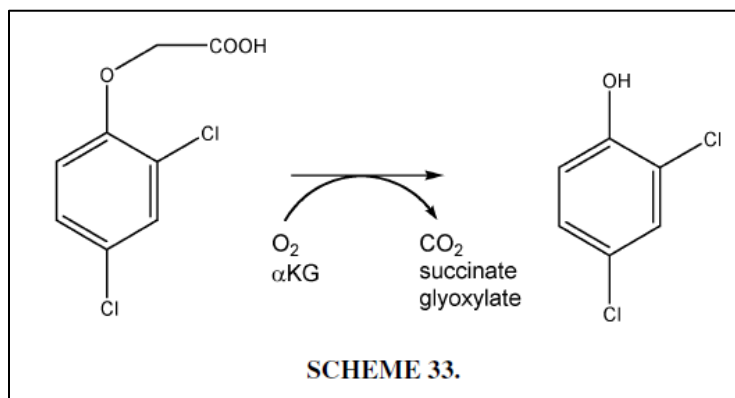
1. Reactions Catalyzed

58. Well before 2005-2006, researchers identified bacteria that could degrade 2,4-D and other phenoxyacetate herbicides. This recognition led to the identification of the gene encoding the TfdA enzyme from the bacterium *Alcaligenes eutrophus* JMP134 in 1997.³ Ex. 1025 (Streber). TfdA was initially mischaracterized as a monooxygenase. Ex. 2001 (Fukumori), 24311. In 1993, Fukumori et al. identified the reaction by which TfdA degrades 2,4-D and established that TfdA belongs to the family of α -ketoglutarate-dependent (“ α KG-dependent”) dioxygenase enzymes. Ex. 2001 (Fukumori), 24311.

59. To degrade 2,4-D, α KG-dependent dioxygenases require the presence of Fe(II) as the metallocenter (metal cofactor), as well as oxygen and α -ketoglutarate (α KG) as co-substrates. Ex. 2001 (Fukumori), 24311. When TfdA acts on 2,4-D as its substrate, the enzymatically catalyzed oxidation reaction produces carbon

³ *A. eutrophus* JMP134 was later renamed *Ralstonia eutropha* JMP134, and then renamed *Cupriavidus necator* JMP134. The *tfdA* gene is encoded by the pJP4 plasmid in this strain. In this declaration, I will refer to the canonical JMP134 TfdA enzyme as “TfdA,” unless otherwise noted.

dioxide, succinate, 2,4-DCP (a phenol), and glyoxylate, as shown in the reaction below. Ex. 2054 (Hausinger), 40 (scheme 33).



60. The α KG-dependent dioxygenase family is large. Ex. 2054 (Hausinger), 21. In addition to enzymes like TfdA that are involved in detoxification, the family includes enzymes involved in plant biosynthetic pathways, antibiotic biosynthesis, and lipid metabolism. Ex. 2054 (Hausinger), 21, 29-38.

61. Given the size of this family, members of the α KG-dependent dioxygenase family were generally classified into one of three groups of related enzymes. Ex. 1019 (Hogan), 12400-401. TauD, a family member that is closely related to TfdA, was classified in the same group (Group II) as TfdA. Ex. 1019 (Hogan), 12401. TauD nonetheless has a different function than TfdA; it catalyzes the conversion of α KG, O_2 and taurine to CO_2 , succinate, aminoacetaldehyde, and sulfite, which is used as a source of sulfur. Ex. 2022 (Elkins), 5185.

2. The α KG Motif and How It Was Determined

62. α KG-dependent dioxygenases are generally defined by their consensus motif, which is a simple way to identify their active sites. By 2005-2006, skilled artisans recognized that enzymes in the α KG-dependent dioxygenase family varied greatly in the exact numbering of the motif residues and the number of amino acids in the spacers separating each pair of consecutive motif residues, reflecting the diversity of reactions catalyzed by these enzymes.

63. Dr. Silverstone acknowledges that a suitable way to analyze the structure of a new protein is to “compare the sequence to [that of] a closely related protein of known structure.” Ex. 1003, ¶ 46. That is precisely how the α KG motif in general, and the specific examples of that motif found in TfdA, TauD, SdpA, and other related enzymes, were identified. In the early 2000s, Hogan et al. identified the metal binding residues in the active site motif of TfdA. Ex. 1019 (Hogan 2000), 12400. They defined the TfdA motif as **HX(D/E)X₁₃₈₋₂₀₇HX₁₀₋₁₃R**, which is distinct from the motif of other α KG-dependent dioxygenases. Ex. 1019 (Hogan 2000), 12401. The letters in the motif represent amino acids: “H” is His (histidine), “D/E” means that this residue must be either Asp (aspartate) or Glu (glutamate), and “R” is Arg (arginine). “X” can be any amino acid, and the numeric subscripts refer to the number of amino acids in the spacers. Hogan et al. also performed an alignment with TauD, revealing that it shared the common HX(D/E)X₁₃₈₋₂₀₇HX₁₀₋₁₃R motif. Ex. 1019

(Hogan 2000), 12401, 12407 (Fig. 8). Other enzymes in the family, such as the Group I enzymes, were found to share a common $\text{HXDX}_{50-70}\text{HX}_{10}(\text{R/K})\text{XS}$ motif and to act on different substrates. Ex. 1019 (Hogan), 12401.

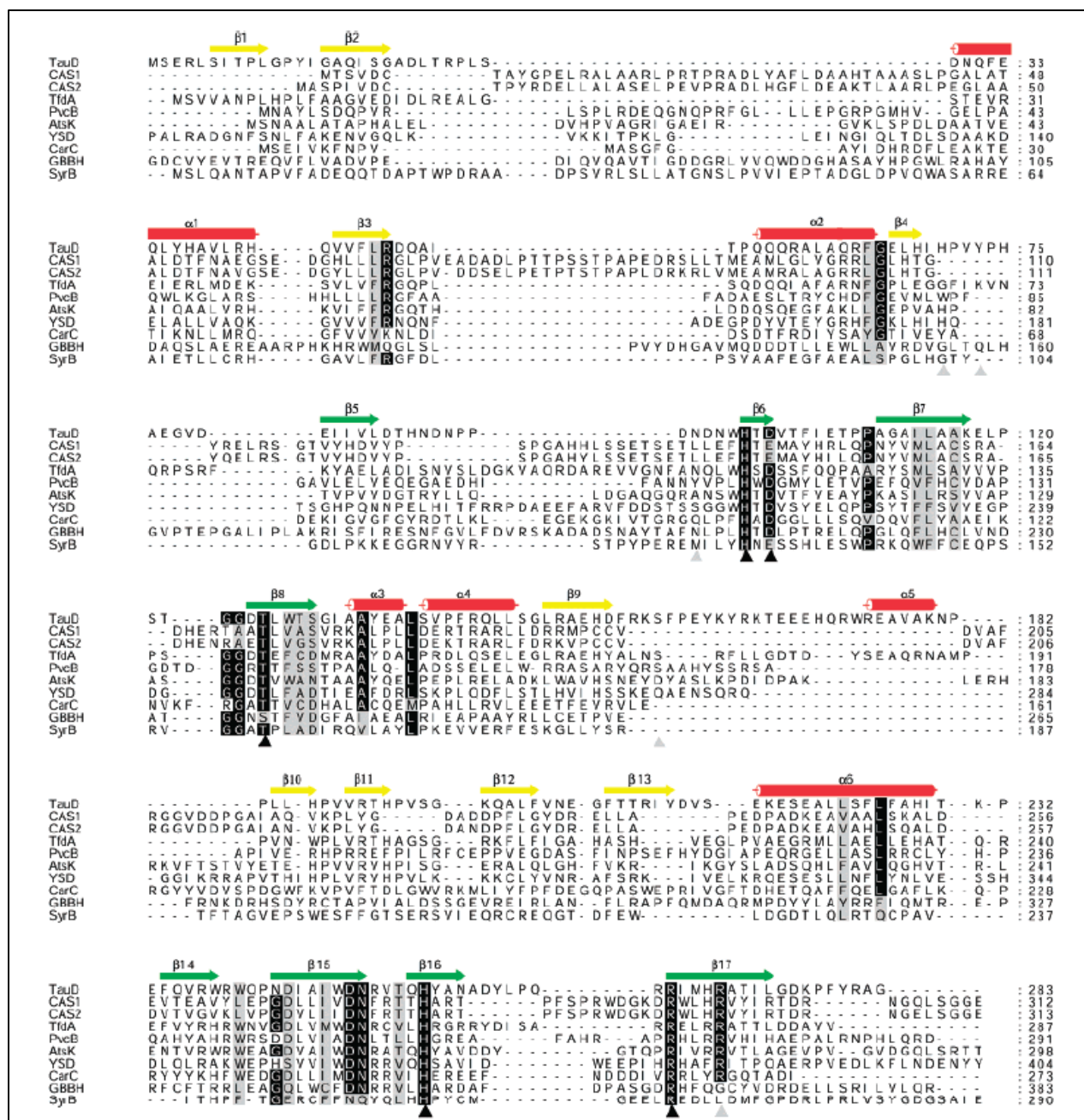
64. In 2002, Elkins et al. reported the crystal structure of TauD and used it to predict the structure of TfdA. Ex. 2022 (Elkins). The TauD motif bound to αKG at two motif sites, T_{126} and R_{266} , through a hydrogen bond and a salt bridge, respectively. Ex. 2022 (Elkins), 5188. The authors found that TauD and related αKG -dependent dioxygenases, including TfdA, share the consensus motif **$\text{HX}(\text{D/E})\text{X}_{23-26}(\text{T/S})\text{X}_{114-183}\text{HX}_{10-13}\text{R}$** . Ex. 2022 (Elkins), 5185. Elkins further explained that since “TfdA is particularly well characterized,” it was “worth modeling to extend our knowledge of the αKG -dioxygenase superfamily” and its predicted overall fold was expected to extend to other “closely related proteins (~30% sequence identity).” Ex. 2022 (Elkins), 5189.

65. A further study by Hotopp et al. in 2002 identified two additional important, conserved residues of TfdA, T_{141} and R_{274} , believed to bind the αKG co-substrate. Ex. 2003 (Hotopp), 9792-93; Ex. 1019 (Hogan 2000), 12407 (Fig. 8). Thus, as reflected in Hogan and Hotopp, skilled artisans understood the structure-function relationships of Group II αKG -dependent dioxygenases and had identified five common, conserved residues (H, D, T, H, R) in those enzymes’ active sites with variable spacing, depending on the specific enzyme.

66. Moreover, a 2004 review article by Hausinger described several α KG-dependent dioxygenases known to have this HX(D/E)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R motif, including TfdA, CarC or carbapenem synthase, shown in Scheme 11; TauD or taurine/ α KG dioxygenase, shown in Scheme 29; and AtsK or alkylsulfatase, shown in Scheme 31. Ex. 2054 (Hausinger), 22, 31, 39. As this article explains, the active site structures of the α KG-dependent dioxygenases exhibit nearly identical arrangements of the three amino acid side chains that bind one face of the metal ion. Ex. 2054 (Hausinger), 45. The '055 patent illustrates these common structural features in Figure 2, which were recognized by persons skilled in the art.

3. Related Structural Features

67. The determination of the crystal structure of TauD by Elkins et al. in 2002 revealed that TfdA and other group II α KG-dependent dioxygenases (generally having ~30% or higher sequence identity) share similar primary and secondary structures. Ex. 2022 (Elkins), 5189. For instance, Figure 2 of Elkins et al. (replicated below) shows the conserved residues and secondary structures in group II α KG-dependent dioxygenases, including shared α -helices and β -strands of the jellyroll motif, among others. The TauD secondary structure is colored red for helix, green for β -strands of the jellyroll motif, and yellow for other β -strands. Ex. 2022 (Elkins), 5188 (Fig. 2).



Ex. 2022 (Elkins), 5190 (Fig. 5).

68. The publication by Elkins et al. regarding the structures and functions of TfdA and other group II α KG-dependent dioxygenases provided guidance with respect to SdpA (AAD-12). In particular, it established that many of the aligned

sequences in Figure 2 had been correlated to a tertiary structure and provided significant guidance to a skilled person in choosing mutations for that family of enzymes.

4. SdpA (AAD-12) and Its Active Site Motif

69. In addition to TfdA, researchers had discovered other enzymes produced by soil bacteria that degraded phenoxy auxin herbicides such as 2,4-D. These enzymes included SdpA and RdpA from the bacteria *Delftia acidovorans* MC1, as well as SdpA and RdpA from *Sphingomonas herbicidovorans* MH. Ex. 1025 (Streber); Ex. 1023 (Müller 2006).

70. By 2006, Müller et al. had reported the specific motif residues in SdpA and RdpA from *Sphingomonas herbicidovorans*. Ex. 2002 (Müller 2006). In particular, sequence alignments between the (*R*)-specific and (*S*)-specific enzymes revealed that the His¹-X-Asp/Glu-X_n-His² motif of RdpA is comprised of residues His111, Asp113, and His270, while that of SdpA involves His102, Asp104, and His257. Ex. 2002 (Müller 2006), 1357. Citing Hogan (Ex. 1019) and its active site motif disclosures, Müller reported that the high degree of active site sequence identity and strong orientation of key side chains by interactions with the Fe^{II} were “supportive of the active site being the most conserved and structurally accurate part of the RdpA and SdpA models.” Ex. 2002 (Müller 2006), 1358.

71. Among enzymes sharing the α KG motif, the exact numbering of the conserved residues and the spacer lengths can vary, but, in any given enzyme, each residue has a precise amino acid number denoting its location in the sequence, and the spacers have well-defined lengths. For example, AAD-12 (SdpA) has a specific motif $\text{HX}_{109}\text{D}(\text{X})_{111-134}\text{T}(\text{X})_{136-261}\text{H}(\text{X})_{263-272}\text{R}$, where the subscripts now give the sequence positions of the amino acids in SEQ ID NO: 2 of the '055 patent. As claimed in the patent, this motif is associated with the dual herbicide-degrading activity of the AAD-12 enzymes. For any given α KG-dependent enzyme, whether or not the AAD-12 motif is present is readily assessed by aligning that enzyme's sequence with SEQ ID NO: 2, such that its first spacer residue (X) between the conserved H and D residues aligns with position 109 (i.e., X_{109}) in SEQ ID NO: 2. If all four of the other motif residues—D, T, H and R—also line up without the need to introduce any gaps in either sequence to make them line up, then the given enzyme possesses the AAD-12 motif and should have the dual herbicide-degrading activity. The motif residues must be unchanged, and the spacers between them must have precisely the specified length (although the sequence position numbering of the actual residues could be different) for the protein in question to possess the AAD-12 motif. A person skilled in the art would have recognized that the location and spacing of the AAD-12 motif residues differ from those in other α KG-dependent dioxygenases having different substrate specificities. Indeed, Dr. Silverstone does

not identify any other enzymes having the particular active site motif present in AAD-12.

VI. Level of Skill in the Art

72. Regarding aspects of the '055 patent relating to enzymology, particularly α KG-dependent dioxygenases (e.g., SdpA/AAD-12) and variants thereof, a person skilled in the art would have generally possessed a Ph.D. in molecular biology, biotechnology, or biochemistry, and 3-5 years of experience in the *development of enzymes and enzyme variants as well as familiarity with α KG-dependent dioxygenases*. This need for a background in enzymology and familiarity with α KG-dependent dioxygenases is reflected in the scientific literature from 2005-2006. E.g., Ex. 1019 (Hogan); Ex. 1020 (Müller); Ex. 2019 (Tian); Ex. 2028 (Rost 2002); Ex. 2025 (Minshull).

VII. The '055 Patent

A. The Claims

73. The '055 patent contains three independent claims and thirty dependent claims, all directed to novel transgenic plants and plant cells comprising a recombinant polynucleotide encoding AAD-12 proteins having required structural features.

74. I have focused my analysis on aspects of the '055 patent claims relating to α KG-dependent dioxygenases (e.g., SdpA/AAD-12), the AAD-12 motif, and sequence identity. I understand that another witness will address aspects of the '055

patent relating to plant transformation and crop science. For this reason, I have not addressed those technical issues here. Claim 1 of the patent reads as follows:

1. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-12 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and a pyridyloxy auxin herbicide, further wherein said AAD-12 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 2; and

ii) an AAD-12 motif having the general formula of:

$\text{HX}_{109}\text{D}(\text{X})_{111-134}\text{T}(\text{X})_{136-261}\text{H}(\text{X})_{263-272}\text{R}$, wherein

X_{109} represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2;

$(\text{X})_{111-134}$ represents a sequence of 24 amino acids;

$(\text{X})_{136-261}$ represents a sequence of 126 amino acids;

and

$(\text{X})_{263-272}$ represents a sequence of 10 amino acids.

Ex. 1001 ('055 Pat.), 125:2-18.

75. The claimed “AAD-12 motif” corresponds to the active site discussed above for SdpA (AAD-12). It specifies five conserved (fixed) amino acids, as well as the particular spacing between these conserved residues.

76. Independent claim 32 recites similar features as independent claim 1 but further requires the AAD-12 motif to have 90% sequence identity with SEQ ID

NO: 2. over the stretch of its sequence that corresponds to positions 108 to 273 of SEQ ID NO: 2. Ex. 1001 ('055 Pat.), 126:52-127:4. Independent claim 33 generally recites a method of controlling at least one weed in a field planted with seeds that comprise an AAD-12 recombinant polynucleotide, and the method comprises applying to the field a pyridyloxy auxin herbicide. Ex. 1001 ('055 Pat.), 127:5-26.

B. The Specification

77. The '055 patent describes transgenic plants expressing the bacterial α KG-dependent dioxygenase enzyme SdpA from *Delftia acidovorans* (i.e., AAD-12) that exhibit resistance to two different classes of herbicides: phenoxy auxins (e.g., 2,4-D) and pyridyloxy auxins (e.g., triclopyr and fluroxypyr). Ex. 1001 ('055 Pat.), 4:44-62, 12:23-58. In view of this dual herbicide-tolerance activity, the inventors termed the enzymes and genes of the '055 patent “AAD-12” (Aryloxy Alkanoate Dioxygenase) proteins and genes. Ex. 1001 ('055 Pat.), 4:48-62. The '055 patent discloses the amino acid sequence of AAD-12 as SEQ ID NO: 2. Ex. 1001 ('055 Pat.), 5:48-51.

78. The '055 patent describes the inventors' discovery of an α KG-dependent dioxygenase (AAD-12) based on searching for homologues that have a significantly lower sequence identity to *tfdA* (within the twilight zone). Ex. 1001 ('055 Pat.), 3:22-33, 32:6-7. Consistent with the function-prediction principles described above (Section V.A.3), the inventors recognized that “highly identical”

sequences generally had the same function. Ex. 1001 ('055 Pat.), 32:1-7. As explained above, by 2005-2006, researchers also recognized that the α KG-dependent dioxygenase family included enzymes with different functions characterized by unique α KG motifs. *See* Section V.C, above; Ex. 1019 (Hogan), 14201.

79. In seeking new homologues with unique functions, the inventors describe in Example 1 that they only looked at sequences having lower than 50% sequence identity to *tfdA* (i.e., extending into the twilight zone). Ex. 1001 ('055 Pat.), 32:6-14. Example 1 reports that the inventors identified SdpA (AAD-12), which had only 31% sequence identity to *tfdA*. Ex. 1001 ('055 Pat.), 32:15-22. In subsequent studies *in planta*, the inventors demonstrated a unique functional capability of the AAD-12 enzymes in their dual herbicide-degrading activity. *E.g.*, Ex. 1001 ('055 Pat.), 45:50-56:38.

80. The '055 patent provides an extensive disclosure that would have supported and guided skilled artisans in making AAD-12 genes and proteins. Ex. 1001 ('055 Pat.), 19:28-22:56. The patent explains, for instance, that this “family of proteins is characterized by three conserved histidine residues in a ‘HX(D/E)X₂₃₋₂₆(T/SX₁₁₄₋₁₈₃HX₁₀₋₁₃R’ motif which comprises the active site.” Ex. 1001 ('055 Pat.), 11:1-16; Ex. 1019 (Hogan), 14200; Ex. 2022 (Elkins), 5185. It further explains that the histidines coordinate Fe⁺² ion in the active site that is essential for catalytic activity (Hogan et al., 2000). Ex. 1001 ('055 Pat.), 11:1-16. When discussing

AAD-12 variants, the '055 patent explains that variants can be prepared “having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins.” Ex. 1001 ('055 Pat.), 19:51-54. These variants may be designed “that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like.” Ex. 1001 ('055 Pat.), 28:57-29:10. These function prediction principles would have been expected to work based on the guidance in the art described above. *See* Section V.A.3; *see, e.g.*, Ex. 2025 (Minshull); Ex. 2019 (Tian).

81. The '055 patent further describes that “[v]arious properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein.” Ex. 1001 ('055 Pat.), 22:11-14. The specification also describes that “[c]onservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule.” Ex. 1001 ('055 Pat.), 22:14-17. By 2005-2006, skilled artisans recognized that substituting similarly charged or sized amino acids represented a useful strategy for making variants having the same or improved activity as the reference enzyme.

82. The patent also describes various techniques, such as directed evolution for generating enzyme variants. Ex. 1001 ('055 Pat.), 20:23-21:32, 28:54-56. As

described above, these techniques were well known and allowed skilled artisans to rapidly produce enzyme variants. *See* Section V.B.2, above. With respect to the active site, the patent provides guidance from others (Müller, Elkins, Chakrabarti) about what specific changes to the active site can be made to avoid negatively affecting the functionality of the enzyme. Ex. 1001 ('055 Pat.), 20:23-25.

83. Figure 2 (replicated below) of the '055 patent shows an amino acid sequence alignment of AAD-12 and other α KG-dependent enzymes, including TfdA, AAD-1, AAD-2, and TauD. Ex. 1001 ('055 Pat.), Fig. 2. A person skilled in the art would recognize that dark highlighting identifies the positions that are either highly conserved or that are generally only conservatively substituted. As detailed above, persons skilled in the art in 2005-2006 would have recognized the value of sequence alignments and relied on this information both for predicting protein structures and functions and for choosing substitutions to make to preserve function. *See* Section V.B.2, above; *see, e.g.*, Ex. 2022 (Elkins), Fig. 5.

		10	20	30	40	50	60	70	80	
AAD-12	1	-----	MQTTLQITPTGATLGRATVIGVHEAT	-----	DDAGFAALHAAWLOHALLT	-----	FGCHLSNDQITHEARRRG	-----		
tfdA	1	-----	MSVVANPPLHPLFAAGVEDIDLREALG	-----	STEVREIERLMDEKSVLVERCOPLS	-----	QDOOTAPARNEGPLEG	-----		
AAD-2	1	-----	MTIATRQLQTHFVGQVSGDLERKPLT	-----	EGEAPVESAMDKYAVLVGHDDIT	-----	TDEQALALNFGOREDA	-----		
AAD-1	1	-----	MHAALSPLSQRFERIAVCPITGVLC	-----	AEITGVDLREPLDDSTWNEILDAF	-----	HTYQVITYECCAITNECHIASPRFG	-----		
tauD	1	-----	MSERLSITPLGPYIGAQISGADITRPL	-----	SDNQFEQLYHAWLRHGVFLPDQAIT	-----	PQQRALDQRRG	-----		
		90	100	110	120	130	140	150	160	
AAD-12	64	-----	AIERIGGGDIVAISNVKADGTVRQHS	-----	PAEWDOMMKVI VGNMAWHADSTY	-----	MPVHAOGAVFSAEVVFAVGGRTCE	-----	ADM	
tfdA	67	-----	GFIKVNQRPSRFKYAELADISNVSL	-----	LDGKVAQDAREVVGNFANQLWHS	-----	DSSEQPPAARYSMLSATVVFPSCG	-----	DTFCOM	
AAD-2	69	-----	RGGTVTKEDYRLQ-SCLNDVSNL	-----	GDKGKPLAKDSRTHLNLGNCLW	-----	HSDSSERPAPKFSLSARVVNINP	-----	CGITFCOM	
AAD-1	75	-----	PVDFVPLKSTIEGYPEVQMRREANES	-----	GR-----	-----	VIGDEWHDDSTLDAPPAAVVMRA	-----	IDVPERGGDTCELSM	
tauD	65	-----	ELHIEHVYPHABGVDEIIIVLDTH	-----	NDNPP-----	-----	DNQWHADVTETETPPAGAILAA	-----	KELPSTGGDTLWTSG	
		*****	*** *	***		*****	** *	***	*	
		170	180	190	200	210	220	230	240	
AAD-12	141	-----	RAAYDALDEATFPLVHQRSAEHS	-----	SLVYSQS-----	KLGHVQOAGSANGIGYGMTTAT	-----	PLPLVKVHEETGRPSLLIGR	-----	HAA
tfdA	147	-----	RAAYDALPRDLQSELEGIRAEHY	-----	ALNSRF-----	LLGDTDSEAQRNAMEPVMVFL	-----	VRTHAGSGRKEFLIGA	-----	HAS
AAD-2	148	-----	RAAYDALDDETHAEIEDIVCEHS	-----	ILMYSRG-----	SLGFTETDEEKQMPKPVLO	-----	RLVRTHPVHRRKSLYLSS	-----	HAG
AAD-1	144	-----	YTWETISPTMOATIEGINVVHS	-----	ATRVFGSLYQAQNRRFSENTSV	-----	KVMDVDAGDRETVE-----	SGRKLQYVNOQVYCO	-----	
tauD	132	-----	IAAYENASVPFRQLLSGIRAEH	-----	DFRKSFP-----	EYKYRKTEEHQRWREAVAKNE	-----	FLLE-----	7SGKQALFVNEGFTT	
		*****	***	***		***		***	*****	
		250	260	270	280	290	300	310		
AAD-12	216	-----	ALTEGMDAESERFLEGLVDWAC	-----	QAPPVHAHQAAAGDVVMDNRC	-----	LLHRAEPWDFKLPVMWHSRLA	-----	GRPETEGAALV	
tfdA	217	-----	HVEGLPVAEGRMLLABLL	-----	-----	PEFVTHRRNVGQIVMDNRC	-----	VLHRRRYDEISAREELRRAT	-----	FLDDAVV
AAD-2	218	-----	KIASMSVPEGRLLLRDLN	-----	-----	PEFVTVHRNKLRLVMDNRC	-----	THRRVRRYDQSQPRDVRAT	-----	VAGTETVQQQAAE
AAD-1	224	-----	RLEGMTDAESKELLQFLY	-----	-----	REDETCEVRRAKKDOVLVMDN	-----	LCVHRAVPDYAGKPELTLT	-----	RTTVCCVRRAR
tauD	209	-----	PLVDVSEKESEALLSELFA	-----	-----	KEEFQVRWRWQPNDAIWDNR	-----	VICHMANADYLEQRRINERAT	-----	ILCDREFYRAG
						*****		*****		

84. The inventors demonstrated their discovery of AAD-12 and its ability to impart resistance to several herbicides through twenty-four examples, extensive data, and three figures.

85. For instance, Example 2 discloses the preparation and analysis of a “plant-optimized” (v1) and *E. coli*-optimized (v2) AAD-12 sequences that encode the AAD-12 protein. Ex. 1001 (’055 Pat.), 32:24-41:2. Example 2 also provides the design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS enzyme having mutations that confer glyphosate tolerance. Ex. 1001 (’055 Pat.), 32:24-41:2. Tables 6 and 7 of the patent disclose codon comparisons for designing AAD-12 variants with optimal translation for expression in *E. coli* and soybean. A

person skilled in the art, reading Example 2, would understand the importance of optimizing the AAD-12 sequence for expression in plants, including cells of monocot crops (e.g., corn) and dicot crops (e.g., soybeans).

86. Example 3 discloses the preparation of expression and transformation vectors for AAD-12 (v2). Ex. 1001 ('055 Pat.), 41:4-43:20. Examples 5 and 6 report enzymatic activity of AAD-12 using high-throughput *in vitro* assays measuring phenol and chloropyridinol production by colorimetric and spectrophotometric assays. Ex. 1001 ('055 Pat.), 45:50-48:60. The specification, for example, describes a 96-well microplate format for performing enzyme assays measuring degradation activity against 2,4-D and triclopyr using incubation times of 5 to 10 minutes. Ex. 1001 ('055 Pat.), 45:50-46:45. The '055 patent reports *in vitro* testing data “show[ing] that AAD-12 (v2) is able to effectively degrade pyridyloxy-alkanoate herbicides such as triclopyr” and 2,4-D. Ex. 1001 ('055 Pat.), 47:4-48:60, Fig. 3.

87. Example 7 describes the transformation of *Arabidopsis* plants with an AAD-12 gene. Ex. 1001 ('055 Pat.), 48:62-50:33. Seeds from those transformed plants were collected, and first-generation plants grown from that seed (i.e., T₁ seed) were tested for dual herbicide-resistance activity. Ex. 1001 ('055 Pat.), 50:32-56:38. A skilled person reading Example 7 would understand that “AAD-12-transformed plants were . . . clearly protected from the triclopyr and fluroxypyr herbicide injury that was seen in the transformed non-resistant controls” Ex. 1001 ('055 Pat.),

54:13-16, 54:23-45 (Table 13). A skilled artisan would also understand that the AAD-12 (v1) gene imparted herbicide resistance to *Arabidopsis* plants against 2,4-D. Ex. 1001 ('055 Pat.), 51:35-52:38 (Table 11).

88. Example 14 reports comparative testing of AAD-12-transformed *Arabidopsis* against *Arabidopsis* transformed to express a different α KG-dependent dioxygenase enzyme (AAD-2). Ex. 1001 ('055 Pat.), 83:22-85:23. The amino acid sequence of AAD-2 is substantially different from that of AAD-12 (only 34% identity), and the two have different active site motifs. See Ex. 1001 ('055 Pat.), Fig. 2. In this study, the inventors found that AAD-12-transformed plants exhibited stronger 2,4-D resistance than AAD-2-transformed plants. Ex. 1001 ('055 Pat.), 84:49-85:23.

89. Examples 8, 10-12, 16, 17, 21, 22 report investigations of monocot and dicot plants transformed with an AAD-12 gene, including soybeans, tobacco, cotton, corn, rice, and canola. For instance, the specification describes how to prepare AAD-12 expression cassettes and transform soybean plants using (1) *Agrobacterium*-mediated transformation, (2) aerosol-beam-mediated transformation, (3) biolistic bombardment using mature seed, (4) WHISKERS-mediated transformation, and (5) particle bombardment using embryogenic callus tissue. Ex. 1001 ('055 Pat.), 72:48-79:61. In Example 11, transformed soybean plants were tested for tolerance to the herbicide 2,4-D. *Id.* The '055 patent discloses that “[a]ll AAD-12 (v1)-containing

plants were clearly resistant to the herbicide application,” while “control plants were severely injured” or killed, depending on the 2,4-D application rate. Ex. 1001 (’055 Pat.), 76:40-50 (“These data are consistent with the fact that AAD-12 (v1) can impart high tolerance (>2x field rates) to a sensitive crop like soybeans.”). The ’055 patent also describes the use of combinations of 2,4-D, triclopyr, and fluroxypyr with AAD-12-transformed dicot crops such as soybeans. Ex. 1001 (’055 Pat.), 86:23-87:20 (“One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by AAD-12 (v1) transformation.”); Ex. 1001 (’055 Pat.), 71:4-9 (“AAD-12 (v1) transformed event lines [in tobacco] displayed a high level of tolerance to 2,4-D, triclopyr and fluroxypyr”). A person skilled in the art, reading these examples, would also understand that the ’055 patent describes successful testing of AAD-12-transformed corn and rice to both classes of herbicides. Ex. 1001 (’055 Pat.), 64:28-65:53, 94:19-95:15.

90. Taken together, these examples teach a person skilled in the art that (1) plants comprising an AAD-12 gene are rendered resistant to a phenoxyacetic acid herbicide (such as 2,4-D) and/or one or more pyridyloxyacetate herbicides (such as triclopyr and fluroxypyr); and (2) a plant expressing an α KG-dioxygenase enzyme with at least 85% sequence similarity to AAD-12 and the claimed AAD-12 motif

was reasonably expected to exhibit dual herbicide resistance. *See, e.g.*, Ex. 1001 ('055 Pat.), 4:44-5:6.

VIII. The '055 Patent Enables Making and Using AAD-12 Enzymes

A. Qualities Common to the Claimed Set of AAD-12 Enzymes

91. As referenced above in Section IV, I have been informed that the '055 patent claims are enabled if there is some general quality, such as a structural feature, running through the claimed set of AAD-12 enzymes that makes them particularly suited for their claimed dual herbicidal activity.

92. The '055 patent discloses two such structural features (“at least 85% sequence identity” and an “AAD-12 motif”) common to the AAD-12 enzymes of the claims. These structural features in the '055 patent are correlated to their function of degrading the two specified classes of herbicides, phenoxy auxins (e.g., 2,4-D) and pyridyloxy auxins (e.g., triclopyr or fluroxypyr), and thus provide significant guidance for making and using AAD-12 enzyme variants. Accordingly, in my opinion, it would have required only a reasonable amount of experimentation to make and use AAD-12 enzymes across the scope of the claims.

1. At Least 85% Sequence Identity to SEQ ID NO: 2

93. The first common structure of the '055 patent claims is the requirement of “at least 85% sequence identity to SEQ ID NO: 2.” Ex. 1001 ('055 Pat.), 125:8-9. SEQ ID NO: 2 is the amino acid sequence of the SdpA enzyme from *D. acidovorans* disclosed in the '055 patent as degrading phenoxy auxin and pyridyloxy auxin

herbicides. Ex. 1001 ('055 Pat.), 5:48-52; Ex. 2013, ¶¶ [0021]-[0022]; Exs. 2014, 2017, ¶¶ [0020]-[0021]; Ex. 2015, ¶¶ [0024]-[0025]; Ex. 2016, ¶¶ [0023]-[0024]. Its plant-optimized variant, AAD-12 (v1), was reported to degrade phenoxy auxin and pyridyloxy auxin herbicides in many plant species. Ex. 1001 ('055 Pat.), Examples 7, 8, 10, 21, 22; Exs. 2013-2017, Examples 7, 8, 10, 21, 22.

94. In my opinion, a skilled artisan would have generally expected enzymes having at least 85% sequence identity to SEQ ID NO: 2 to possess the dual herbicide-degrading activity of AAD-12. My opinion is supported by the '055 patent's disclosures as well as the skilled person's background knowledge of enzymology in 2005-2006.

95. The '055 patent discloses that proteins "having the same or similar activity of the exemplified proteins," such as SEQ ID NO: 2, will typically be within a sequence identity of "at least 60%." Ex. 1001 ('055 Pat.), 21:42-50; Ex. 2013, ¶ [0093]; Exs. 2014, 2017, ¶ [00102]; Ex. 2015, ¶ [00105]; Ex. 2016, ¶ [00108]. It also specifies higher sequence identities, including 85% identity and above. Ex. 1001 ('055 Pat.), 21:52-59; Ex. 1001 ('055 Pat.), 21:42-50; Ex. 2013, ¶ [0093]; Exs. 2014, 2017, ¶ [00102]; Ex. 2015, ¶ [00105]; Ex. 2016, ¶ [00108]. Among α KG-dependent dioxygenases, the specification further discloses that enzymes having 85-98% identity are "highly identical" and that "sequences with high homology would be expected to retain similar properties." Ex. 1001 ('055 Pat.), 31:61-32:14; Ex. 2013,

¶ [00123]; Exs. 2014, 2017, ¶ [00150]; Ex. 2015, ¶ [00153]; Ex. 2016, ¶ [00156]. A skilled artisan would have understood that high levels of sequence identity between enzymes are generally correlated with having the same or similar function.

96. Indeed, the '055 patent's disclosures are consistent with the background knowledge of a skilled artisan. By 2005-2006, several research groups had independently analyzed many thousands of protein sequences to determine threshold levels of sequence identity at which common function can be reliably predicted, as well as the levels of sequence identity where enzyme function begins to differ. Ex. 2019 (Tian), 863; Ex. 2024 (Wilson), 233; Ex. 2026 (Devos), 98; Ex. 2027 (Todd), 1113; Ex. 2028 (Rost 2002), 595. These studies demonstrated that at sequence identities of about 60-70% and higher, the function of a known enzyme can be reliably transferred to (i.e., predicted for) an enzyme whose function has not yet been experimentally confirmed.

97. Tian et al., for instance, explained in 2003:

[B]ecause the percentage of sequence identity is *simple, quick and widely accepted* by the biologist, *several groups have examined the extent to which pairwise sequence identity [to] function similarity can be inferred*. Devos, Wilson, and Todd [i.e., three separate studies] conducted an all-against-all pairwise enzyme sequence comparison, concluded that *when the sequence identity is above 40%, above 90% accuracy can be*

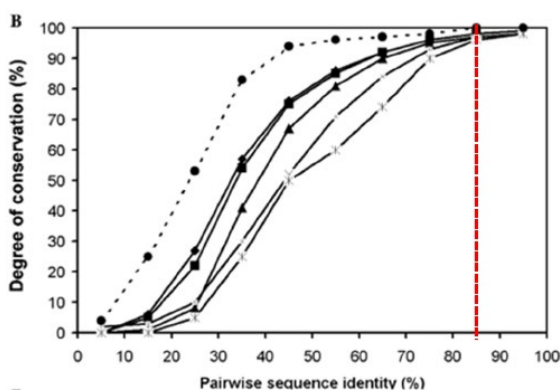
achieved when transferring the full four digits [of an] EC number.

Ex. 2019 (Tian), 872. Tian et al. reviewed and built upon prior studies by these other groups (Devos (Ex. 2026), Wilson (Ex. 2024), and Todd (Ex. 2027)). Ex. 2019 (Tian), 864. Tian's further analysis reported that "above 60% sequence identity is needed to have above 90% accuracy" for transferring all four digits of an EC number. Ex. 2019 (Tian), 876; *see* Section V.A.3, above.

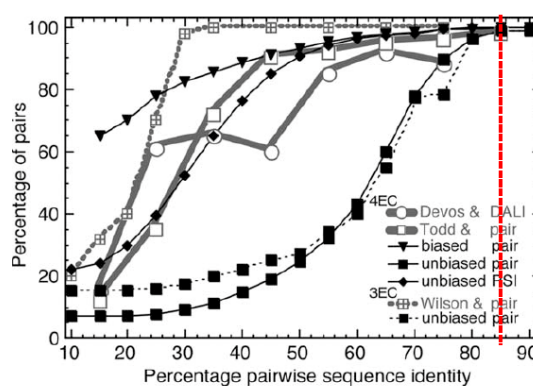
98. Another study by Rost in 2002 (also considered by Tian et al.) found that at least 70% sequence identity was required to predict enzyme function. Ex. 2028 (Rost 2002); *see* Section V.A.3, above. Rost reported estimates for sequence conservation of enzyme function that were "more cautious" than previous studies but provided "important benchmarks for everyday sequence analysis." Ex. 2028 (Rost 2002), 595; Ex. 2024, 245 ("The sequence and function similarity thresholds potentially establish minimum thresholds of sequence similarity for reliable function prediction.").

99. These studies collectively confirmed that the minimum threshold for reliably predicting enzyme function was well below the 85% sequence identity threshold recited in the '055 patent claims. This conclusion is illustrated by the figures below from Rost and Tian, to which I have added the red line annotation to indicate 85% sequence identity. Both figures show that at 85% sequence identity, the

degree of enzyme function conservation (shown on the y-axis) is well above 90% and nearing 100%. Thus, skilled artisans would have considered the “at least 85% sequence identity” feature of the ’055 patent claims as conservatively defining a narrow sequence space, in which proteins would be expected to have the same function as SEQ ID NO: 2.



Ex. 2019 (Tian), 866 (Fig. 1(B)).



Ex. 2028 (Rost 2002), 604 (Fig. 5).

100. Dr. Silverstone states that “there would be no indication or guidance as to whether an amino acid sequence with an accepted predicted tertiary structure would have any functionality at all, much less the ability to degrade both phenoxy and pyridyloxy auxin herbicides.” Ex. 1003, ¶ 87. I disagree. His statement is contradicted by the studies discussed above, which confirmed the reliability with which skilled artisans in 2005-2006 predicted protein function based on sequence. Moreover, a skilled artisan would have appreciated that the sequence-based conservation of enzyme function was related to the strong conservation of enzyme structure at high levels of sequence identity. *See, e.g.*, Ex. 2028 (Rost 2002), 596

(“In general, transfer of structure is almost guaranteed to be correct above the twilight zone, e.g., all known pairs of proteins with more than 33 residues in 100 identical have similar structure.”); Ex. 2021 (Rost 1999), 85 (“Sequence alignments unambiguously distinguish between protein pairs of similar and non-similar structure when the pairwise sequence identity is high (> 40% for long alignments).”); Ex. 2026 (Devos), 105 (“Structural similarity is conserved even at very low levels of sequence similarity.”).

101. Indeed, skilled artisans understood in 2005-2006 that an enzyme’s tertiary structure is at least as conserved as (if not more conserved than) an amino acid sequence based on the structure-and-function principles discussed above. Because enzyme structure can be quite forgiving to amino acid substitutions, skilled artisans generally based protein-function predictions on sequence identity rather than other structural comparisons. Ex. 2024 (Wilson), 238 (“Structure is always more highly conserved than sequence, so usually a given amount of structural similarity is not as significant as a corresponding amount of sequence similarity.”); Ex. 1033 (Lehninger), 141 (“protein tertiary structure is more reliably conserved than primary sequence”); Ex. 2028 (Rost 2002), 604 (“When two sequences are very similar we can safely infer that they have similar structures.”). Accordingly, persons skilled in the art would have predicted that at or above the claimed threshold of at least 85% identity to SEQ ID NO: 2, the tertiary structure of a given enzyme would

also be sufficient to maintain the function of degrading phenoxy auxin and pyridyloxy auxin herbicides.

102. Dr. Silverstone also speculates that enzymes with 85% sequence identity to SEQ ID NO: 2 might catalyze entirely different reactions. Ex. 1003, ¶ 48 (referencing desaturation, epoxidation, halogenation and other reactions that can be catalyzed by other α KG-dependent dioxygenases). He suggests that one could not predict that an enzyme within 85% sequence identity “would be an α -KG-dependent dioxygenase at all.” Ex. 1003, ¶ 50. I disagree. A skilled artisan would have understood that at least 85% sequence identity defines a narrow sequence space well within established ranges for conserving enzyme function. Ex. 2019 (Tian). Indeed, Dr. Silverstone’s speculative assertion is unsupported. He identifies no enzyme with 85% (or higher) identity to SEQ ID NO: 2 that would have a different enzymatic function or that would fall into another enzyme class. Contrary to Dr. Silverstone’s opinion, as discussed above, Tian, Rost, and other researchers demonstrated that, for enzymes having 85% or more sequence identity, a skilled artisan would have expected conserved function. Ex. 2019 (Tian), Fig. 1(b); Ex. 2028 (Rost 2002), Fig. 5. Identifying other types of enzymes within 85% sequence identity would have been considered highly unusual.

103. Moreover, by 2005-2006, persons skilled in the art used data from known sequence-activity relationships not just to predict the function of newly

discovered sequences, but also to make new protein variants. Ex. 2035 (Chaparro-Riggers), 187. For example, Minshull et al. reported that the sequences of “orthologous” proteins—i.e., related proteins from a different organism and typically having lower sequence similarity—are a “potentially rich source of information regarding sequence changes that are acceptable without drastically disrupting protein function.” Ex. 2025 (Minshull), 1-3 (citing the creation of new enzymes as a main reason to “predict an enzyme’s function from its sequence”); Ex. 2035 (Chaparro-Riggers), 185 (orthologs “can be used to identify residues that can tolerate mutation without radically altering function”). Thus, skilled artisans were using sequence comparisons in 2005-2006 to identify important residues and provide guidance about which mutations would likely be tolerated or not tolerated. Ex. 2035 (Chaparro-Riggers), 187.

104. Accordingly, a sequence identity of 85% or greater, as disclosed in the ’055 patent, was recognized to be correlated to enzyme function and used for making enzyme variants that maintain function. Skilled artisans would therefore have expected AAD-12 enzyme variants to retain their dual herbicide-degrading activity when they shared the common structural feature of having at least 85% sequence identity to SEQ ID NO: 2.

2. The AAD-12 Active Site Motif

105. The second common structure of the '055 patent claims is the AAD-12 motif, $\text{HX}_{109}\text{D}(\text{X})_{111-134}\text{T}(\text{X})_{136-261}\text{H}(\text{X})_{263-272}\text{R}$. Ex. 1001 ('055 Pat.), 125:10-18. Thus, in addition to requiring highly similar sequences (“at least 85% sequence identity to SEQ ID NO: 2”), the claims require the AAD-12 enzymes to have the same active site motif as in the dual herbicide-degrading protein sequence of SEQ ID NO: 2.

106. Dr. Silverstone states that “it is impossible to ascertain substrate specificity from presence of the general motif attributed to the family, of which the ‘AAD-12 motif’ belongs” because enzymes across the α KG-dependent dioxygenase family catalyze a wide range of reactions. Ex. 1003, ¶48. His concern is misplaced. The AAD-12 motif of the '055 patent claims is well-differentiated from the general motif characterizing the entire enzyme family. One key example of how the AAD-12 and general motifs differ is in the number of amino acids in the “spacers” (the stretches of unknown residues X) between each pair of consecutive amino acids that are specified (H, D, T, H, and R). In the AAD-12 motif, all spacers must have an exact, specified length (1, 24, 126, and 10 amino acids). In the general α KG-dependent dioxygenase motif, the spacers can have varied lengths. In the case of the last three spacers, the specified lengths of 24, 126, and 10 amino acids in the AAD-12 motif in the patent differ from the spacer lengths found in most other α KG-

dependent enzymes. Dr. Silverstone fails to identify any α KG-dependent enzyme that has all four specified spacer lengths, at least 85% identity to SEQ ID NO: 2, and catalyzes a different type of reaction. I am unaware of any such enzyme. It is irrelevant that other enzymes within the α KG-dependent enzyme family that have one or more different spacer lengths or less than 85% sequence identity can catalyze other reactions, because such enzymes do not fall within the claimed genus of AAD-12 enzymes.

107. A skilled artisan would have recognized that the AAD-12 motif differs from the corresponding sequence patterns in other α KG-dependent dioxygenases having different substrate specificities. As discussed above, by 2005-2006, prior studies by Hogan, Hotopp, and Elkins had characterized the active site motif for α KG-dependent dioxygenases generally (the “ α KG motif”). *See* Section V.C.2, above; Ex. 1019 (Hogan); Ex. 2003 (Hotopp); Ex. 2022 (Elkins). The α KG motif described in those publications, however, did not have fixed spacer lengths but rather allowed for significant variation in the spacing between the specified residues.

108. Hogan et al., for instance, characterized the variable motif for Group II α KG-dependent dioxygenases as: $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$. Ex. 1019 (Hogan), 12401. A person skilled in the art would have understood that this α KG motif requires a histidine (H), followed by any amino acid (X), followed by either an aspartic acid (D) or a glutamic acid (E), followed by a sequence (spacer) of any

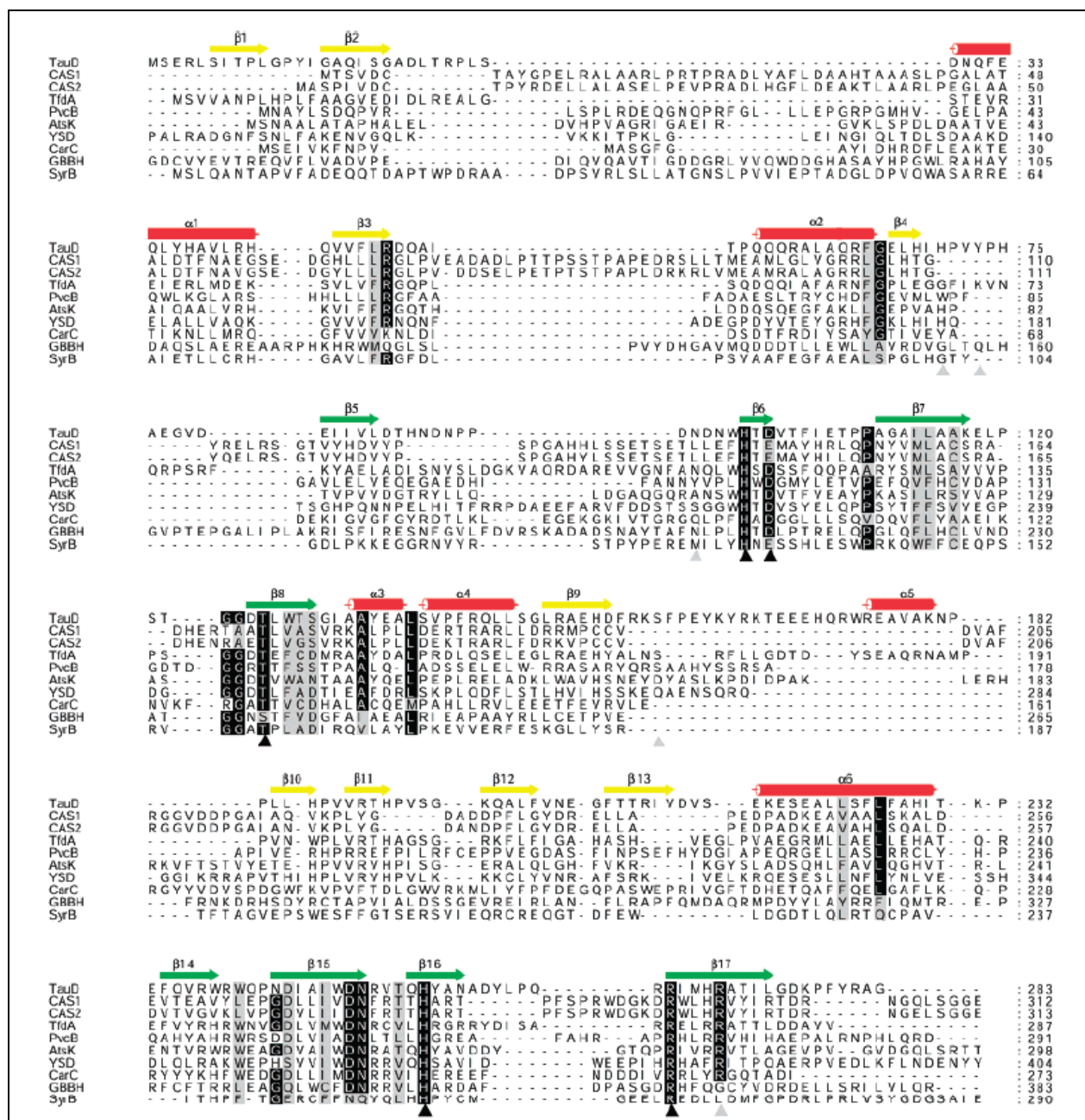
length between 23 and 26 amino acids, followed by either a threonine (T) or a serine (S), followed by a sequence (spacer) of any length between 114 and 183 amino acids, followed by a histidine (H), followed by a sequence (spacer) of any length between 10 and 13 amino acids, followed by an arginine (R).

109. Compared to the variable α KG motif, the AAD-12 motif defines specific residues and fixed spacing anchored to position 109 of SEQ ID NO: 2, as summarized below.

Group II αKG Motif Requirements (HX(D/E) ₂₃₋₂₆ (T/S) ₁₁₄₋₁₈₃ HX ₁₀₋₁₃ R)	AAD-12 Motif Requirements (HX ₁₀₉ D(X) ₁₁₁₋₁₃₄ T(X) ₁₃₆₋₂₆₁ H(X) ₂₆₃₋₂₇₂ R)
Histidine (H)	Histidine (H)
X (anywhere)	X (anchored relative to position 109 in SEQ ID NO: 2)
Aspartic acid (D) or glutamic acid (E)	Aspartic acid (D)
Anywhere from 23 to 26 variable amino acids (X ₂₃₋₂₆)	24 variable amino acids (i.e., from positions 111 to 134)
Threonine (T) or serine (S)	Threonine (T)
Anywhere from 114 to 183 variable amino acids (X ₁₁₄₋₁₈₃)	126 variable amino acids (i.e., from positions 136 to 261)
Histidine (H)	Histidine (H)
Anywhere from 10 to 13 variable amino acids (X ₁₀₋₁₃)	10 variable amino acids (i.e., from positions 263 to 272)
Arginine (R)	Arginine (R)

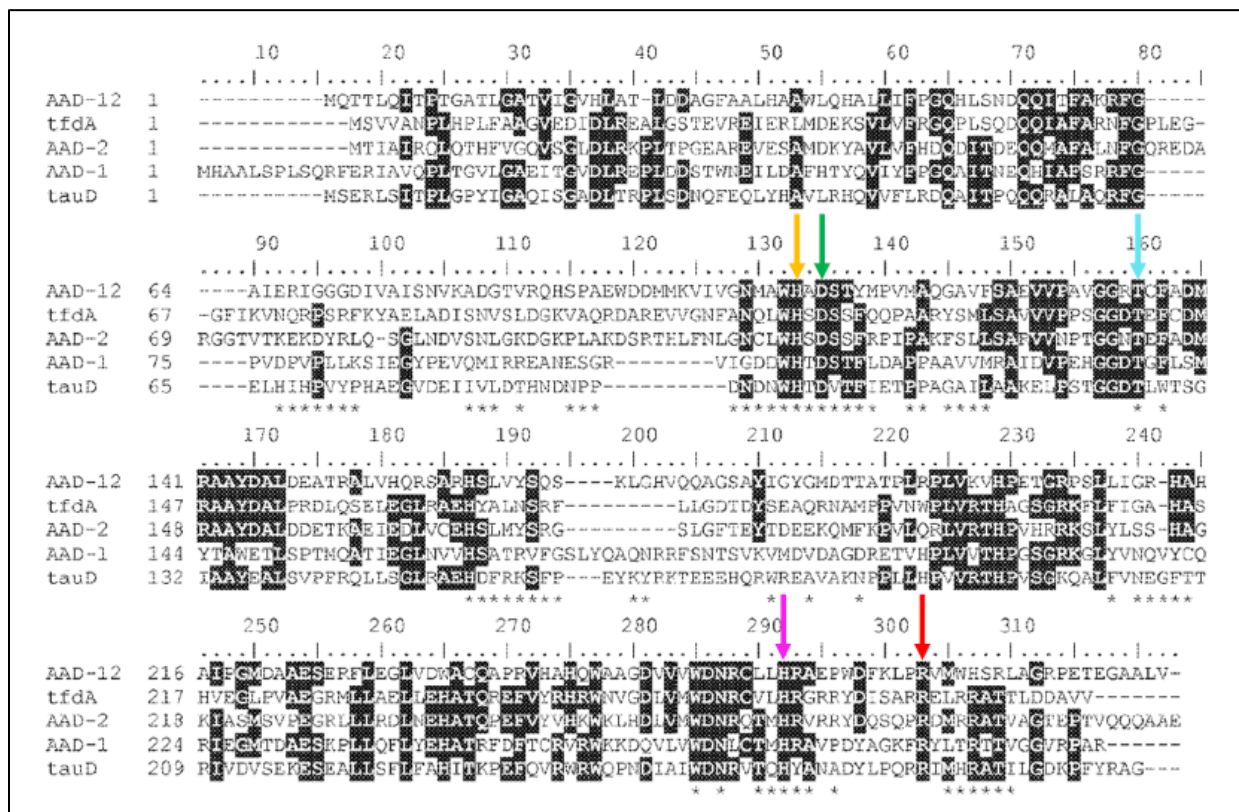
110. Persons skilled in the art also recognized that compared to other α KG-dependent dioxygenases having a close relationship to AAD-12, the AAD-12 motif and its dual activity remain distinctive. In 2002, following the work of Hogan, Elkins

et al. published the crystal structure of TauD and used it both to model the structure of TfdA and to interpret the structures of other α KG-dependent dioxygenases. Ex. 2022 (Elkins), 5185. In Figure 5 of Elkins, the authors illustrated secondary structures and conserved active site motif residues of several Group II α KG-dependent dioxygenases based on a sequence alignment to TauD.



Ex. 2022 (Elkins), 5190 (Fig. 5) (with secondary structures including α -helices colored red, β -strands of the jellyroll motif colored green, and other β -strands colored yellow).

111. Citing Elkins and its published TauD crystal structure, the '055 patent specification further compared the sequence of AAD-12 to related enzymes, including TfdA, AAD-2, AAD-1, and TauD. Ex. 1001 ('055 Pat.), Fig. 2, 20:23-34 (directing skilled artisans to Figure 2 regarding the active site); Exs. 2014-2017, Fig. 2. In the annotated figure below, colored arrows indicate where a skilled artisan would have recognized the conserved active site residues of these enzymes.



Ex. 1001 ('055 Pat.), Fig. 2 (annotations in color added).

112. The alignments between AAD-12, TfdA, AAD-2, AAD-1, and TauD, which have some gaps, confirm that none of these other Group II α KG-dependent dioxygenases have the exact, specified spacer lengths recited in the AAD-12 motif

of the '055 patent (*see* table below). Moreover, the sequence identities between AAD-12 and the other Group II α KG-dependent dioxygenases are low (~26-37%). These differences further illustrate the importance of the AAD-12 motif and having at least 85% sequence identity to SEQ ID NO: 2 for the unique dual herbicide-degrading activity of AAD-12 enzymes. Indeed, Dr. Silverstone does not assert that the other enzymes in Figure 2 exhibit degrading activity against pyridyloxy auxin herbicides, which is not a specific substrate for tfdA (phenoxy auxins), AAD-2 (phenoxy auxins), AAD-1 (phenoxy auxins and (R)-aryloxyphenoxypropionate herbicides), or TauD (taurine).

Enzyme	Active-Site Motif	% Seq Identity to AAD-12
AAD-12	HX ₁₀₉ D(X) ₁₁₁₋₁₃₄ T(X) ₁₃₆₋₂₆₁ H(X) ₂₆₃₋₂₇₂ R	100%
tfdA	HX ₁₁₅ D(X) ₁₁₇₋₁₄₀ T(X) ₁₄₂₋₂₆₂ H(X) ₂₆₄₋₂₇₃ R	37%
AAD-2	HX ₁₁₆ D(X) ₁₁₈₋₁₄₁ T(X) ₁₄₃₋₂₆₃ H(X) ₂₆₅₋₂₇₄ R	35%
AAD-1	HX ₁₁₂ D(X) ₁₁₄₋₁₃₇ T(X) ₁₃₉₋₂₆₉ H(X) ₂₇₁₋₂₈₀ R	30%
TauD	HX ₁₀₀ D(X) ₁₀₂₋₁₂₅ T(X) ₁₂₇₋₂₅₄ H(X) ₂₅₆₋₂₆₅ R	26%

113. Accordingly, as the '055 patent discloses, the conserved residues and fixed spacing of the AAD-12 motif differ from the active-site motif of other α KG-dependent dioxygenases and correlate with the claimed dual herbicide-degrading function.

B. Dr. Silverstone's *Wands* Factors Analysis Is Incorrect

114. Dr. Silverstone addresses a set of *Wands* factors from which he concludes that significantly more support would be necessary to enable the '055

patent claims. Ex. 1003, ¶¶ 56-93. I have also considered those factors and disagree with his analysis.

1. Level of Skill in the Art

115. Dr. Silverstone states that a “very high level of skill in the biochemical arts is required for the genetic modification of plants.” Ex. 1003, ¶¶ 62, 71-75. But he does not appear to address the knowledge of enzymes and capabilities that a highly skilled artisan in the field would have had in 2005-2006. *Id.* Indeed, as discussed above in Section VI, his proposed level of skill omitted any background in enzymology or, more specifically, in α KG-dependent dioxygenases.

116. In my opinion, Dr. Silverstone’s failure to account for the substantial background knowledge of a skilled artisan in the field of enzymology renders his opinions deficient and unsupported. For example, he did not take into account a skilled artisan’s understanding that multiple studies before 2005-2006 had shown that high levels of sequence identity, such as 60% (Ex. 2019 (Tian)) or a “more cautious” 70% (Ex. 2028 (Rost 2002)), reliably predicted common function between enzymes. Nor did he take into account skilled artisans’ use of such correlations in making enzyme variants. Ex. 2025 (Minshull). He also failed to address the significance of the specification’s Figure 2, which shows the active site residues of SEQ ID NO: 2 (AAD-12) and other highly conserved residues with the 3D-mapped sequence of TauD as well as other related enzymes. Ex. 1001 (’055 Pat.), 20:23-34,

Fig. 2; Exs. 2014-2017, Fig. 2. He also failed to address the known and established techniques that could be used to generate enzyme variants in 2005-2006, if desired or needed. These techniques included directed evolution, which speeds up and mimics natural protein evolution to produce large numbers of variants according to evolutionarily favored amino acid substitution principles that had been known since the 1970s. Ex. 2038 (Chica).

2. Nature of the Invention and Scope of the Claims

117. The '055 patent claims are directed to transgenic plants and plant cells having a recombinant polynucleotide that encodes an AAD-12 protein that exhibits dual herbicide-degrading activity against phenoxy auxin and pyridyloxy auxin herbicides. The AAD-12 proteins are required to have at least 85% sequence identity to SEQ ID NO: 2, which was demonstrated to have the claimed function in the specification. The AAD-12 proteins must also have the AAD-12 motif, HX₁₀₉D(X)₁₁₁₋₁₃₄T(X)₁₃₆₋₂₆₁H(X)₂₆₃₋₂₇₂R.

118. The scope of AAD-12 proteins encompassed by the '055 patent claims is narrow. The at least 85% sequence identity requirement limits the AAD-12 proteins to those falling within a closely confined sequence space at which skilled artisans would expect to maintain the same or similar function, just as the specification discloses. Ex. 1001 ('055 Pat.), 21:41-60, 32:1-6; Ex. 2013, ¶¶ [0093], [00123]; Exs. 2014, 2017, ¶¶ [00102], [00150]; Ex. 2015, ¶¶ [00105], [00153]; Ex.

2016, ¶¶ [00108], [00156]. Indeed, skilled persons in 2005-2006 recognized that high levels of sequence identity, including 85%, were predictive of common function. Ex. 2019 (Tian); Ex. 2028 (Rost 2002); Ex. 2024 (Wilson). Enzymes having at least 85% sequence identity would have been considered highly identical and would have been presumed to be essentially the same enzyme having the same function.

119. The scope of AAD-12 proteins is further narrowed by the required AAD-12 active site motif and especially the spacing between the active site motif residues. Absent this limitation, the active site residues and their spacing would be subject to modification. Instead, the AAD-12 motif requirement imposes a fixed active site requirement (anchored to position 109 of SEQ ID NO: 2), forecloses sequence modifications at those highly function-correlated residues, and requires such residues to have specific spacing requirements. Ex. 2025 (Minshull), 6 (“changes at key active site residues or other highly conserved residues are generally deleterious”).

120. Dr. Silverstone fails to evaluate the scope of AAD-12 proteins from the perspective of a skilled person with background knowledge of enzymology, who would have had experience working with far wider sequence identity ranges than at least 85% identity. Instead, his position boils down to a purely mathematical calculation without accounting for how skilled artisans understood enzyme amino

acid sequences and made variants. Ex. 1003, ¶¶ 79-80, n.1. Indeed, in 2005-2006, skilled artisans did not randomly substitute any of the 19 possible amino acids at any location when seeking variants that retain function, which is the hypothetical approach upon which his analysis is based. Such an approach assumes a low level of skill (or no skill at all), not the high level of skill that Dr. Silverstone admits a skilled person would have possessed by 2005-2006.

121. In my opinion, the AAD-12 proteins encompassed by the claims do not represent a structurally or functionally diverse genus—they represent a narrow set of well-defined enzymes that are highly identical in sequence and that maintain the same motif residues and precise spacing that comprise their active site.

3. The Specification’s Significant Guidance for Making AAD-12 Variants in View of the State of the Art

122. In my opinion, the ’055 patent provides sufficient direction, guidance, and experimental data to allow a skilled artisan to routinely make AAD-12 enzymes that retain their dual herbicide-degrading function.

123. First, the specification provides significant structural guidance to skilled artisans in making AAD-12 variants of SEQ ID NO: 2 “having the same or similar activity of the exemplified proteins.” Ex. 1001 (’055 Pat.), 21:42-52; Ex. 2013, ¶ [0093]; Exs. 2014, 2017, ¶ [00102]; Ex. 2015, ¶ [00105]; Ex. 2016, ¶ [00108]. The specification discloses that the amino acid identity of such variants “will typically be at least 60%,” including 85% or higher in identity to SEQ ID

NO: 2. Ex. 1001 ('055 Pat.), 21:41-60; Ex. 2013, ¶ [0093]; Exs. 2014, 2017, ¶ [00102]; Ex. 2015, ¶ [00105]; Ex. 2016, ¶ [00108]. These disclosures were consistent with several studies that established minimum thresholds for sequence identity-based conservation of function. Ex. 2028 (Rost 2002), 596 (despite the “variety of methods and scores explored, all groups agreed that variations of function largely occurred below levels of 50% pairwise sequence identity, and that EC classifications are almost as conserved as structure”); Ex. 2019 (Tian), 877 (“Typically, enzymes that catalyze the same reaction often show significant sequence and structural similarity.”).

124. Second, the specification provides additional structural guidance by disclosing where to identify the AAD-12 active site residues, which a skilled artisan would understand to retain when seeking to retain function. Ex. 1001 ('055 Pat.), 11:1-14, SEQ ID NO: 2, Figure 2; Exs. 2014-2017, Fig. 2. The specification also directs skilled artisans to the known TauD crystal structure, where secondary 3D structures such as conserved three-dimensional α -helices and jellyroll β -strand structures were identified. Ex. 1001 ('055 Pat.), 20:23-34; Ex. 2022 (Elkins), 5190 (Fig. 5). The specification provides an alignment of AAD-12 (SEQ ID NO: 2) to the 3D-mapped sequence of TauD as well as with other α KG-dependent dioxygenases. Skilled artisans in 2005-2006, as addressed above, were capable of avoiding disruptions to these structures and maintaining active site residues.

125. Third, Figure 2 of the '055 patent provides a comparison of the sequence of AAD-12 (SEQ ID NO: 2) to the sequences of four related α KG-dependent dioxygenases, highlighting their conserved residues using shading. Ex. 1001 ('055 Pat.), Fig. 2; Exs. 2014-2017, Fig. 2; Ex. 2025 (Minshull), 2-3. Those shaded residues are presumptively structurally and functionally important, and thus skilled artisans would typically avoid making substitutions at those positions or would make substitutions to residues of similar side-chain structure (e.g., L to I, V, L, F or M). Skilled artisans would readily identify in Figure 2 the residues that are identical across all five enzymes and generally avoid making substitutions to those strictly conserved residues. In the annotated Figure 2 below, green arrows are used to indicate the strictly conserved residues across AAD-12, tfdA, AAD-2, AAD-1, and TauD disclosed in the '055 specification. Indeed, skilled artisans understood that the sequences of related proteins are often a “rich source of information regarding sequence changes that are acceptable without drastically disrupting protein function.” Ex. 2025 (Minshull), 2-3.

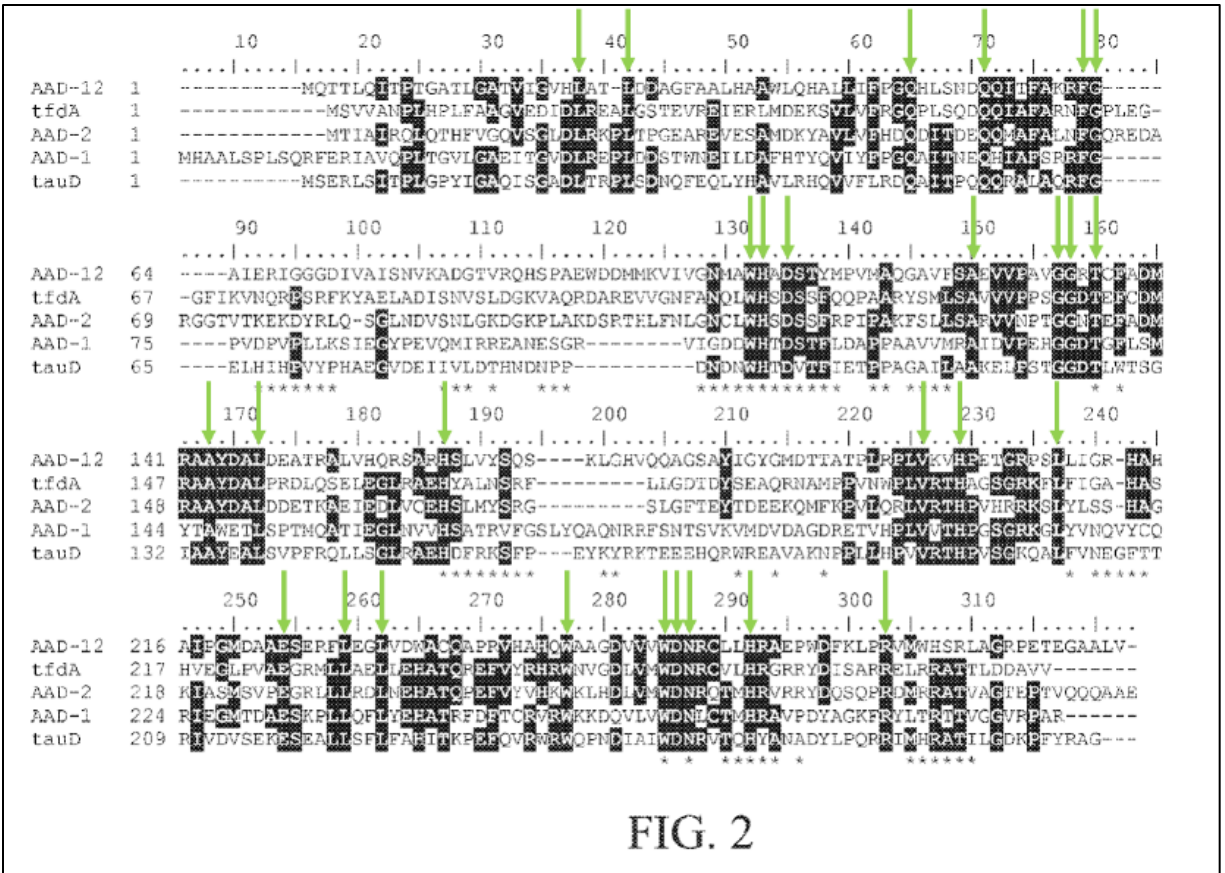


FIG. 2

Ex. 1001 ('055 Pat.), Fig. 2 (annotations in color added).

126. A skilled artisan would also understand from Figure 2 that there are many shaded residues (marked in red below) that are not strictly conserved across the sequences of all five enzymes. These residues are somewhat conserved but have been shown to tolerate conservative changes to similar amino acids as shown in the sequence alignment (e.g., L to I or V; S to G; etc.), providing guidance to skilled artisans that these residues may be preferentially left unsubstituted or conservatively substituted.

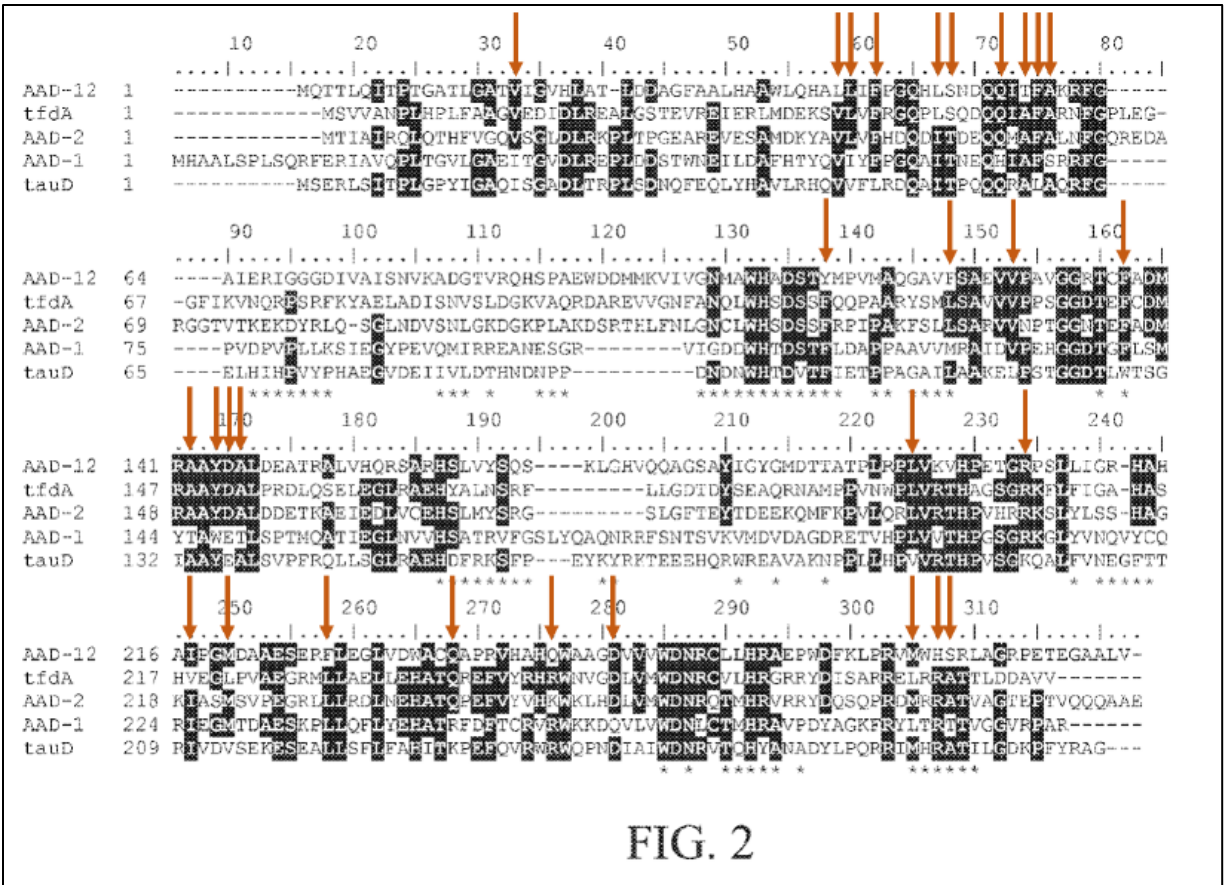


FIG. 2

Ex. 1001 ('055 Pat.), Fig. 2 (annotations in color added).

127. A skilled artisan would further understand from Figure 2 that the unshaded residues would likely tolerate a broader range of amino acid substitutions, particularly when overall sequence identity is kept at 85% or higher. For example, as shown among the unshaded residues in Figure 2, position 51—H, E, L or Y (different charge and L is non-polar and hydrophobic); position 112—R, D, E or H (different charge); position 215—M, R, K, D or V (hydrophobic, positive charge, positive charge, negative charge, hydrophobic).

128. Fourth, the '055 patent specification provides further guidance that was consistent with recognized principles for making enzyme variants in 2005-2006. For instance, the specification discloses that variant enzymes may “differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like.” Ex. 1001 ('055 Pat.), 28:57-60. This guidance comports with a skilled artisan’s understanding that positively charged amino acids (i.e., K, R) and negatively charged amino acids (i.e., D, E) generally should not be exchanged at positions with a degree of conservation in sequence alignments of related proteins. Similarly, a skilled artisan seeking to retain an enzyme’s overall three-dimensional structure and function would aim to not dramatically change steric bulk by an amino acid substitution, as in by changing a smaller amino acid (i.e., A, S) to a significantly larger one (i.e., W, Q) or vice versa (i.e., L to G or A). The '055 patent also explains that “[c]onservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule.” Ex. 1001 ('055 Pat.), 22:11-24; Ex. 2013, ¶ [0095]; Exs. 2014, 2017, ¶ [00104]; Ex. 2015, ¶ [00107]; Ex. 2016, ¶ [00110]. Conservative substitutions are those made within groups of amino acids that have similar properties and can often be interchanged without significant detriment to structure or function. *See* Ex. 2055 (EP 1,073,676), Table 1. A skilled

artisan would have recognized these categories of similarly charged and similarly sized amino acids by 2005-2006.

TABLE 1: Overview of conservative amino acid substitutions.

Amino acid	Conservative substitution by
Ser (S)	Thr, Gly, Asn
Arg (R)	His, Lys, Glu, Gln
Leu (L)	Ile, Met, Phe, Val, Tyr
Pro (P)	Ala, Thr, Gly
Thr (T)	Pro, Ser, Ala, Gly, His, Gln
Ala (A)	Pro, Gly, Thr
Val (V)	Met, Ile, Tyr, Phe, Leu
Gly (G)	Ala, Thr, Pro, Ser
Ile (I)	Met, Leu, Phe, Val, Tyr
Phe (F)	Met, Tyr, Ile, Leu, Trp, Val
Tyr (Y)	Phe, Trp, Met, Ile, Val, Leu
Cys (C)	Ser, Thr, Met
His (H)	Gln, Arg, Lys, Glu, Thr
Gln (Q)	Glu, His, Lys, Asn, Thr, Arg
Asn (N)	Asp, Ser, Gln
Lys (K)	Arg, Glu, Gln, His
Asp (D)	Asn, Glu, Gln
Glu (E)	Gln, Asp, Lys, Asn, His, Arg
Met (M)	Ile, Leu, Phe, Val

Ex. 2055 (EP 1,073,676), Table 1.

129. To make variants, the specification directs skilled artisans to known and established techniques such as directed evolution. Ex. 1001 ('055 Pat.), 28:54-29:10, 29:43-30:9. By 2005-2006, these techniques were, in the hands of skilled artisans, capable of mimicking natural protein evolution in a high-throughput manner to make variants having evolutionarily favored changes in sequence that did not disrupt and

could even improve the protein's function. The specification also discloses exemplary assays (e.g., available since at least 1993) that skilled artisans would have been able to use to efficiently confirm the activity of variants, including colorimetric and spectrophotometric assays that were established in the art and could be implemented in a high-throughput manner. Ex. 1001 ('055 Pat.), 45:50-48:59 (disclosing a modified protocol from a 1993 publication deployed in a 96-well microplate format); Exs. 2013-2017, Examples 5, 6.

130. Even Petitioner's declarant, Dr. Silverstone, and his colleagues have relied on high-throughput techniques for generating improved enzyme variants, including synthetic ligation reassembly (SLR) and optimized directed evolution. *See, e.g.*, Ex. 2008 (US 8,343,747), 70:58-75:21. With respect to SLR, they explain that it allows researchers to generate libraries of over 10^{100} to 10^{1000} different progeny chimeras that can "be examined systemically in smaller groups." Ex. 2008 (US 8,343,747), 71:18-72:36. Dr. Silverstone fails to address any of these methods that were routinely used to generate and test enzyme variants.

131. Accordingly, the '055 patent provides significant direction and structural guidance for making AAD-12 variants with 85% or greater sequence identity to SEQ ID NO: 2 that skilled artisans would expect to retain the dual herbicide-degrading activity.

4. The Quantity of Experimentation Necessary Was Not Undue

132. In my opinion, in 2005-2006, the amount of experimentation required to make and use the full scope of AAD-12 proteins would have been reasonable, not undue. Dr. Silverstone fails to account for the guidance provided in the '055 patent, how skilled artisans made enzyme variants in 2005-2006, and how quickly and routinely enzyme variants were made and evaluated for function.

133. The '055 patent directs skilled artisans to a narrow sequence space for making AAD-12 enzymes. As Example 1 discloses, the inventors already completed the work of investigating a broad range of α KG-dependent dioxygenase sequences. Ex. 1001 ('055 Pat.), 31:55-32:22; Ex. 2013, ¶¶ [00123]-[00124]; Exs. 2014, 2017, ¶¶ [00150]-[00151]; Ex. 2015, ¶¶ [00153]-[00154]; Ex. 2016, ¶¶ [00156]-[00157]. Starting with the sequence of TfdA, the inventors found other enzyme sequences ranging from “highly identical (85-98%) to very low identity (23-32%).” Ex. 1001 ('055 Pat.), 32:1-6; Ex. 2013, ¶ [00123]; Exs. 2014, 2017, ¶ [00150]; Ex. 2015, ¶ [00153]; Ex. 2016, ¶ [00156]. In other words, their investigation extended down into what skilled artisans referred to as the “twilight zone” of sequence identity (e.g., approximately 20-35%), where sequence-function relationships are less predictable. Ex. 2021 (Rost 1999), 85.

134. Among those lower identity sequences, the inventors identified SdpA (AAD-12), reported its unique dual herbicide-degrading activity, and directed

skilled artisans to make variants having the same or similar activity at high levels of sequence identity that were at or above established thresholds for strong sequence-function conservation (e.g., typically at least 60%, including 85% and higher identity). Ex. 1001 ('055 Pat.), 32:1-22, 12:23-58, 21:41-60, Examples 5-8, 10, 21, 22; Ex. 2013, ¶¶ [0093], [00123]; Exs. 2014, 2017, ¶¶ [00102], [00150]; Ex. 2015, ¶¶ [00105], [00153]; Ex. 2016, ¶¶ [00108], [00156]; Ex. 2019 (Tian); Ex. 2028 (Rost 2002). Accordingly, there was no need for skilled artisans to investigate unpredictable ranges of sequence identity to SEQ ID NO: 2 in search of enzymes having the dual herbicide-degrading activity. Instead, the inventors had already completed that work and the '055 patent guides skilled artisans directly to a narrow range of sequences where conservation of function is expected.

135. Dr. Silverstone fails to acknowledge how skilled artisans made enzyme variants in 2005-2006. Researchers often sought to make proteins having targeted improvements in certain properties, such as increased thermostability or a higher catalytic rate. Ex. 2038 (Chica), 378. Those efforts often involved investigating lower ranges of sequence identity (e.g., as in Example 1) or modifying active site residues in search of larger, unpredictable differences in properties. Skilled artisans would then focus their attention solely on the top-performing proteins, colloquially referred to as “winners” in the field of enzymology and protein engineering. Here, by contrast, a skilled artisan seeking to make AAD-12 enzymes across the scope of

the claims would face a simpler task. All variants exhibiting any level of the dual herbicide-degrading activity would broadly be “winners” because they would fall within the scope of the claims. Ex. 2035 (Chaparro-Riggers), 185 (discussing use of sequence comparisons to identify residues “that can tolerate mutation without radically affecting function”). A skilled artisan would only need to avoid total loss of activity. The ’055 patent significantly guards against loss of activity by precluding substitution of the AAD-12 active site motif residues, which a skilled artisan would expect to be deleterious to function. Ex. 2025 (Minshull), 6. In addition, a skilled artisan would be guided by the ’055 patent’s sequence alignments to avoid deleterious changes to other strictly conserved residues. Ex. 1001, Fig. 2; Exs. 2014-2017, Fig. 2.

136. I thus disagree with Dr. Silverstone’s position, for example, that making AAD-12 enzymes would require carrying out “the entire inventive process” anew. Ex. 1003, ¶ 83. Guided by the ’055 patent’s disclosures to a narrow, presumptively function-conserved sequence space (at least 85% identity) with a required active site motif, skilled artisans would have been able to routinely make AAD-12 genes and enzymes across the full scope of the claims.

137. Indeed, skilled artisans had established principles to follow in making functional protein variants. Evolutionarily favored amino acid substitutions had been known since the 1970s, which were originally published in the form of matrices

cataloguing probabilities that one amino acid will be substituted for another amino acid over time. For example, W is the residue least likely to be substituted, as its side chain has a unique shape. It has the highest diagonal probability score in all the substitution scoring matrices (meaning most likely to be substituted by itself, i.e., conserved) and lowest total of off-diagonal elements (meaning least combined probability of being substituted by one of the 19 other amino acids over evolutionary distance). Residues such as A, S and T are more tolerant to mutual substitutions, as are V, I, and L. By 2005-2006, rational design and directed evolution techniques were incorporating these principles into largely automated practice to rapidly make variants across ranges of sequence that retained function and even improved an enzyme's properties. Ex. 2038 (Chica); Ex. 2039 (Fox 2007).

138. Skilled artisans often further restricted the extent, types, and locations of mutations to further ensure retention of function. These principles included:

- a. Staying at high sequence identity in view of the known sequence-function correlations for enzymes. Ex. 2019 (Tian); Ex. 2028 (Rost 2002); Ex. 2025 (Minshull).
- b. Maintaining the active site motif residues. Ex. 2025 (Minshull), 6 (“changes at key active site residues or other highly conserved residues are generally deleterious”).

- c. Retaining or only cautiously modifying residues that are conserved across related enzymes. Ex. 2025 (Minshull), 6.
- d. Retaining overall surface charge (i.e., avoiding changing a charged amino acid to an oppositely charged amino acid) and steric bulk in the sequence. Ex. 1001 ('055 Pat.), 28:57-60; Ex. 2035 (Chaparro-Riggers), 186 (recommending restricting mutation diversity where small amino acids can create additional space in a binding pocket or large amino acids can narrow the binding pocket).

139. The first two of these principles would have significantly guided a skilled artisan to stay within a highly function-correlated sequence space and to maintain the function-correlated active site residues, both of which are required by the '055 patent claims. Figure 2 of the '055 patent also discloses the highly conserved residues across enzymes related to AAD-12 (SEQ ID NO: 2), which a skilled artisan would generally either not mutate (highly conserved residues) or mutate cautiously (conservative changes) when seeking to maintain activity. Ex. 2025 (Minshull), 6. This figure also discloses where highly variable residues are located (residues with no shading), which a skilled artisan would expect to mutate more freely. The '055 patent's disclosures to maintain surface charge distribution and overall structure also comport with a skilled artisan's understanding to avoid

dramatic changes in surface charge and steric bulk within a sequence. Ex. 1001 ('055 Pat.), 28:57-60. As I discussed above in Section V.B.2, skilled artisans also understood that these protein engineering principles may be implemented in automated directed evolution techniques and other computational techniques, requiring relatively little hands-on input.

140. A skilled artisan would thus understand that following the structural guidance in the '055 patent and known principles and techniques for making functional variants would significantly narrow the amount of experimentation necessary. As explained above, Dr. Silverstone's enablement analysis fails to account for a skilled person's background knowledge of enzymology and protein engineering principles. This is a critical deficiency because the purely mathematical 10^{106} sequence calculation he repeatedly relies upon incorrectly assumes that a skilled artisan would use no skill and randomly use each of the 19 possible substitutions at each possible residue location other than the 5 specified AAD-12 motif residues. Ex. 1003, ¶¶ 79-80, n. 1, ¶ 96. His false assumption is entirely at odds with how skilled persons made enzyme variants in 2005-2006.

141. I also disagree with Dr. Silverstone's position that "there is inadequate resolution necessary to determine the precise configuration of the active site, which is necessary to predict activity...." Ex. 1003, ¶ 88. Skilled artisans predicted activity much more easily by staying at high sequence identity and retaining active-site motif

residues, as discussed above. While crystal structures or 3D coordinates could be useful for making enzyme variants, they were far from necessary. *See, e.g.*, Ex. 2035 (Chapparo-Riggers), 180 (“Crystal structures of the protein of interest (with or without the substrate bound) are, of course, useful, but data-driven protein engineering is not limited to such cases.”). Skilled artisans routinely relied on amino acid sequence information and recognized principles to successfully make protein variants, particularly within high levels of sequence identity. Ex. 2035 (Chaparro-Riggers), 180 (“Besides crystal structures, a wide variety of tools can be applied,” including “sequence alignments”).

142. I thus disagree with Dr. Silverstone’s opinions regarding the making of enzyme variants, which have no citation support. Ex. 1003, ¶¶ 79-88. As illustrated above, established principles and techniques for making enzyme variants in 2005-2006 did not depend on “spatial coordinates,” “predicted secondary and predicted tertiary structures,” or mapping all potential residues within an active site. Ex. 1003, ¶ 79, 83-84; Ex. 2024 (Wilson), 238 (“Structure is always more highly conserved than sequence, so usually a given amount of structural similarity is not as significant as a corresponding amount of sequence similarity.”). Nor did they require predicting in advance which variants would be necessarily better, equivalent, or worse—particularly when all AAD-12 variants that did not have a total loss of activity would be within the scope of the claims, provided they retained a minimum of 85% identity

to SEQ ID NO: 2 and the AAD-12 motif. Ex. 1003, ¶¶ 81-82, 85. Nor did making enzyme variants require 3D-modeling. Ex. 1003, ¶¶ 85, 87-88. Dr. Silverstone thus bases his opinions on steps and techniques that a skilled artisan would have understood were not required. Instead, he appears to impose an inappropriate commercial development-type standard. Ex. 1003, ¶ 93 (demanding “commercially-relevant” performance), ¶ 86 (“guarantee of success”), ¶ 91 (“guarantee performance”).

143. I also disagree with Dr. Silverstone’s opinions regarding protein expression and *in vitro* testing. Ex. 1003, ¶¶ 79-91. The ’055 patent discloses detailed examples of expression in both *E. coli* and *P. fluorescens*, as well as *in vitro* activity testing of AAD-12 on various substrates. Ex. 1001 (’055 Pat.), Examples 3-6; Exs. 2013-2017, Examples 3-6. A skilled artisan would have recognized that these examples provide significant guidance for designing and testing AAD-12 variants.

144. Performing *in vitro* assays also was not undue in 2005-2006. Examples 5 and 6 of the ’055 patent disclose exemplary colorimetric and spectrophotometric assays, with the colorimetric assay having been available since 1993, to measure the herbicide-degrading activating of AAD-12. These are rapid assays, requiring only 5- to 10-minute incubation times. Ex. 1001 (’055 Pat.), 45:50-48:62; Exs. 2013-2017, Examples 5, 6. Skilled artisans would have understood that these standard

assays were conducted in high-throughput micro-well plates for measuring enzymatic activity based on detection of a colorimetric or spectrophotometric marker. By 2005-2006, these types of assays were further automated and could be run with little or no hands-on intervention. Accordingly, these assays, as well as other computational screening techniques, were readily available and widely used in the field to limit the amount of experimentation. *See* Ex. 2038 (Chica), 378; Ex. 2039 (Fox), 338; Ex. 2008 (US 8,343,747), 71:18-72:36.

145. Accordingly, in my opinion, it would have taken only a reasonable amount of experimentation by skilled artisans in 2005-2006 to make and use AAD-12 genes and enzymes across the full scope of the claims. Those genes would then be readily used for plant transformation, which I understand another witness will address in this proceeding.

IX. The '055 Patent Specification Adequately Describes AAD-12 Enzymes

146. As stated in Section IV, I have been informed that the disclosure of the '055 patent (and its priority applications) provides written description support for the claims if the specification describes the claimed subject matter, such as by common structural features correlated to function or representative species (e.g., enzymes), so that a skilled artisan could recognize the claimed set of AAD-12 enzymes having the dual herbicidal activity.

147. Here, the '055 patent describes two common structural features (at least 85% sequence identity and an AAD-12 motif) that a skilled artisan would have recognized are correlated to the AAD-12 enzyme's dual herbicide function. The patent also discloses two exemplary AAD-12 enzymes that are representative of the AAD-12 enzymes of the claims, which are not structurally or functionally diverse. Accordingly, in my opinion, the specification provides sufficient written description support for the claimed set of AAD-12 enzymes.

A. The '055 Patent Describes AAD-12 Enzymes

1. The Disclosed Structural Features of at Least 85% Sequence Identity and the AAD-12 Motif Are Correlated to the Dual Herbicide-Degrading Function

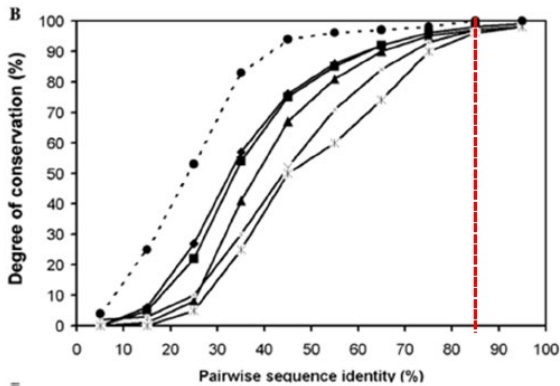
148. In my opinion, the '055 patent discloses two structural features of AAD-12 proteins that are correlated to function: (1) at least 85% sequence identity with SEQ ID NO: 2 and (2) the AAD-12 motif.

149. The patent states that enzymes having the “same or similar activity” (e.g., as compared to AAD-12) typically have at least 60% sequence identity, including 85% or higher identity. Ex. 1001, 21:41-60, 32:1-6; Ex. 2013, ¶¶ [0093], [00123]; Exs. 2014, 2017, ¶¶ [00102], [00150]; Ex. 2015, ¶¶ [00105], [00153]; Ex. 2016, ¶¶ [00108], [00156]. The '055 patent also correctly states that among α KG-dependent dioxygenases like AAD-12 (SdpA), sequence identities of 85% and higher are “highly identical” and expected to have similar properties (i.e., sequence-

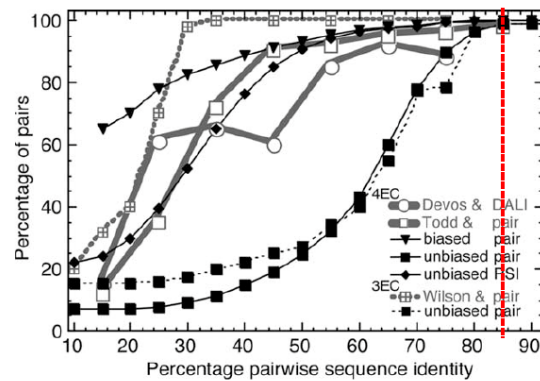
function correlation). Ex. 1001 ('055 Pat.), 31:61-32:6, 21:41-60; Ex. 2013, ¶¶ [0093], [00123]; Exs. 2014, 2017, ¶¶ [00102], [00150]; Ex. 2015, ¶¶ [00105], [00153]; Ex. 2016, ¶¶ [00108], [00156].

150. These disclosures in the specification are consistent with the understanding in the art as of 2005-2006 that sequence identities above 40-60% are predictive of common enzymatic function. *See* Section V.A, above. As summarized in the annotated figures below, several research groups consistently found that high levels of sequence identity correlate to enzymatic function.

151. For instance, Rost reported, using a “cautious” approach, that at least 70% identity was required to predict protein function. Ex. 2028 (Rost 2002), 596; Ex. 2024 (Wilson), 245; Section V.A.3, above. Tian et al. then reported in 2003 that only “60% sequence identity is needed to have at least 90% accuracy” in predicting common function and substrate specificity, defined as “all four digits of an EC number.” Ex. 2019 (Tian), 865. The '055 patent's claimed threshold of at least 85% sequence identity thus significantly exceeded the thresholds reported in the art for inferring an enzyme's function from its level of sequence identity to a known enzyme.



Ex. 2019 (Tian), 866 (Fig. 1(B)).



Ex. 2028 (Rost 2002), 604 (Fig. 5).

152. A skilled person in 2005-2006 therefore would have considered the '055 patent's claimed 85% sequence identity threshold as "highly identical" among α KG-dependent dioxygenases and as conferring similar properties to a reference sequence (e.g., SEQ ID NO: 2). Ex. 1001 ('055 Pat.), 32:3-6 ("highly identical (85-98%)"); Ex. 2013, ¶ [00123]; Exs. 2014, 2017, ¶ [00150]; Ex. 2015, ¶ [00153]; Ex. 2016, ¶ [00156]. Indeed, the claimed minimum of 85% sequence identity comports with the specification's disclosure to stay above 60% for sequence identity-based conservation of function. Ex. 1001 ('055 Pat.), 21:49-50 ("amino acid identify will typically be at least 60%"), 21:41-60; Ex. 2013, ¶ [0093]; Exs. 2014, 2017, ¶ [00102]; Ex. 2015, ¶ [00105]; Ex. 2016, ¶ [00108]; Ex. 2028 (Rost 2002), 596; Ex. 2019 (Tian), 877; Ex. 2008 (US 8,343,747), 48:55-66 (Dr. Silverstone's patent stating that "substantially identical" for two or more sequences refers to at least about 50%, 51%, and up to 99% or more sequence identity). Accordingly, a skilled artisan

would have viewed an enzyme that was at least 85% identical to AAD-12 as essentially the same enzyme with the same enzymatic function.

153. In my opinion, in view of the '055 patent disclosures and background knowledge discussed above, a skilled artisan would not have needed to see specific examples of AAD-12 proteins at or near 85% identity to SEQ ID NO: 2 to recognize that enzymes having 85% or more sequence identity would exhibit the dual herbicide-degrading activity of AAD-12 (SEQ ID NO: 2). Instead, skilled artisans would have understood that variants of AAD-12 with at least 85% sequence identity were reasonably expected to maintain the claimed function.

154. The '055 patent also describes the AAD-12 active site motif, which correlates to the enzyme's dual herbicide-degrading activity. As detailed above, the claimed motif is specific to AAD-12 and defines specific residues and fixed spacing of the active site residues, anchored to position 109 of SEQ ID NO: 2. The location and spacing of the motif residues differ from those in other α KG-dependent dioxygenases having different substrate specificities. *See* Section VIII.A.2, above. A skilled artisan would have understood that the conserved residues and fixed spacing of the AAD-12 motif represent the active site and correlate to the claimed dual activity.

155. For instance, the AAD-12 motif differs from other α KG-dependent oxygenase motifs by the number of amino acids in the spacers. In this case, the

specified lengths of 24, 126, and 10 in the last three spacers in the AAD-12 motif differ from the spacer lengths found in most other α KG-dependent enzymes. *See* Section VIII.A.2, above. A skilled artisan would understand that an AAD-12 enzyme that has all four specified spacer lengths and motif residues, and at least 85% identity to SEQ ID NO: 2, would be expected to catalyze the same type of reaction as AAD-12 (SEQ ID NO: 2) because the active site motif determines the correct fit and binding for its particular substrate.

156. By 2005-2006, skilled artisans understood that *active site* residues are, as their name reflects, correlated to an enzyme's *activity*. Indeed, it had been recognized that "changes at key active site residues . . . are generally deleterious" to enzyme function. Ex. 2025 (Minshull), 6. A skilled artisan would therefore have understood the importance of the claimed AAD-12 motif for conferring its herbicide-degrading activity. *See* Section VIII.A.2, above.

157. Dr. Silverstone asserts that "the final three-dimensional structure of a protein, which is critical for its function, is determined by a variety of factors that cannot be predicted, based on our current level of understanding, solely from the primary sequence of amino acids." Ex. 1003, ¶ 42. I disagree. First, Dr. Silverstone fails to account for the state of the art as of 2005-2006, which taught that three-dimensional structure is highly conserved at 85% or more sequence identity. *See* Section V.A.3, above; Ex. 2028 (Rost 2002), 604 ("When two sequences are very

similar we can safely infer that they have similar structures.”); Ex. 2028 (Rost 2002), 596 (“In general, transfer of structure is almost guaranteed to be correct above the twilight zone, e.g., all known pairs of proteins with more than 33 residues in 100 identical have similar structure.”); Ex. 2021 (Rost 1999), 85 (“Sequence alignments unambiguously distinguish between protein pairs of similar and non-similar structure when the pairwise sequence identity is high (> 40% for long alignments).”); Ex. 2026 (Devos), 98 (“The comparison of protein sequences is widely used to infer protein structure and function.”). Based on these principles, while not guaranteed in every case, a skilled artisan would have expected that AAD-12 enzymes at 85% and higher sequence identity would exhibit similar three-dimensional structures based on their high levels of sequence identity and specific motif residues.

158. Second, Dr. Silverstone’s requirement for a three-dimensional structure was not necessary in 2005-2006 for predicting enzyme function. Instead, skilled artisans recognized that there were alternatives to using crystal structures for making enzyme variants. Ex. 2035 (Chaparro-Riggers), 180 (“Besides crystal structures, a wide variety of tools can be applied, ranging from sequence alignments or phylogenetic trees to computational models, and small, experimental libraries used to ‘scout’ for interesting residues.”). While crystal structures were useful, the art recognized that “protein engineering is not limited to such cases.” Ex. 2035 (Chaparro-Riggers), 180.

159. As Dr. Silverstone acknowledges, one established protein engineering technique was to “compare the sequence to a closely related protein of known structure.” Ex. 1003, ¶ 46. This approach was widely used in the art in 2005-2006. For example, Elkins et al. modeled the structure of TfdA in 2002 based on the known crystal structure of another α KG-dependent dioxygenase, TauD. Citing Elkins, the ’055 patent discloses a sequence alignment between AAD-12 and three-dimensionally characterized TauD, as well as tfdA, AAD-1, and AAD-2. Ex. 1001 (’055 Pat.), Figure 2, 20:23-34; Exs. 2014-2017, Fig. 2. Accordingly, the ’055 patent provides comparisons of AAD-12 to a previously 3D-mapped α KG-dependent dioxygenase, consistent with Dr. Silverstone’s demands.

160. I also disagree with Dr. Silverstone’s hypothetical concerns regarding potential protein misfolding. Ex. 1003, ¶ 47. By 2005-2006, researchers followed established principles (discussed above) for making protein variants to reduce issues such as protein misfolding or total loss of activity. *See* Section V.B.2, above; Ex. 2025 (Minshull), 6 (“changes at key active site residues or other highly conserved residues are generally deleterious”). Dr. Silverstone fails to address the knowledge and capabilities that a skilled artisan would have applied when making AAD-12 enzymes within the scope of the claims.

161. Dr. Silverstone also repeatedly relies on a generalized biochemistry textbook for his misfolding arguments. *E.g.*, Ex. 1003, ¶¶ 42-46, 49. But the cited

textbook does not support his positions. For instance, Dr. Silverstone states that “[a]lthough there is flexibility in the amino acids between the histidine residues in the motif, there is still an ultimate three-dimensional arrangement which may not be predicted by a given primary amino acid sequence.” Ex. 1003, ¶ 49 (citing pp. 120-44 of Ex. 1033). Nothing in the more than 20 pages that he cites, however, supports his proposition. If anything, the textbook is consistent with the general conservation of structure at high sequence identities (tertiary structure more conserved). Ex. 1033 (Lehninger), 141 (“protein tertiary structure is more reliably conserved than primary sequence”). Where a skilled artisan sought to conserve (not destroy) enzyme function and structure, there were known scientific principles for how to accomplish that. Dr. Silverstone, however, fails to address those guiding principles for making functional enzyme variants.

162. Accordingly, in my opinion, the structural AAD-12 motif and 85% sequence identity features of the claims (individually and collectively) are sufficiently correlated to the claimed function and provide adequate written description support for the claims of the '055 patent.

2. The Disclosed AAD-12 Enzymes Are Representative of the AAD-12 Enzymes of the Claims

163. I have been informed that species are representative when they reflect the structural and functional diversity of the genus in the claims. In my opinion, the genus (set) of AAD-12 enzymes is neither structurally nor functionally diverse, and

the AAD-12 enzymes disclosed in the '055 patent are amply representative of the genus.

164. The two exemplary AAD-12 enzymes described in the '055 patent are representative of the claimed AAD-12 enzymes. The claims are narrowly tailored to AAD-12 enzymes having at least 85% sequence identity to SEQ ID NO: 2 and containing the AAD-12 motif, resulting in little variation in the structural features of the AAD-12 enzymes. As detailed above, those structural features are also strongly correlated with the dual herbicide-degrading function. Accordingly, a person skilled in the art would have understood that the disclosed AAD-12 enzymes are sufficiently representative of the claimed set of AAD-12 enzymes.

165. A skilled artisan would consider the claimed set of AAD-12 enzymes as essentially the same enzyme with the same function based on their high sequence similarity (85% or more) to SEQ ID NO: 2 and conserved AAD-12 motif. With respect to high sequence identity, the '055 patent accurately describes enzymes having 85% or higher sequence identity as “highly identical” and as “expected to retain similar properties.” Ex. 1001 ('055 Pat.), 32:3-6; Ex. 2013, ¶ [00123]; Exs. 2014, 2017, ¶ [00150]; Ex. 2015, ¶ [00153]; Ex. 2016, ¶ [00156]. This was consistent with a skilled artisan’s understanding in 2005-2006 that enzyme function and structure are strongly correlated at 85% and higher sequence identity. *See* Section V.A.3, above; Ex. 2019 (Tian), 863 (“several studies suggested that when the

sequence identity is above 40%, enzyme function is well-conserved”); Ex. 2028 (Rost 2002), 604 (“When two sequences are very similar we can safely infer that they have similar structures.”); Ex. 2021 (Rost 1999).

166. Also, as summarized above (Section VIII), a skilled artisan would have known how to generate AAD-12 variants within the scope of the claims that would have reasonably been expected to possess the claimed activity. In addition to staying at high sequence identities (above 85%) and including an AAD-12 motif as the claims require, a skilled artisan would know to follow established principles for making AAD-12 variants, including retaining or cautiously modifying highly conserved residues and retaining overall surface charge. *See* Section V.B.2, above. Following these recognized protein engineering principles, a skilled artisan would have reasonably expected that any AAD-12 enzyme within the claimed set would exhibit the claimed dual herbicide activity.

167. I disagree with Dr. Silverstone’s assertions that enzymes within 85% sequence identity to SEQ ID NO: 2 might catalyze entirely different reactions or even fall into enzyme families other than α KG-dependent dioxygenases. Ex. 1003, ¶¶ 48, 50. A skilled artisan would have understood at least 85% sequence identity to define a narrow sequence space well within established ranges for conserving enzyme function. Dr. Silverstone does not identify any enzyme having at least 85% identity to SEQ ID NO: 2 and an AAD-12 motif that has a different enzymatic

function than AAD-12 (SEQ ID NO: 2) or that falls into another enzyme class. In fact, such an enzyme would have been unlikely since Tian, Rost, and others had already demonstrated by 2005-2006 that, for enzymes having 85% or more sequence identity, enzyme function is highly conserved. Ex. 2019 (Tian), Fig. 1(b); Ex. 2028 (Rost 2002), Fig. 5.

168. Accordingly, in my opinion, the disclosed AAD-12 enzymes in the '055 patent would have been considered representative of the full set of claimed AAD-12 enzymes.

X. Conclusion

169. For the reasons set forth above, it is my opinion that the '055 patent claims are adequately described and enabled, particularly in view of the patent's extensive disclosure and known enzyme structure-function correlations, including for α KG-dependent dioxygenases such as SdpA (AAD-12), in 2005-2006 as well as in 2017.

170. I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true. I further declare that all statements made herein were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code.

Date: 1/21/2024

By: J. Martin Bollinger, Jr.
J. Martin Bollinger, Jr., Ph.D.

APPENDIX A

JOSEPH MARTIN BOLLINGER, JR.

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BORN: August 2, 1964

EDUCATION

Postdoctoral

N.I.H. Postdoctoral Fellow, January 1993 to May 1995
Harvard Medical School,
Department of Biological Chemistry and Molecular Pharmacology
Advisor: Christopher T. Walsh
"Enzymes in Biosynthesis of the Trypanosomatid Metabolite, Trypanothione."

Graduate

Doctor of Philosophy, Biochemistry, 1993
Massachusetts Institute of Technology (1987-1992)
Advisor: JoAnne Stubbe
"On the Chemical Mechanism of Assembly of the Tyrosyl Radical-Dinuclear Iron Cluster Cofactor of *E. coli* Ribonucleotide Reductase."

Undergraduate

Bachelor of Science with distinction, Chemistry, 1986
The Pennsylvania State University (1982-1986)

PROFESSIONAL EXPERIENCE

- Russell and Mildred Marker Professor of Natural Products Chemistry
The Pennsylvania State University 2021-present
- Professor, Department of Chemistry
The Pennsylvania State University 2008-present
- Professor, Department of Biochemistry and Molecular Biology
The Pennsylvania State University 2008-present
- Associate Professor, Department of Chemistry,
The Pennsylvania State University 2004-2008
- Associate Professor, Department of Biochemistry and Molecular
Biology, The Pennsylvania State University 2002-2008
- Assistant Professor, Department of Biochemistry and Molecular
Biology, The Pennsylvania State University 1995-2002

AWARDS

- ACS BIOL Abeles and Jencks Award for the Chemistry of Biological Processes 2023
- ACS Nobel Laureate Signature Award for Graduate Education in Chemistry 2023
- American Society for Biochemistry and Molecular Biology William C. Rose Award 2022

- Penn State Eberly College of Science Distinguished Faculty Mentoring Award 2021
- Howard B. Palmer Faculty Mentoring Award 2012
- Fellow, American Association for the Advancement of Science 2010
- Penn State University Faculty Scholar Medal for Outstanding Achievement 2009
- Society for Biological Inorganic Chemistry Early Career Award 2008
- Searle Scholar Award of the Chicago Community Trust 1996-1999
- Camille and Henry Dreyfus New Faculty Award 1995
- National Institutes of Health Post-Doctoral Fellowship 1993-1994
- Whitaker Health Sciences Fund Fellowship 1989-1991

EDITORIAL ADVISORY BOARDS

- *Journal of Biological Chemistry* 2007-2011
- *Journal of Biological Inorganic Chemistry* 2007-2012
- *Biochemistry* 2013-2015

PROFESSIONAL MEMBERSHIPS

- American Association for the Advancement of Science
- American Chemical Society
- American Society for Biochemistry and Molecular Biology
- Society of Biological Inorganic Chemistry

OFFICES IN PROFESSIONAL ORGANIZATIONS

- Program Committee, American Chemical Society, Division of Biological Chemistry 2008-2012
- Co-chair of the Program Committee, ACS, Division of Biological Chemistry 2010-2012

RESEARCH INTERESTS

- Enzyme structures and reaction mechanisms
- Oxygen and C-H activation by metalloenzymes
- Control of high-valent metalloenzyme intermediates
- Design of enzyme inhibitors and inactivators
- Oxidation reactions in natural product biosynthesis
- Iron and oxygen homeostasis
- Epigenetic regulation of transcription by redox metalloenzymes
- Oxidations in neurotransmitter and bioactive peptide biosynthesis

ACTIVE RESEARCH SUPPORT

- Department of Energy DE-SC0016255, Production of Ethylene and 3-Hydroxypropanoate from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE), March 1, 2023 to February 28, 2026 (M.P.I. with Carsten Krebs, Amie Boal, and Elvira Sayfutyarova)
- National Science Foundation 2108583, Exploration of the chemical and mechanistic diversity of mixed-valent iron oxygenases and oxidases, October 1, 2021 to September 30, 2024 (M.P.I. with Carsten Krebs and Alexey Silakov)
- National Institutes of Health R01 GM132653-02, Diverse Transition-Metal and Free-Radical Chemistry Enabling 2'-Deoxyribonucleotide Production by Bacteria in Restrictive Environments, June 1, 2019 to May 31, 2024 (P.I.)

- National Institutes of Health R01 GM138580-01, Structures and Mechanisms of “Heme-oxygenase-like” Non-heme Di-iron Enzymes that Catalyze Complex N-oxygenation and Olefin-installing C–C-Fragmentation Reactions, July 10, 2020 to June 30, 2024 (P.I.)

COMPLETED RESEARCH SUPPORT

- The Camille and Henry Dreyfus Foundation, Mechanisms and Consequences of Iron-Sulfur Cluster Assembly and Disassembly in Enzymes and Regulatory Proteins, July, 1995 to June, 2000 (P.I.)
- Searle Scholars/Chicago Community Trust, Mechanisms and Consequences of Iron-Sulfur Cluster Assembly and Disassembly in Enzymes and Regulatory Proteins, July 1, 1996 to June 30, 2000 (P.I.)
- National Institutes of Health R01 GM55365-01-04, Ribonucleotide Reductase: Iron-Radical Cofactor Assembly, January 1, 1997 to December 31, 2000 (P.I.)
- National Science Foundation, Structure-Function and Physiology of Novel Enzymes from Diverse Microbes, Research and Training Grant with J. G. Ferry, P. I., August 15, 1996 to August 14, 2001
- Tobacco Settlement Formula Fund, Mechanisms of Iron-Sulfur Cluster Assembly, July 1, 2002 to June 30, 2003 (co-P.I. with Carsten Krebs)
- Innovative Biotechnology Fund, Huck Institutes for the Life Sciences, Penn State University, The Mechanism of Oxygen Activation by Taurine:α-Ketoglutarate Dioxygenase, June 1, 2002 to May 31, 2004 (co-P.I. with Carsten Krebs)
- National Institutes of Health R01 GM55365, Ribonucleotide Reductase: Iron-Radical Cofactor Assembly, January 1, 2001 to December 31, 2005 (P.I.)
- National Science Foundation MCB-0235979, Structure, Mechanism, and Physiology of Two Sulfur-Mobilizing Enzymes from *Synechocystis* 6803, April 1, 2003 to March 30, 2006 (P.I.)
- Johnson and Johnson and Huck Institutes for the Life Sciences Innovative Biotechnology Seed Grant, Mechanism, Kinetics, and Inter-protein Interactions of Myo-Inositol Oxygenase, a Target for Treatment of Complications from Diabetes, July 1, 2005 to June 30, 2006 (co-P.I. with K. Sandeep Prabhu)
- National Institutes of Health R01 GM069657, Mechanism of Taurine:α-Ketoglutarate Dioxygenase, January 1, 2004 to December 31, 2009 (co-investigator; P.I. Carsten Krebs)
- National Science Foundation MCB-0642058, Non-Heme Fe(IV)-oxo Intermediates: Structure and Function, July 1, 2007 to June 30, 2010 (co-P.I. with Carsten Krebs)
- American Chemical Society, Petroleum Research Fund – Type AC grant Oxygen Activation by Isopenicillin N Synthase, September 1, 2007 to August 31, 2010 (co-P.I. with Carsten Krebs)
- National Science Foundation CHE-0724084, An Integrative Approach to Metalloenzyme-Catalyzed C-H Activation (joint program with the Deutsche Forschungsgesellschaft), January 1, 2008 to December 31, 2010 (co-P.I. with Carsten Krebs and Sharon Hammes-Schiffer)
- National Institutes of Health R01 DK074641, Structure and Mechanism of myo-Inositol Oxygenase, February 1, 2007 to January 31, 2012 (P.I.)
- National Institutes of Health, Administrative Supplement to GM55365 Oxygen Activation and Radical Transfer in Ribonucleotide Reductase from Pathogens, June 1, 2010 to May 31, 2012 (lead P.I.; multiple P.I.s Carsten Krebs and Michael T. Green)
- National Institutes of Health R01 GM55365, Oxygen Activation and Radical Transfer in Ribonucleotide Reductase from Pathogens, August 15, 2008 to July 31, 2013 (lead P.I.; multiple P.I.s Carsten Krebs and Michael T. Green)
- National Science Foundation CHE-1058931, Structure and Function of Intermediates Involved in Metalloenzymatic N-Oxygenation and Oxidation Reactions, February 15, 2011 to January 31, 2015 (co-P.I. with Carsten Krebs)

- National Science Foundation MCB-1122079, Identification of the Dinuclear Metallo-Cofactor and Elucidation of the Reaction Mechanism of the Aldehyde Decarbonylase from the Alkane-Producing Cyanobacterium, *Nostoc punctiforme*, July 1, 2011 to June 30, 2014 (co-P.I. with Carsten Krebs and Squire Booker)
- Industrial – LS9 Inc., South San Francisco, CA, Fundamental Biochemistry and Mechanism of Aldehyde Decarbonylase in Conjunction with Acyl-ACP Reductase in the Synthesis of Alkanes, Olefins, and Reaction Byproducts, one-time unrestricted gift on December 1, 2011.
- National Institutes of Health R01 GM103268, Functionalization of Unactivated sp^2 -hybridized Carbon Atoms, July 1, 2012 to June 30, 2017 (M.P.I. with Squire Booker and Carsten Krebs)
- National Science Foundation MCB-1330784, Mapping the Hydrolase-oxygenase Boundaries and Range of Catalytic Capabilities in the HD-domain Dinuclear Metalloenzyme Superfamily, August 1, 2013 to July 31, 2017 (M.P.I. with Carsten Krebs)
- National Institutes of Health R01 GM069657-06, Mechanism of Taurine: alpha-Ketoglutarate Dioxygenase, July 1, 2013 to June 30, 2018 (M.P.I. with Carsten Krebs)
- National Science Foundation CHE-1610676, Structures and Mechanisms of Two Enzymes that Produce Hydrocarbon Fuels from Abundant Metabolites, September 1, 2016 to August 31, 2019 (M.P.I. with Carsten Krebs and Amie Boal)
- National Institutes of Health R01 GM113106, Mechanisms of oxacycle- and olefin-installing iron/2-(oxo)glutarate oxygenases, September 10, 2015 to June 30, 2020 (M.P.I. with Carsten Krebs)
- National Institutes of Health R01 GM118812-05, Mechanisms and Reprogramming of Iron/2-Oxoglutarate Desaturases and Oxacyclases, June 1, 2016 to May 31, 2021 (M.P.I. with Carsten Krebs)
- Department of Energy DE-SC0016255, Mechanism of Ethylene Production from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE), September 1, 2016 to August 31, 2021 (M.P.I. with Carsten Krebs and Amie Boal)
- Department of Energy DE-SC0016255, Mechanism of Ethylene Production from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE), September 1, 2021 to February 28, 2023 (M.P.I. with Carsten Krebs and Amie Boal)

PUBLICATIONS

Refereed Original Research Articles

- 140)** Burke, E. J.; Copeland, R. A.; Dixit, Y.; Krebs, C.; and **Bollinger, J. M., Jr.** "Steric Perturbation of the Grob-like Final Step of Ethylene-Forming Enzyme Enables 3-Hydroxypropionate and Propylene Production," *J. Am. Chem. Soc.* **2024**, e-pub ahead of print, doi.org/10.1021/jacs.3c09733.
- 139)** Slater, J. W.; Neugebauer, M. E.; McBride, M. J.; Sil, D.; Lin, C. Y.; Katch, B. J.; Boal, A. K.; Chang, M. C. Y.; Silakov, A.; Krebs, C.; **Bollinger, J. M., Jr.** "Synergistic Binding of the Halide and Cationic Prime Substrate of the L-Lysine 4-Chlorinase, BesD, in Both Ferrous and Ferryl States," *Biochemistry* **2023**, 62, 2480-2491.
- 138)** Maio, N.; Raza, M. K.; Li, Y.; Zhang, D. L.; **Bollinger, J. M. Jr.**; Krebs, C.; Rouault, T. A. "An iron-sulfur cluster in the zinc-binding domain of the SARS-CoV-2 helicase modulates its RNA-binding and -unwinding activities," *Proc Natl Acad Sci U S A.* **2023**, 120(33):e2303860120.
- 137)** Manesis, A. C.; Slater, J. W.; Cantave, K.; **Bollinger, J. M., Jr.**; Krebs, C.; Rosenzweig, A. C.; "Capturing a bis-Fe(IV) State in *Methylosinus trichosporium* OB3b MbnH," *Biochemistry* **2023**, 62, 1082–1092.

- 136) Panth, N.; Wenger, E. S.; Krebs, C.; **Bollinger, J. M., Jr.**; Grossman, R. B.; "Synthesis of 6,6- and 7,7-difluoro-1-acetamidopyrrolizidines and their oxidation catalyzed by the nonheme Fe oxygenase LoLO," *ChemBioChem* **2022**, 23, (13) e202200081.
- 135) Park, Y. J.; Jodts, R. J.; Slater, J. W.; Reyes, R. M.; Winton, V. J.; Montaser, R. A.; Thomas, P. M.; Dowdle, W. B.; Ruiz, A.; Kelleher, N. L.; **Bollinger, J. M., Jr.**; Krebs, C.; Hoffman, B. M.; Rosenzweig, A. C.; "A mixed-valent Fe(II)Fe(III) species converts cysteine to an oxazolone/thioamide pair in methanobactin biosynthesis," *Proc. Natl. Acad. Sci. U.S.A.* **2022**, 119, (13) e2123566119.
- 134) Lin, C.-Y.; Hernández, A. L. M.; Laremore, T. N.; Silakov, A.; Krebs, C.; Boal, A. K.; **Bollinger, J. M., Jr.** "Use of Noncanonical Tyrosine Analogues to Probe Control of Radical Intermediates during Endoperoxide Installation by Verruculogen Synthase (FtmOx1)," *ACS Catal.* **2022**, 12, 6968-6979.
- 133) McBride, M. J.; Nair, M. A.; Sil, D.; Slater, J. W.; Neugebauer, M. E.; Chang, M. C. Y.; Boal, A. K.; Krebs, C. **Bollinger, J. M., Jr.**; "Substrate-Triggered μ -Peroxodiiron(III) Intermediate in the 4-Chloro-L-Lysine-Fragmenting Heme-Oxygenase-like Diiron Oxidase (HDO) BesC: Substrate Dissociation from, and C4 Targeting by, the Intermediate," *Biochemistry* **2022**, 61, 689–702.
- 132) Copeland, R. A.; Zhou, S.; Schaperdorth, I.; Tokufu, K. S.; **Bollinger, J. M., Jr.**; Krebs, C. "Hybrid Radical-Polar Pathway for Excision of Ethylene from 2-Oxoglutarate by an Iron Oxygenase," *Science* **2021**, 373, 1489-1493.
- 131) Maio, N.; Lafont, A. P.; Sil, D.; Li, Y.; **Bollinger, J. M., Jr.**; Krebs, C.; Pierson, T. C.; Linehan, M.; Rouault, T. A. "Fe-S cofactors in the SARS-CoV-2 RNA-dependent RNA polymerase are potential antiviral targets," *Science* **2021**, 373, 236-241.
- 130) Copeland, R. A.; Davis, K.; Shoda, T.; Blaesi, E.; Boal, A.; Krebs, C.; **Bollinger, J. M., Jr.** "An Iron(IV)-oxo Intermediate Initiating L-Arginine Oxidation but not Ethylene Production by the 2-Oxoglutarate-dependent Oxygenase, Ethylene-Forming Enzyme," *J. Am. Chem. Soc.* **2021**, 143, 2293-2303.
- 129) McBride, M. J.; Pope, S. R.; Hu, K.; Okafor, C. D.; Balskus, E. P.; **Bollinger, J. M., Jr.**; Boal, A. K. "Structure and Assembly of the Diiron Cofactor in the Heme-oxygenase-like Domain of the *N*-nitrosourea-producing Enzyme SznF," *Proc. Natl. Acad. Sci. U.S.A.* **2021**, 118 (4), e2015931118.
- 128) Liu, G.; Sil, D.; Maio, N.; Tong, W.H.; **Bollinger, J. M., Jr.**; Krebs, C. Rouault, T. A.; "Heme Biosynthesis Depends on Previously Unrecognized Acquisition of Iron-Sulfur Cofactors in Human Aminolevulinic Acid Dehydratase," *Nat. Commun.* **2020**, 11, 6310.
- 127) Srnc, M.; Iyer, S. R.; Dassama, L. M. K.; Park, K.; Wong, S. D.; Sutherlin, K. D.; Yoda, Y.; Kobayashi, Y.; Kurokuzu, M.; Saito, M.; Seto, M.; Krebs, C.; **Bollinger, J. M., Jr.**; Solomon, E. I. "Nuclear Resonance Vibrational Spectroscopy Definition of the Facial Triad Fe^{IV}=O Intermediate in Taurine Dioxygenase: Evaluation of Structural Contributions to Hydrogen Atom Abstraction," *J. Am. Chem. Soc.* **2020**, 142, 18886–18896.
- 126) Mocniak, L. E.; Elkin, K.; **Bollinger, J. M., Jr.** "Lifetimes of the Aglycone Substrates of Specifier Proteins, the Autonomous Iron Enzymes That Dictate the Products of the Glucosinolate-Myrosinase Defense System in Brassica Plants," *Biochemistry*, **2020**, 59, 2432-2441.
- 125) McBride, M. J.; Sil, D.; Ng, T. L.; Crooke, A. M.; Kenney, G. E.; Tysoe, C. R.; Zhang, B.; Balskus, E. P.; Boal, A. K.; Krebs, C.; **Bollinger, J. M., Jr.** "A Peroxodiiron(III/III) Intermediate Mediating Both *N*-Hydroxylation Steps in Biosynthesis of the *N*-Nitrosourea Pharmacophore of Streptozotocin by the Multi-domain Metalloenzyme SznF," *J. Am. Chem. Soc.* **2020**, 142, 11818-11828.
- 124) Cutsail, G. E. III; Blaesi, E. J.; Pollock, C. J.; **Bollinger, J. M. Jr.**; Krebs, C.; DeBeer S. "High-resolution iron X-ray absorption spectroscopic and computational studies of non-heme diiron peroxo intermediates," *J. Inorg. Biochem.* **2020**, 203, 110877.
- 123) Zhou, S.; Pan, J.; Davis, K. M.; Schaperdorth, I.; Wang, B.; Boal, A. K.; Krebs, C.; **Bollinger, J. M., Jr.** "Steric Enforcement of *cis*-Epoxide Formation in the Radical C–O-Coupling Reaction by Which (S)-2-Hydroxypropylphosphonate Epoxidase (HppE) Produces Fosfomycin," *J. Am. Chem. Soc.*, **2019**, 141, 20397–20406.

- 122) Davis K. M.; Altmyer, M.; Martinie, R. J.; Schaperdorth, I.; Krebs, C.; **Bollinger, J. M., Jr.**; Boal, A. K. "Structure of a Ferryl Mimic in the Archetypal Iron(II)- and 2-(Oxo)-glutarate-Dependent Dioxygenase, TauD," *Biochemistry*, **2019**, *58*, 4218–4223.
- 121) Zhang, B.; Rajakovich, L. J.; Van Cura, D.; Blaesi, E. J.; Mitchell, A. J.; Tysoe, C. R.; Zhu, X.; Streit, B. R.; Rui, Z.; Zhang, W.; Boal, A. K.; Krebs, C.; **Bollinger, J. M., Jr.** "Substrate-triggered Formation of a Peroxo-Fe₂(III/III) Intermediate during Fatty Acid Decarboxylation by UndA," *J. Am. Chem. Soc.*, **2019**, *141*, 14510–14514.
- 120) Pan, J.; Wenger, E. S.; Matthews, M. L.; Pollock, C. J.; Bhardwaj, M.; Kim, A. J.; Allen, B. D.; Grossman, R. B.; Krebs, C.; **Bollinger, J. M., Jr.** "Evidence for Modulation of Oxygen-Rebound Rate in Control of Outcome by Iron(II)- and 2-Oxoglutarate-Dependent Oxygenases," *J. Am. Chem. Soc.* **2019**, *141*, 15153–15165.
- 119) Chekan, J. R.; Ongpipattanakul, C.; Wright, T. R.; Zhang, B.; **Bollinger, J. M., Jr.**, Rajakovich, L. J.; Krebs, C.; Cicchillo, R. M.; Nair, S. K. "Molecular Basis for Enantioselective Herbicide Degradation Imparted by Aryloxyalkanoate Dioxygenases (AADs) in Transgenic Plants," *Proc. Natl. Acad. Sci. U.S.A.*, **2019**, *116*, 13299–13304.
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Invited and Refereed Review Articles

- 11) Krebs, C.; Dassama, L.M.K.; Matthews, M.L.; Jiang, W.; Price, J.C.; Korboukh, V.; Li, N.; **Bollinger, J.M., Jr.** "Novel Approaches for the Accumulation of Oxygenated Intermediates to Multi-Millimolar Concentrations," *Coord. Chem. Rev.*, **2013**, 257, 234-243
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Invited Perspectives

- 5) Boal, A. K.; **Bollinger, J. M., Jr.**; Chang, W.-c. "Assembly of the Unusual Oxacycles in the Orthosomycin Antibiotics," *Proc. Natl. Acad. Sci. U.S.A.*, **2015**, *112*, 11989-11990.
- 4) **Bollinger, J. M. Jr.**, "Biochemistry: Getting the Metal Right," *Nature*, **2010**, *465*, 40-41.
- 3) **Bollinger, J. M., Jr.**, Matthews, M. L. "Biochemistry: Remote Enzyme Microsurgery," *Science*, **2010**, *327*, 1337-1338.
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Invited Review Articles

- 1) **Bollinger, J. M., Jr.**, Tong, W. H., Ravi, N., Huynh, B. H., Edmondson, D. E., and Stubbe, J. "Use of Rapid Kinetics Methods to Study the Assembly of the Diferric-Tyrosyl Radical Cofactor of *E. coli* Ribonucleotide Reductase," *Meth. Enzymol.*, **1995**, *258*, 278-303.

Book Chapters

- 3) Rajakovich, L. R.; Zhang, B.; McBride, M. J.; Boal, A. K.; Krebs, C.; **Bollinger, J.M., Jr.** (2020) "Emerging Structural and Functional Diversity in Proteins with Dioxygen-Reactive Dinuclear Transition Metal Cofactors," In: Hung-Wen (Ben) Liu, Tadhg P. Begley (eds.) *Comprehensive Natural Products III: Chemistry and Biology*, *5*, 215-250, Elsevier.
- 2) **Bollinger, J.M., Jr.**; Chang, W.-c.; Matthews, M.L.; Martinie, R.; Boal, A.K.; Krebs, C. (2015) "Mechanisms of 2-Oxoglutarate-Dependent Oxygenases: The Hydroxylation Paradigm and Beyond" in *RSC Metallobiology Series No. 3*. R.P. Hausinger and C.J. Schofield, eds. pp. 95-122. Royal Society of Chemistry, Washington, D.C.
- 1) Huynh, B. H.; **Bollinger, J. M., Jr.**; and Edmondson, D. E. (1998) "Reaction intermediates in oxygen activation by enzymes containing carboxylate-bridged binuclear iron clusters" in *Spectroscopic Methods in Bioinorganic Chemistry*, E. I Solomon and K. O. Hodgson, eds. pp. 403-422. American Chemical Society, Washington, D.C.

INVITED LECTURES

At Regional, National and International Meetings

- 1) "Control of the Outcome of Oxygen Activation by the Diiron-Carboxylate Proteins," **Annual Conference on Enzymes in Deoxyribonucleotide Synthesis**, September 4-7, 1998, Sandhamn, Sweden.

- 2) "Structural Determinants of the Mechanisms and Outcomes of O₂ Activation by the Diiron Carboxylate Proteins," 1999 **International Conference on Biological Inorganic Chemistry**, July 11-16, 1999, Minneapolis, MN. Published abstract: *J. Inorg. Biochem.* 1999, 74, 13.
- 3) "An Fe(IV) Intermediate in the Reaction of Taurine/Alpha-ketoglutarate Dioxygenase from *E. coli*," 2003 **International Conference on Biological Inorganic Chemistry**, July 19-23, 2003, Cairns, Australia. Published abstract: *J. Inorg. Biochem.* 2003, 96, 223.
- 4) "Identification of the Precursor to Cluster X in Oxygen Activation by the Diiron(II) Center in Protein R2 of Ribonucleotide Reductase from *Escherichia coli*." **227th ACS National Meeting**, March 28-April 1, 2004, Anaheim, CA. Published abstract Abstracts of Papers, 2004, INOR-805.
- 5) "Precursors to Cluster X in Oxygen Activation by the Diiron(II) Cluster in Protein R2 of Ribonucleotide Reductase from *Escherichia coli*." **19th Enzyme Mechanisms Conference**, January 5-9, 2005, Pacific Grove, CA.
- 6) "New Tricks from an Old Dog: Novel Mechanisms of Oxygen Activation and C-H Cleavage by a Dinuclear Iron Cluster." **Gordon Research Conference on Protein Derived Cofactors, Radicals and Quinones**, January 22-27, 2006, Ventura, CA (cancelled due to family medical emergency).
- 7) "New Tricks from an Old Dog: Novel Mechanisms of Oxygen Activation and C-H Cleavage by a Dinuclear Iron Cluster." **Gordon Research Conference on Metals in Biology**, January 29-February 3, 2006, Ventura, CA (cancelled due to family medical emergency).
- 8) "Novel Mechanisms of Oxygen Activation and C-H Cleavage by a Dinuclear Iron Cluster." **German Research Foundation Meeting on Radicals in Enzymatic Catalysis**, March 6-8, 2006, Marburg, Germany (cancelled due to family medical emergency).
- 9) "Oxygen activation by a mixed-valent diiron cluster and C-H cleavage by an iron-superoxide intermediate in the glycol cleavage reaction catalyzed by *myo*-inositol oxygenase" **25th Summer Symposium in Molecular Biology: Frontiers in Metallobiochemistry**, June 7-10, 2006, University Park, PA
- 10) "Mechanism of Protein and Substrate Radical Generation in the Class Ic Ribonucleotide Reductase from *Chlamydia trachomatis*" **Gordon Research Conference on Metals in Biology**, January 28-February 2, 2007, Ventura, CA
- 11) "Formation and Function of a Radical-Initiating Mn(IV)/Fe(III) Cofactor in Ribonucleotide Reductase from *Chlamydia trachomatis*" **Gordon Research Conference on Enzymes, Co-enzymes, and Metabolic Pathways**, July 8-13, 2007, Biddeford, ME
- 12) "Formation and Function of a Radical-Initiating Mn(IV)/Fe(III) Cofactor in Ribonucleotide Reductase from *Chlamydia trachomatis*" **Gordon Research Conference on Protein Cofactors, Radicals And Quinones**, January 20-25, 2008, Ventura, CA
- 13) "Stabilization of Fe(IV)-oxo Intermediates in Proteins: Tactics and Limits" **Pre-ICBIC14 Symposium on Dioxygen Activation by Metalloenzymes and Models**, March 20-21, 2008, Nagoya, Japan (cancelled for health reasons)
- 14) "Strategies for and Intermediates in O₂ and C-H Activation by Iron Enzymes" **235th National Meeting and Exposition of the American Chemical Society** (Alfred Bader Award Symposium for Lawrence Que, Jr.), April 6-10, 2008, New Orleans, LA
- 15) "Mechanistic and Evolutionary Aspects of Radical Initiation by the Mn(IV)/Fe(III) Cofactor of *Chlamydia trachomatis* Ribonucleotide Reductase," **21st Enzyme Mechanisms Conference Loews Ventana Canyon Resort**, January 3-6, 2009, Tucson, AZ

- 16) "Why the iron- and α -ketoglutarate-dependent aliphatic halogenases are not hydroxylases," Plenary Lecture in Honor of 2008 Society for Biological Inorganic Chemistry Early Career Award, **14th International Conference on Biological Inorganic Chemistry**, July 25-30, 2009, Nagoya, Japan
- 17) "How the Aliphatic Halogenases Avoid Competing Hydroxylation," **24th Annual Symposium of The Protein Society**, August 1-5, 2010, San Diego, CA
- 18) "How the Aliphatic Halogenases Avoid Competing Hydroxylation," **The 2010 International Chemical Congress of Pacific Basin Societies (Pacifichem)**, December 15-20, 2010, Honolulu, HI (sent student, Megan Matthews, in my place due to family health emergency)
- 19) "The Mn/Fe Redox Cofactor of *Chlamydia trachomatis* Ribonucleotide Reductase: One of a Kind, or the First of Many?" **Gordon Research Conference on Inorganic Reaction Mechanisms**, March 6-11, 2011, Galveston, TX
- 20) "Cyanobacterial Aldehyde Decarbonylase Expands the Catalytic Repertoire of the Ferritin-like Dimetal Carboxylate Proteins," **Gordon Research Conference on Enzymes, Coenzymes, and Metabolic Pathways**, July 10-15, 2011, Waterville Valley, NH
- 21) "Cyanobacterial Aldehyde Decarbonylase: A Surprising New Catalytic Capability for the Ferritin-Like Dimetal-Carboxylate Proteins," **Society for Industrial Microbiology (SIM) Annual Meeting and Exposition**, July 24-28, 2011, New Orleans, LA
- 22) "Protein-Gated, Intersubunit, Long-Distance Hole Transfer (PCET) in the Manganese- and Iron-Dependent Class Ic Ribonucleotide Reductase from *Chlamydia trachomatis*," **1st International Conference on Proton-Coupled Electron Transfers: PCET, from Biology to Catalysis**, October 9-13, 2011, Loire Valley, France
- 23) "Cyanobacterial Aldehyde Decarbonylase Expands the Catalytic Repertoire of the Ferritin-like Dimetal Carboxylate Proteins," **Zing Conference on Enzymes, Coenzymes, and Metabolic Pathways**, November 17-21, 2011, Xcaret, Mexico (sent Ph.D. student Ning Li due to family medical emergency)
- 24) "The Novel, Cryptically Redox Monooxygenation Reaction Catalyzed by Cyanobacterial Aldehyde Decarbonylase and Evidence Against the Proposed Dioxygen-Independent Pathway," **Gordon Research Conference on Isotopes in Biological and Chemical Sciences**, February 5-10, 2012, Galveston, TX (sent Prof. Carsten Krebs in my place due to family medical emergency)
- 25) "Dissection of Mechanisms of O₂-Utilizing Metalloenzymes by Direct Observation of Reactive Intermediates," **243rd National Meeting and Exposition of the American Chemical Society** (Special Symposium on Electronic Structure in Honor of Edward I. Solomon), March 25-29, 2012, San Diego, CA
- 26) "O₂-Evolving Chlorite Dismutase as a Tool for Studying O₂-Utilizing Enzymes and Selected Applications," **Gordon Research Conference on Protein Cofactors, Radicals and Quinones**, July 29 – August 3, 2012, Mount Holyoke College, Holyoke, MA.
- 27) "Mechanism of the Aliphatic Halogenases," **Society of Industrial Microbiology and Biotechnology Annual National Meeting and Exposition**, August 12-15, 2012, Washington, DC (sent Dr. Megan Matthews in my place due to a family medical emergency)
- 28) "The Mechanism of the Cryptically-Redox Oxygenation Reaction Catalyzed by Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," **Gordon Research Conference on Metals in Biology**, January 20 – 25, 2013, Ventura, CA.

- 29) "The Mechanism of the Cryptically-Redox Oxygenation Reaction Catalyzed by Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," **245th American Chemical Society National Meeting**, April 7 – 11, 2013, New Orleans, LA.
- 30) "Non-haem Iron Enzymes in the Biosyntheses of Bioactive Natural Products," **International Conference on Bioinorganic Chemistry**, July 22 – 26, 2013, Grenoble, France (sent Dr. W.-c. Chang in my place due to a family medical emergency).
- 31) "Mapping the Hydrolase-oxygenase Boundaries and Range of Catalytic Capabilities in the HD-domain Dinuclear Metalloenzyme Superfamily," **23rd Solvay Conference on Chemistry**, October 16 – 19, 2013, Brussels, Belgium (cancelled due to a family medical emergency).
- 32) "Fatty Acids to Diesel Fuel: the Mechanism of the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," **247th American Chemical Society National Meeting**, March 16-20, 2014, Dallas, TX.
- 33) "Frontiers Beyond the Hydroxylation Paradigm in the Diverse Chemistry of Non-heme Fe(IV)-oxo (Ferry) Enzyme Intermediates," **2014 ASBMB Annual Meeting**, April 26-30, 2013, San Diego, CA.
- 34) "Demystifying the Chemical Magic of Non-heme-iron Enzymes in Natural Product Biosynthesis," **2014 Gordon Research Conference on Iron-Sulfur Enzymes**, June 15-20, 2014, Easton, MA.
- 35) "The Remarkable Versatility of Hydrogen-Abstracting Iron(IV)-oxo (Ferry) Enzyme Intermediates in the Biosynthesis of Bioactive Natural Products," **International Conference on Hydrogen Atom Transfer**, June 22-26, 2014, Rome, Italy.
- 36) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," **2014 Gordon Research Conference on Enzymes, Coenzyme, and Metabolic Pathways**, July 13-18, 2014, Waterville Valley, NH.
- 37) "Synthesis of Natural-Product Drugs by Iron and 2-(Oxo)glutarate Oxygenases," **2015 GTC Conference on Enzymes in Drug Discovery**, February 26-27, 2015, San Diego, CA.
- 38) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes in the Biosynthesis of Bioactive Natural Products," **2015 Southeast Enzymes Meeting**, April 11, 2015, Atlanta, GA.
- 39) "Fatty Acids to Diesel Fuel: the Mechanism of the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," **Pacificchem 2015**, December 15-20, 2015, Honolulu, HI.
- 40) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," **Pacificchem 2015**, December 15-20, 2015, Honolulu, HI.
- 41) "Mechanisms and Purview of C-H-Bond Activation by Iron(III)-Superoxide Complexes," **251st American Chemical Society National Meeting**, March 12-16, 2016, San Diego, CA.
- 42) "Mechanism and Outcome Control in the Petrodiesel-Producing Diiron Enzyme, Aldehyde Deformylating Oxygenase," **A Sojourn in Biochemistry: RNR (R&R) Was Anything But (symposium to honor JoAnne Stubbe's 70th birthday and career)**, June 11-12, 2016, Cambridge, MA
- 43) "Mechanism and Reaction Control in the Petrodiesel-Producing Diiron Enzyme, Aldehyde Deformylating Oxygenase," **5th Symposium for Advanced Biological Inorganic Chemistry**, January 7-11, 2017, Kolkata, India.
- 44) "Emerging Diversity in the Chemistry of Non-Heme Diiron Oxidases and Oxygenases," **Gordon Research Conference on Metals in Biology**, January 22-27, 2017, Ventura, CA.

- 45) "Novel Outcomes and Mechanisms Emerging from Dioxygen Activation by Non-Heme Di-Iron Enzymes," **253rd American Chemical Society National Meeting**, April 2-6, 2017, San Francisco, CA.
- 46) "Mechanistic Pathways to Unusual Outcomes in Reactions of Iron-Dependent Oxygenases," **"Experimental Biology 2017"** Symposium of the **2017 Annual Meeting of the American Society of Biochemistry & Molecular Biology**, April 22-26, 2017, San Francisco, CA.
- 47) "Capabilities and Limitations of Mid-Valent Iron-Superoxide Complexes for Hydrogen Atom Transfer," **International Conference on Hydrogen Atom Transfer**, July 2-6, 2017, Rome, Italy.
- 48) "How Iron- and 2-(Oxo)glutarate-dependent (Fe/2OG) Oxygenases Direct Their Common Ferryl Intermediate to Different Outcomes," **26th Enzyme Mechanisms Conference**, January 6-9, 2019, New Orleans, LA
- 49) Title to be determined, **19th International Conference on Biological Inorganic Chemistry**, August 11-16, 2019, Interlaken, Switzerland (withdrew for health reasons)
- 50) "Mechanistic and Structural Analysis of How Nonheme-Iron Enzymes Direct Different Oxidation Reactions," **258th American Chemical Society National Meeting**, August 25-29, 2019, San Diego, CA
- 51) Title to be determined, **International Conference on Hydrogen Atom Transfer**, June 21-25, 2020, Rome, Italy (cancelled due to Covid-19 pandemic).
- 52) "How Nonheme-Iron Enzymes Achieve Such Remarkable Versatility in Biological C–H Bond Activation" (keynote lecture), **Midwest Enzyme Chemistry Conference**, October 24, 2020, remote.
- 53) Plenary Lecture, **EuroBIC**, July 17-22, 2022, Grenoble France (declined due to Covid-19 risk)
- 54) Title to be determined, **International Conference on Hydrogen Atom Transfer**, June 9-13, 2024, Monteporzio Catone, Italy.

At Companies

- 1) "Mutagenesis to Probe Protein Control of Dioxygen Activation at the Diiron Cluster in Protein R2 of Ribonucleotide Reductase," 3M Corporation, Minneapolis, MN, June 21, 1996
- 2) "Why the Iron- and α -Ketoglutarate-dependent Aliphatic Halogenases are not Hydroxylases," New England Biolabs, Ipswich, MA, October 8, 2009
- 3) "Cyanobacterial Aldehyde Decarbonylase Expands the Catalytic Repertoire of the Ferritin-like Dimetal Carboxylate Proteins," LS9, Inc., South San Francisco, CA, June 17, 2011
- 4) "Monooxygenases and Dioxygenases: Nomenclature and General Principles," Orrick, Herrington, and Sutcliffe, LLP, San Francisco, CA, September 27, 2011
- 5) "The Mechanism of the Cryptically-Redox Oxygenation Reaction Catalyzed by Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," New England Biolabs, Ipswich, MA, June 27, 2013.
- 6) "Cyanobacterial Conversion of Abundant Fatty Acids to Petrodiesel via the Cryptically Redox Reaction of Aldehyde Deformylating Oxygenase," ExxonMobil Research and Engineering Company, Annandale, NJ, May 21, 2018.

At Universities

- 1) "Control of the Outcome of Oxygen Activation by the Diiron-Carboxylate Proteins," Program in Molecular Medicine, Penn State University College of Medicine, October 15, 1997

- 2) "Control of the Outcome of Oxygen Activation by the Diiron-Carboxylate Proteins," Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, November 7, 1997
- 3) "Control of the Outcome of Oxygen Activation by the Diiron-Carboxylate Proteins," Department of Chemistry, Duquesne University, January 30, 1998
- 4) "Structural Determinants of Reaction Mechanism and Outcome In Oxygen Activation by the Di-Iron Proteins," Department of Biophysics, Johns Hopkins University, May 4, 1998
- 5) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Departments of Chemistry and Physics, Emory University, September 29, 1998
- 6) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Biochemistry, Temple University School of Medicine, October 9, 1998
- 7) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Biochemistry, McMaster University (Canada) October 27, 1998
- 8) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Chemistry, Northwestern University, November 13, 1998
- 9) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Chemistry, University of Minnesota, December 14, 1998
- 10) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Interdepartmental Program in Medicinal Chemistry, University of Michigan, January 28, 1999
- 11) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Chemistry, Stanford University, February 2, 1999
- 12) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Chemistry, Oregon Graduate Institute, February 4, 1999
- 13) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Biochemistry and Biophysics, Oregon State University, February 5, 1999
- 14) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Biochemistry and Biophysics, University of Pennsylvania, "George Raiziss Biochemical Rounds: Enzymology at the Interface of Chemistry and Medicine" September 14, 1999
- 15) "Structural Determinants of Reaction Outcome in Oxygen Activation by the Di-Iron-Carboxylate Proteins," Department of Biochemistry, University of Wisconsin, November 1, 1999
- 16) "Structural and Mechanistic Bases for the Divergent Outcomes of Dioxygen Activation by the Diiron-Carboxylate Oxygenase and Oxidase Proteins," Department of Chemistry, Ohio State University, January 10, 2000
- 17) "Structural and Mechanistic Bases for the Divergent Outcomes of Dioxygen Activation by the Diiron-Carboxylate Oxygenase and Oxidase Proteins," Department of Chemistry, Columbia University, January 20, 2000
- 18) "Structural and Mechanistic Bases for the Divergent Outcomes of Dioxygen Activation by the Diiron-Carboxylate Oxygenase and Oxidase Proteins," Department of Molecular Biology, The Scripps Research Institute, February 2, 2000
- 19) "Mechanism of Rapid Electron Transfer During Oxygen Activation at the Diiron-Carboxylate Center of Ribonucleotide Reductase Protein R2," Department of Biochemistry, Stockholm University (Sweden), September 21, 2000

- 20) "Aiming a Molecular Cannon: Protein Control of Di-Oxygen Activation at Carboxylate-Bridged Dinuclear Iron Clusters," Department of Chemistry, Penn State University, September 26, 2000
- 21) "Aiming a Molecular Cannon: Protein Control of Di-Oxygen Activation at Carboxylate-Bridged Dinuclear Iron Clusters," Department of Chemistry, University at Buffalo (SUNY), February 28, 2001
- 22) "Aiming a Molecular Cannon: Protein Control of Di-Oxygen Activation at Carboxylate-Bridged Dinuclear Iron Clusters," Department of Biochemistry, Virginia Polytechnical Institute and State University, September 10, 2001
- 23) "Aiming a Molecular Cannon: Protein Control of Di-Oxygen Activation at Carboxylate-Bridged Dinuclear Iron Clusters," Department of Chemistry, California Institute of Technology, October 22, 2001
- 24) "Probing the Structural and Mechanistic Bases for the Divergent Control of Oxygen Activation by the Diiron-Carboxylate Proteins." Department of Chemistry, Massachusetts Institute of Technology, December 3, 2001
- 25) "Aiming a Molecular Cannon: Protein Control of Di-Oxygen Activation at Carboxylate-Bridged Dinuclear Iron Clusters," Program in Complex Biological Systems, Montana State University, December 7, 2001
- 26) "Aiming a Molecular Cannon: Protein Control of Di-Oxygen Activation at Carboxylate-Bridged Dinuclear Iron Clusters," Department of Chemistry, Bucknell University, March 27, 2002
- 27) "Dissecting the Mechanism of Oxygen Activation by Taurine:α-Ketoglutarate Dioxygenase," Williams College, September 26, 2003
- 28) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," Department of Chemistry, Utah State University, October 15, 2003
- 29) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," Department of Chemistry, Penn State University, November 11, 2003
- 30) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," Department of Chemistry, University of Minnesota, March 3, 2004
- 31) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," Department of Chemistry, Montana State University, April 30, 2004
- 32) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," University of Kansas, May 11, 2004
- 33) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," University of Pennsylvania, Department of Chemistry Inorganic Seminar Series, October 26, 2004
- 34) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," University of Delaware, Department of Chemistry, February 7, 2005
- 35) "Stalking Intermediates in Oxygen Activation by Iron Enzymes," Texas A. & M. University, Department of Chemistry, September 29, 2005
- 36) "Stalking Intermediates in Oxygen Activation by Iron Proteins," Duquesne University, Mini-symposium in convocation of their Center for Biological Inorganic Chemistry, November 4, 2005
- 37) "Novel Mechanism of *myo*-Inositol Oxygenase," University of Michigan, Department of Biological Chemistry, May 2, 2006
- 38) "Novel Mechanism of *myo*-Inositol Oxygenase," Emory University, Department of Chemistry, Biomedical Division, December 11, 2006

- 39) "Novel Mechanisms of O₂ and C-H Activation by a Diiron Cluster in *myo*-Inositol Oxygenase," King's College, April 4, 2007 (Lecture at the 369th meeting of the American Chemical Society Susequehanna Valley Section)
- 40) "Novel Mechanisms of O₂ and C-H Activation by a Diiron Cluster in *myo*-Inositol Oxygenase," Moravian College, April 11, 2007
- 41) "Bioinorganic Chemistry: the Coming Decade and Beyond," Emory University, June 13, 2007 (This was a three-hour "vision" presentation given jointly with Profs. Carsten Krebs, Squire Booker, and Michael Green)
- 42) "Dissecting Mechanisms of Metalloenzymes by Kinetics, Spectroscopy, and Protein and Substrate Mutagenesis," Department of Chemistry, Technical University of Denmark, Kgs. Lyngby, Denmark, June 19, 2007 (This was a three-hour tutorial delivered at a Ph. D. school as part of their Summer school program with expenses paid by the host.)
- 43) "Strategies for O₂ and C-H Activation by Metalloenzymes," R. B. Woodward Lectures in the Chemical Sciences, Harvard/M.I.T. Inorganic Chemistry Seminar, Harvard University, September 19-20, 2007
- 44) "Formation, function, and evolution of a radical-initiating manganese-iron cofactor in ribonucleotide reductase from *Chlamydia trachomatis*," Chemical Biology/Inorganic Chemistry Series, University of Illinois at Urbana-Champaign, November 29, 2007
- 45) "Formation and Function of a Radical Initiating Mn(IV)/Fe(III) Cofactor in the Ribonucleotide Reductase from *Chlamydia trachomatis*," Department of Chemistry, University of California, Irvine, May 9, 2008
- 46) "Formation and function of a stable high-valent, manganese-iron redox cofactor in the ribonucleotide reductase from *Chlamydia trachomatis*," Department of Chemistry, Yale University, September 23, 2008 (postponed due to family medical emergency)
- 47) "The Mid-Valent Metal-Superoxo Manifold for Enzymatic O₂ and C-H Activation," University of Minnesota Chemical Biology Training Grant Symposium, May 27, 2009
- 48) "The Basis for the Strict Selectivity of Aliphatic Halogenases against Substrate Hydroxylation," Department of Chemistry, Princeton University, September 10, 2009
- 49) "Why the Iron- and Alpha-Ketoglutarate-Dependent Aliphatic Halogenases are not Hydroxylases," Department of Chemistry, Case Western Reserve University, October 1, 2009
- 50) "Cyanobacterial Aldehyde Decarbonylase Expands the Catalytic Repertoire of the Ferritin-like Dimetal Carboxylate Proteins," Department of Chemistry, University of California at Berkeley, September 26, 2011
- 51) "Cyanobacterial Aldehyde Decarbonylase Expands the Catalytic Repertoire of the Ferritin-like Dimetal Carboxylate Proteins," Department of Chemistry, Loyola University of Chicago, September 29, 2011
- 52) "Cyanobacterial Aldehyde Decarbonylase Expands the Catalytic Repertoire of the Ferritin-like Dimetal Carboxylate Proteins," Department of Chemistry, Carnegie Mellon University, November 17, 2011
- 53) "Cyanobacterial Aldehyde Decarbonylase: A Surprising New Catalytic Capability for the Ferritin-Like Dimetal-Carboxylate Proteins," Department of Biochemistry and Molecular Biology, Michigan State University, January 19, 2012

- 54) "Cyanobacterial Aldehyde Decarbonylase: A Surprising New Catalytic Capability for the Ferritin-Like Dimetal-Carboxylate Proteins," Department of Chemistry, University of Chicago, January 30, 2012
- 55) "Cyanobacterial Aldehyde Decarbonylase: A Surprising New Catalytic Capability for the Ferritin-Like Dimetal-Carboxylate Proteins," Department of Chemistry, University of Florida, February 20, 2012 (postponed due to family health emergency)
- 56) "Cyanobacterial Aldehyde Decarbonylase: A Surprising New Catalytic Capability for the Ferritin-Like Dimetal-Carboxylate Proteins," Department of Chemistry, Florida State University, February 21, 2012 (postponed due to family health emergency).
- 57) "Making Diesel Fuel in Bacteria: the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," Department of Chemistry, University of California at Davis, February 5, 2013.
- 58) "Making Diesel Fuel in Bacteria: the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," Department of Chemistry, Texas A&M, February 21, 2013 (postponed due to family health emergency)
- 59) "Making Diesel Fuel in Bacteria: the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," Department of Chemistry, Ohio State University, February 25, 2013 (postponed due to family health emergency).
- 60) "Making Diesel Fuel in Bacteria: the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," Department of Chemistry, Northwestern University, April 11, 2013.
- 61) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Departments of Chemistry and Biochemistry, University of Texas, Austin, February 28, 2014 (postponed due to family health emergency).
- 62) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Chemistry, University of California, San Diego, May 2, 2014 (postponed; rescheduled for January 9, 2015).
- 63) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," **2014 Baker Symposium**, Cornell University, May 2-3, 2014.
- 64) "Fatty Acids to Diesel Fuel: the Mechanism of the Cryptically Redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase (Formerly Aldehyde Decarbonylase)," Energy Biosciences Institute, University of California, Berkeley, September 30, 2014.
- 66) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Chemistry, University of California, San Diego, January 9, 2015.
- 67) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Chemistry, Wake Forest University, January 21, 2015.
- 68) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Chemistry, University of Minnesota, Twin Cities, February 12, 2015.
- 69) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Macalester College, Minneapolis, Minnesota, February 13, 2015.
- 70) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Chemistry, Brigham Young University, February 23, 2015.
- 71) "Making Diesel Fuel in Bacteria via the Cryptically-redox Oxygenation Reaction of Aldehyde Deformylating Oxygenase," Department of Chemistry, Texas A & M University, College Station, TX, March 4, 2015.

- 72) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, May 6, 2015.
- 73) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes in Biosynthetic Pathways to Natural Product Drugs," Molecular Life Sciences Seminar Series, The Ohio State University, Columbus, Ohio, February 16, 2016.
- 74) "Demystifying the Chemical Magic of Non-Heme-Iron Enzymes Involved in Biosynthesis of Bioactive Natural Products," Department of Chemistry, Princeton University, Princeton, NJ, March 24, 2016.
- 75) "The Remarkable Versatility of High-valent-iron Intermediates in Biosynthesis of Bioactive Natural Products," M.I.T Department of Chemistry T.Y. Shen Lectureship, Massachusetts Institute of Technology, Cambridge, MA, April 9, 2018.
- 76) "Unique Capabilities (and Limitations) of C–H-Activation by Iron-Superoxide Enzyme Intermediates," M.I.T Department of Chemistry T.Y. Shen Lectureship, Massachusetts Institute of Technology, Cambridge, MA, April 10, 2018.
- 77) "The Remarkable Versatility of High-Valent Iron Intermediates in Biosynthesis of Bioactive Natural Products" Departments of Chemistry and Pharmacy, University of Kentucky, Lexington, KY, April 13, 2018.
- 78) "The Remarkable Versatility of High-Valent Iron Intermediates in Biosynthesis of Bioactive Natural Products," Departments of Chemistry, North Carolina State University, Raleigh, NC, April 20, 2018.
- 79) "Capabilities and Limitations of Iron(III)-superoxo Intermediates in Enzymatic Transformation of Aliphatic Carbons," Department of Chemistry, University of Washington, Seattle, WA, January 28, 2020.
- 80) "Protein Control of Outcome in Activation of Dioxygen by Nonheme Iron Enzymes," Department of Chemistry, University of California, Santa Barbara, Santa Barbara, CA, March 05, 2024 (scheduled).
- 79) "Protein Control of Outcome in Activation of Dioxygen by Nonheme Iron Enzymes," Department of Chemistry, University of California, Irvine, Irvine, CA, March 07, 2024 (scheduled).

OTHER PARTICIPATION IN INTERNATIONAL MEETINGS

- 1) Discussion Leader, Gordon Research Conference on Protein Cofactors, Radicals and Quinones, January 13-18, 2002, Ventura, CA.
- 2) Discussion Leader, Gordon Research Conference on Enzymes Coenzymes and Metabolic Pathways, July 21-26, 2002, Kimball Union Academy, NH (declined for family reasons).
- 3) Discussion Leader, Gordon Research Conference on Metals in Biology, January 27 - February 1, 2008, Ventura, CA.
- 4) Co-Vice-Chair, Gordon Research Conference on Enzymes, Coenzymes and Metabolic Pathways, July 13-18, 2008, Biddeford, ME.
- 5) Co-Chair, Gordon Research Conference on Enzymes Coenzymes and Metabolic Pathways, July 12-17, 2009, Waterville Valley, NH.
- 6) Discussion Leader, Gordon Research Conference on Protein Cofactors, Radicals and Quinones, January 24-29, 2010, Ventura, CA.
- 7) Co-Chair, 29th Annual Penn State Summer Symposium in Molecular Biology: Frontiers in Metallobiochemistry, June 1-5, 2010, University Park, PA
- 8) Chair, Division of Biological Chemistry Program of the American Chemical Society, Fall National Meeting, August 22-26, 2010, Boston, MA

- 9) Chair, Division of Biological Chemistry Program of the American Chemical Society, Spring National Meeting, March 27-31, 2011, Anaheim, CA
- 10) Discussion Leader, Gordon Research Conference on Protein Cofactors, Radicals and Quinones, July 29 - August 3, 2012, Holyoke, MA.
- 11) Co-Chair, 33rd Annual Penn State Summer Symposium in Molecular Biology: Frontiers in Metallobiochemistry, June 4-7, 2014, University Park, PA
- 12) Discussion Leader, Gordon Research Conference on Metals in Biology, January 24-29, 2016, Ventura, CA
- 13) Co-Chair, "A Sojourn in Biochemistry: RNR (R&R) Was Anything But" (symposium to honor JoAnne Stubbe's 70th birthday and career), June 11-12, 2016, Cambridge, MA
- 14) Symposium Chair, "At the Leading Edge of Biocatalysis," 2017 ASBMB Annual Meeting, April 22-26, 2017, Chicago, IL.
- 15) Vice-Chair, Gordon Research Conference on Metals in Biology, January 21-26, 2018, Ventura, CA.
- 16) Co-Chair, 37th Annual Penn State Summer Symposium in Molecular Biology: Frontiers in Metallobiochemistry, June 6-8, 2018, University Park, PA
- 17) Chair, Gordon Research Conference on Metals in Biology, January 27 – February 1, 2019, Ventura, CA.
- 18) Session Chair, Virtual Biocatalysis and Protein Engineering Meetup, March 18, 2021.
- 19) Discussion Leader, Gordon Research Conference on Metals in Biology, January 21-26, 2024, Ventura, CA.

SELECTED SERVICE ACTIVITIES

To Industry

Expert Witness for PureCircle SDN BHD and PureCircle USA Inc., in Case No. SA CV 18-01679 NS (JD Ex) PureCircle USA Inc. et al. v. SweeGen, Inc. et al.	Aug. 2020 – June 2022
Expert Witness for Dow AgroSciences, ICC Case No. 18892/VRO/AGF BAYER CROPSCIENCE, AG and BAYER CROPSCIENCE NV v. DOW AGROSCIENCES LLC, MYCOGEN PLANT SCIENCE, INC., AGRIGENETICS, INC. d/b/a MYCOGEN SEEDS, LLC, and PHYTOGEN SEED COMPANY, LLC	July 2014 – Aug. 2014
Expert Witness for Dow AgroSciences, LLC, ICC Case No. 18892/VRO/AGF/ZF	
Expert Witness for the Defendant, Bayer CropScience AG v Dow AgroSciences, LLC, C.A. No. 10-1045-RMB-JS, U.S. Dist. Ct. Del. (decided in favor of the defendant Sept. 27, 2012)	June 2011 – Sept. 2012
Member, Scientific Advisory Board, Renewable Energy Group (formerly, LS9 Incorporated), South San Francisco, CA	Dec. 2011 – 2015

To Government Agencies

Faculty Participant, NSF Workshop on Science Policy at The Pennsylvania State University	March 12, 1996
Member, Special Emphasis Panel to Review Post-Doctoral Fellowship Applications Biochemistry Initial Review Group (IRG), NIH	June 30, 2000
Member (<i>ad hoc</i>), Biochemistry IRG, NIH	June 14-15, 2001
Member, Special Emphasis Panel to Review Grant Application, Physiological Chemistry IRG, NIH	July 19, 2001
Review of Proposals (9) for the National Science Foundation	2002-2007
Member, IRG for Post-Doctoral Fellowship Applications, NIH	March 1, 2007
Member (<i>ad hoc</i>), Macromolecular Structure and Function E IRG, NIH	June 4-5, 2007
Member (<i>ad hoc</i>), Molecular Biochemistry Review Panel, NSF	October 29-31, 2007
Permanent Member, Macromolecular Structure and Function E IRG, NIH	Oct. 2008-July 2012
Member (<i>ad hoc</i>), Macromolecular Structure and Function E IRG, NIH	October 17, 2012
Member, DOE ARPA-E REMOTE (Reducing Emissions using Methanotrophic Organisms for Transportation Energy) Review Panel	August 1, 2013
Co-Chair, Special Emphasis Panel to Review P01 Application, NIH	April 4, 2014
Member, Special Emphasis Panel F04A-W(20)L for fellowship review, NIH	Feb. 17-18, 2015
Member, Special Emphasis Panel to Review Applications for New "Maximizing Investigator's Research Award" (MIRA) Program, NIGMS, NIH	Nov. 9-10, 2015
Member, Mechanistic Molecular Biophysics II Review Panel, NSF	March 28-29, 2016
Member, Special Emphasis Panel 2017/05 ZRG1 BCMB-G (02) M, NIH	February 22-23, 2017
Member, Enzyme Structure and Function Review Panel for DE-FOA-0001664, DOE	March 2, 2017
Member, Special Emphasis Panel to Review Applications for "Early Stage Investigator Maximizing Investigator's Research Award" (ESI-MIRA) Program, NIGMS, NIH	March 8-9, 2018
Member, Special Emphasis Panel to Review Applications for "Early Stage Investigator Maximizing Investigator's Research Award" (ESI-MIRA) Program, NIGMS, NIH	March 14-15, 2019
Member, Special Emphasis Panel to Review K99 "Pathway to Independence" Applications, NIGMS, NIH	March 11, 2021
Member, Special Emphasis Panel to Review Applications for "Early Stage Investigator Maximizing Investigator's Research Award" (ESI-MIRA) Program, NIGMS, NIH	March 8-9, 2022
To Journals and Reference Book Series	
Contributing Member, <i>Faculty of 1000</i>	2014-2016
Editorial Advisory Board Member, <i>Biochemistry</i>	2013-2015
Editorial Advisory Board Member, <i>Journal of Biological Chemistry</i>	2007-2011
Editorial Advisory Board Member, <i>Journal of Biological Inorganic Chemistry</i>	2007-2012
Special Guest Editor, <i>Proceedings of the National Academy of Sciences U. S. A.</i>	2007,8,9,11,15,19
Section Co-Editor (with V. Bandarian and S. Booker), <i>Comprehensive Natural Products III: Chemistry and Biology</i> (Volume 5 – Enzymes and Enzyme	2020

Mechanisms: Radicals and Metalloenzymology)

To the Metallobiochemistry Community

Co-chair, Biennial Penn State Bioinorganic Workshops (visit <http://sites.psu.edu/bioinorganic/>)

1st Workshop - 36 trainees, 46 participants	June 1-2, 2010
2nd Workshop - 78 trainees, 123 participants	May 29 - June 6, 2012
3rd Workshop - 80 trainees, 162 participants	May 27 - June 4, 2014
4th Workshop - ~ 70 trainees, 154 participants	June 2-10, 2016
5th Workshop - ~ 80 trainees, 160 participants	June 1-5, 2018

SELECTED TRAINEES

Ph.D. Graduates

- Dr. John C. Price, Ph.D. 2005, Assistant Professor of Chemistry and Biochemistry, Brigham Young University, 2013-2019; Associate Professor 2019-present
- Dr. Lana Saleh, Ph.D. 2005, Division Head, New England BioLabs (2004 Alumni Association Dissertation Award)
- Dr. Megan L. Matthews, Ph.D. 2011, Assistant Professor of Chemistry, University of Pennsylvania, 2017-present (2010 Alumni Association Dissertation Award; 2018 Penn State Alumni Achievement Award)
- Dr. Laura M. K. Dassama, Ph.D. 2013, Assistant Professor of Chemistry, Stanford University, 2018-present (2013 Alumni Association Dissertation Award; 2019 Penn State Alumni Achievement Award)
- Dr. Lauren Rajakovich, Ph.D. 2017, Assistant Professor of Chemistry, University of Washington, 2022-present
- Dr. Ryan Martinie, Ph. D. 2018, Assistant Professor of Chemistry, Hamilton College, 2021-present
- Dr. Noah Dunham, Ph.D. 2018, Senior Scientist, Merck Biocatalysis 2021-present
- Dr. Rachelle Copeland, Ph.D. 2021, Scientist I, Automation and Analytical Sciences Codexis, Inc. 2021-present

Post-doctoral Fellows

- Dr. Gang Xing, Senior Principal Scientist, Pfizer, 2018-present
- Dr. Yisong Guo, Associate Professor of Chemistry, Carnegie Mellon University, 2014-present
- Dr. Maria-Eirini Pandelia, Assistant Professor of Biochemistry, Brandeis University, 2016-present
- Dr. Wei-chen Chang, Associate Professor of Chemistry, North Carolina State University, 2016-present
- Dr. Alexey Silakov, Assistant Professor of Chemistry, Penn State University, 2017-present

APPENDIX B

APPENDIX B

Exhibit	Description
1001	US Patent No. 11,371,055 (“the ’055 patent”).
1003	Dr. Silverstone’s Declaration.
1019	Hogan, D.A. et al., <i>Site-directed Mutagenesis of 2, 4-Dichlorophenoxyacetic Acid/α-Ketoglutarate Dioxygenase: Identification of Residues Involved in Metallocenter Formation and Substrate Binding</i> , J. of Biol. Chem., Vol. 275, No. 17, pp. 12400-12409 (2000) (“Hogan”).
1020	Müller, T.A., et al., <i>Structural basis for the enantiospecificities of R- and S-specific phenoxypropionate/α-ketoglutarate dioxygenases</i> , Protein Science, Vol. 15, No. 6, pp. 1356-1368 (2006) (“Müller”).
1023	Müller, T.A., et al., <i>Purification and characterization of two enantioselective α-ketoglutarate-dependent dioxygenases, RdpA and SdpA, from Sphingomonas herbicidovorans MH</i> , Applied and Environ. Microbiol., Vol. 72, No. 7, pp. 4853-4861 (2006). 72(7) (“Müller 2006”).
1025	Streber, W.R. et al., <i>Analysis, cloning, and high-level expression of 2, 4-dichlorophenoxyacetate monooxygenase gene tfdA of Alcaligenes eutrophus JMP134</i> , J. of Bacteriol., Vol. 169, No. 7, pp. 2950-2955 (1987) (“Streber”).
1033	Nelson, D.L, and Cox, M.M., <i>Lehninger Principles of Biochemistry</i> , 4th edition, pp. 116-156 (2005) (“Lehninger”).
2001	Fumiyasu Fukumori and Robert P. Hausinger, <i>Purification and Characterization of 2,4-Dichlorophenoxyacetate/α-Ketoglutarate Dioxygenase</i> , J. of Biol. Chem., Vol. 268, No. 32, pp. 24311-17 (1993) (“Fukumori”).
2002	Tina A. Müller et al., <i>Structural basis for the enantiospecifics of R- and S-specific phenoxypropionate/α-ketoglutarate dioxygenases</i> , Protein Sci., Vol. 15, No. 6, pp. 1356-68 (“Müller”) (library stamped copy of Ex. 1020).
2003	Julie C. Dunning Hotopp and Robert P. Hausinger, <i>Probing the 2,4-Dichlorophenoxyacetate/α-Ketoglutarate Dioxygenase Substrate-Binding Site by Site-Directed Mutagenesis and Mechanism-Based Inactivation</i> , Biochem., Vol. 41, No. 31, pp. 9787-94 (2002) (“Hotopp”).
2008	U.S. Patent No. 8,343,747 to Silverstone et al. (issued Jan. 1, 2013)
2013	Provisional U.S. App. No. 60/731,044

Exhibit	Description
2014	Certified File History for U.S. App. No. 12/091,896
2015	Certified File History for U.S. App. No. 13/647,081
2016	Certified File History for U.S. App. No. 14/491,197
2017	WO 2007/053482 A2, published May 10, 2007
2018	Lewis and Stone, <i>Biochemistry, Proteins Enzymes</i> , StatPearls (NCBI Bookshelf) (2023)
2019	Tian and Skolnick, <i>How Well is Enzyme Function Conserved as a Function of Pairwise Sequence Identity?</i> 333 J. MOL. BIOL. 863–882 (2003) (“Tian”)
2020	Tipton and Boyce, <i>History of the enzyme nomenclature system</i> , 16(1) BIOINFORMATICS 34–40 (2000) (“Tipton”)
2021	Rost, <i>Twilight zone of protein sequence alignments</i> , 12(2) PROTEIN ENGINEERING 85–94 (1999) (“Rost 1999”)
2022	Elkins et al., <i>X-ray Crystal Structure of Escherichia coli Taurine/α-Ketoglutarate Dioxygenase Complexed to Ferrous Iron and Substrates</i> , 41 BIOCHEMISTRY 5185–92 (2002) (“Elkins”)
2023	Chung and Subbiah, <i>A structural explanation for the twilight zone of protein sequence homology</i> , 4(10) STRUCTURE 1123–27 (1996) (“Chung”)
2024	Wilson et al., <i>Assessing Annotation Transfer for Genomics: Quantifying the Relations between Protein Sequence, Structure and Function through Traditional and Probabilistic Scores</i> , 297 J. MOL. BIOL. 233–249 (2000) (“Wilson”)
2025	Minshull et al., <i>Predicting enzyme function from protein sequence</i> , 9(2) CURR. OPIN. CHEM. BIOL. 202-09 (2005) (“Minshull”)
2026	Devos and Valencia, <i>Practical Limits of Function Prediction</i> , 41 PROTEINS 98–107 (2000) (“Devos”)
2027	Todd et al., <i>Evolution of Function in Protein Superfamilies, from a Structural Perspective</i> , 307 J. MOL. BIOL. 1113-43 (2001) (“Todd”)
2028	Rost, <i>Enzyme Function Less Conserved than Anticipated</i> , 318 J. MOL. BIOL. 595–608 (2002) (“Rost 2002”)
2029	desJardins, <i>Prediction of Enzyme Classification from Protein Sequence without the use of Sequence Similarity</i> , 5 PROC. INT. CONF. INTELL. SYST. MOL. BIOL. 92–99 (1997) (“desJardins”)
2030	Price and Arkin, <i>PaperBLAST: Text Mining Papers for Information about Homologs</i> , 2(4) MSYSTEMS e00039 1–10 (2017) (“Price”)
2032	Galperin et al., <i>Analogous Enzymes: Independent Inventions in Enzyme Evolution</i> , 8 GENOME RESEARCH 779–90 (1998) (“Galperin”)

Exhibit	Description
2033	Dym and Eisenberg, <i>Sequence-structure analysis of FAD-containing proteins</i> , 10 PROTEIN SCIENCE 1712–28 (2001) (“Dym”)
2034	Minarik et al., <i>Malate Dehydrogenases – Structure and Function</i> , 21 GEN. PHYSIOL. BIOPHYS. 257–65 (2002) (“Minarik”)
2035	Chaparro-Riggers et al., <i>Better library design: data-driven protein engineering</i> , 2 BIOTECHNOL. J. 180–91 (2007) (“Chaparro-Riggers”)
2037	Aziz et al., <i>Physiology, Antibody</i> , StatPearls (NCBI Bookshelf) (2023)
2038	Chica et al., <i>Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design</i> , 16(4) CURR. OPIN. BIOTECHNOL. 378–84 (2005) (“Chica”)
2039	Fox et al., <i>Improving catalytic function by ProSAR-driven enzyme evolution</i> , 25(3) Nature Biotechnology 338–44 (2007) (“Fox 2007”)
2040	Altschul et al., <i>Gapped BLAST and PSI-BLAST: a new generation of protein database search programs</i> , 25(17) NUCLEIC ACIDS RESEARCH 3389–3402 (1997) (“Altschul”)
2041	Müller and Vingron, <i>Modeling Amino Acid Replacement</i> , 7(6) JOURNAL OF COMPUTATIONAL BIOLOGY 761–76 (2000) (“Müller”)
2044	Ng and Henikoff, <i>SIFT: predicting amino acid changes that affect protein function</i> , 31(13) NUCLEIC ACIDS RESEARCH 3812–14 (2003) (“Ng”)
2054	Hausinger, <i>Fe(II)/α-Ketoglutarate-Dependent Hydroxylases and Related Enzymes</i> , 39(21) CRITICAL REVIEWS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY 21–68 (2004) (“Hausinger”)
2055	European Patent No. 1,073,676 B1
2083	Dayhoff et al., <i>A Model of Evolutionary Change in Proteins</i> , 5(3) ATLAS OF PROTEIN SEQUENCE AND STRUCTURE 345–52 (1978) (“Dayhoff”)
2084	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2085	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2086	Protein sequences (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2087	Protein sequences (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2088	Protein assay analysis (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)

Exhibit	Description
2089	Protein sequences (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2090	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2091	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2092	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2093	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2094	Protein presentation (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)
2095	Protein sequences and analysis (CONFIDENTIAL – PROTECTIVE ORDER MATERIAL)