RetroBioCat: Computer-aided synthesis planning for biocatalytic reactions and cascades

William Finnigan[1], Lorna J. Hepworth[1], Sabine L. Flitsch[1]* & Nicholas J. Turner[1]*

[1] Department of Chemistry, University of Manchester, Manchester Institute of Biotechnology, 131 Princess Street, M1 7DN, Manchester, UK

ABSTRACT: As the enzyme toolbox for biocatalysis has expanded, so has the potential for the construction of powerful enzymatic cascades for efficient and selective synthesis of target molecules. Additionally, recent advances in computer-aided synthesis planning (CASP) are revolutionizing synthesis design in both synthetic biology and organic chemistry. However, the potential for biocatalysis is not well captured by tools currently available in either field. Here we present RetroBioCat, an intuitive and accessible tool for computer-aided design of biocatalytic cascades, freely available at retrobiocat.com. Our approach uses a set of expertly encoded reaction rules encompassing the enzyme toolbox for biocatalysis, and a system for identifying literature precedent for enzymes with the correct substrate specificity where this is available. Applying these rules for automated biocatalytic retrosynthesis, we show our tool to be capable of identifying promising biocatalytic pathways to target molecules, validated using a test-set of recent cascades described in the literature.

Biocatalysis is at the nexus of rapidly expanding sequence data, cheaper DNA synthesis, advances in enzyme engineering and a strong need for more sustainable manufacturing processes [1]. Increasingly this means biocatalysis is an attractive option for organic synthesis, particularly where exquisite selectivity is required [2,3]. Mild operating conditions afford enzymes further advantages, in that they can be combined easily into multi-step cascades without costly purification steps, often in a single reactor [4]. Recent industrial examples include cascades for the production of the investigational HIV treatment drug islatravir, as well as a directed evolution campaign towards the synthesis of the Phase II clinical trial drug LSD1 inhibitor GSK2879552 [5,6].

In both organic chemistry and synthetic biology, computer-aided design tools are increasingly used to plan synthesis routes. Tools such as RetroPath have been deployed to develop new metabolic routes to molecules of interest in synthetic biology [7], whilst in chemistry, tools such as Chematica or ASKCOS have been shown to make useful suggestions for synthetic routes to a number of target molecules [8–10]. Despite successes in both of these fields, which biocatalysis spans, computer aided synthesis planning of biocatalytic cascades remains underdeveloped. Enzymatic steps are not well represented in chemical CASP tools, if they appear at all. In contrast, biological CASP tools predominantly feature biosynthetic enzymes, yet the objective of reaching a metabolic starting point, and the use of reaction rules describing transformations in metabolism, does not align well the use of enzymes for organic synthesis (**Figure 1A**).

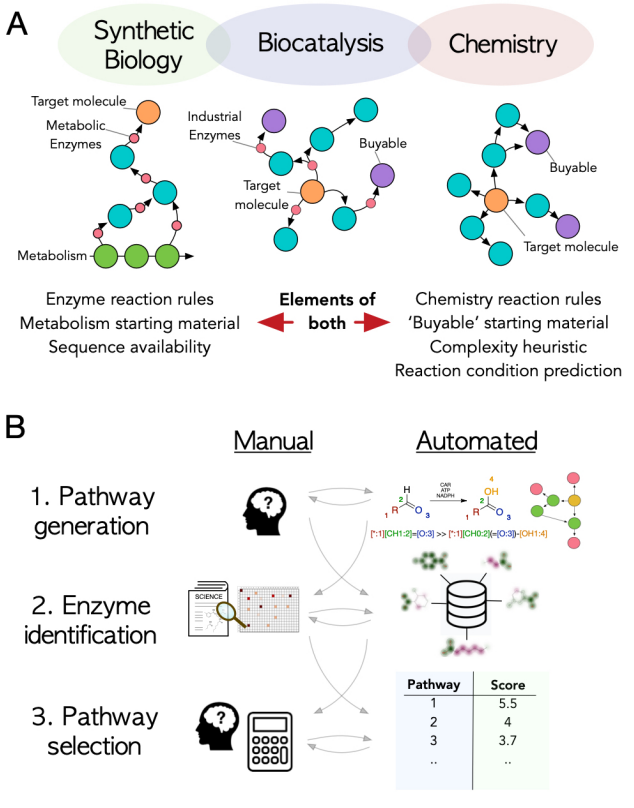

Figure 1. A. A CASP tool for biocatalysis requires elements of both Synthetic Biology and Chemistry. **B.** In the process of generating a pathway, manual and automated processes can be used synergistically for maximum benefit.

Here we present RetroBioCat (available at retrobiocat.com), a tool for computer-aided synthesis planning of biocatalytic cascades. We began the development of RetroBioCat by considering the process a scientist undergoes when planning a new biocatalytic pathway, which typically follows three stages (**Figure 1B**). In the first step, pathways are generated by biocatalytic retrosynthesis. Secondly, specific enzymes are identified for each step, before finally, potential pathways are evaluated based on factors such as the availability of starting materials or the number of steps required. In this work we describe tools which attempt to automate parts of this workflow, seeking to augment the abilities of chemists wishing to exploit the power of biocatalysis.

For the automated generation of pathways to a target molecule, CASP tools typically rely on the use of reaction rules or templates to describe potential retrosynthetic steps, which are iteratively applied until a suitable stopping point is reached (**Figure 1B, Step 1**) [11,12]. Rules can be manually entered [13], or automatically extracted from a database of known reactions [14,15]. Critical to the success of this approach is the level of generalization applied to the reaction rules. Rules which are too specific limit the potential to predict new routes, whilst rules which are too general can lead to unrealistic suggestions [16,17].

Rules to describe biosynthetic reactions have previously been automatically extracted from databases of metabolic reactions, with the option to specify the level of generalization through the selection of a diameter from the reaction center [15]. Integrating these rules into RetroBioCat, we found that whilst this method has clearly been successful in generating new biosynthetic routes [18], literature examples of biocatalytic cascades were either not represented, or required the most extreme promiscuity setting in order to be captured. Crucially, treating all enzymes in metabolism as very promiscuous is unrealistic, yielding a large number of unhelpful results.

As an alternative, we developed a smaller set of expertly encoded reaction rules to describe the enzyme toolbox for biocatalysis (**Figure 2A**) [19–21]. These rules were made relatively general in most cases to reflect established substrate promiscuity. In addition, we included some necessary spontaneous chemical steps. Importantly, in many cases the enzymes these rules represent have been shown to be amenable to enzyme engineering, for the acceptance of highly synthetic substrates [2,22–25]. The current rule set consists of 83 reactions, described using 107 reaction SMARTS.

A system for identifying specific enzyme sequences to carry out each step is also necessary for automated cascade design. Manually, scientists typically rely on extensive literature searches or enzyme screening panels to compete this step (**Figure 1B, Step 2**). To automate this process, a database of literature precedents for synthetic biotransformations is required. However, whilst there are many well-established enzyme databases, these tend to focus on biosynthetic reactions rather than examples of synthetic biotransformations utilized in biocatalysis. Therefore, to demonstrate this step we have begun the construction of a database of synthetic biotransformations, including an approach to handle the comparison of data of different types (**Supplementary Figure 2**). We created a module within RetroBioCat to score reactions based on their similarity to recorded reactions, through the use of fingerprint similarity (**Figure 2B**) [26]. Where many enzymes have been shown to catalyze a specific reaction, our approach selects the best as ranked by activity. Whilst the determinants of substrate specificity may be more complex than can be captured by fingerprint similarity alone, the selection of similar substrates allows a chemist to quickly access the relevant information to make the final decision.

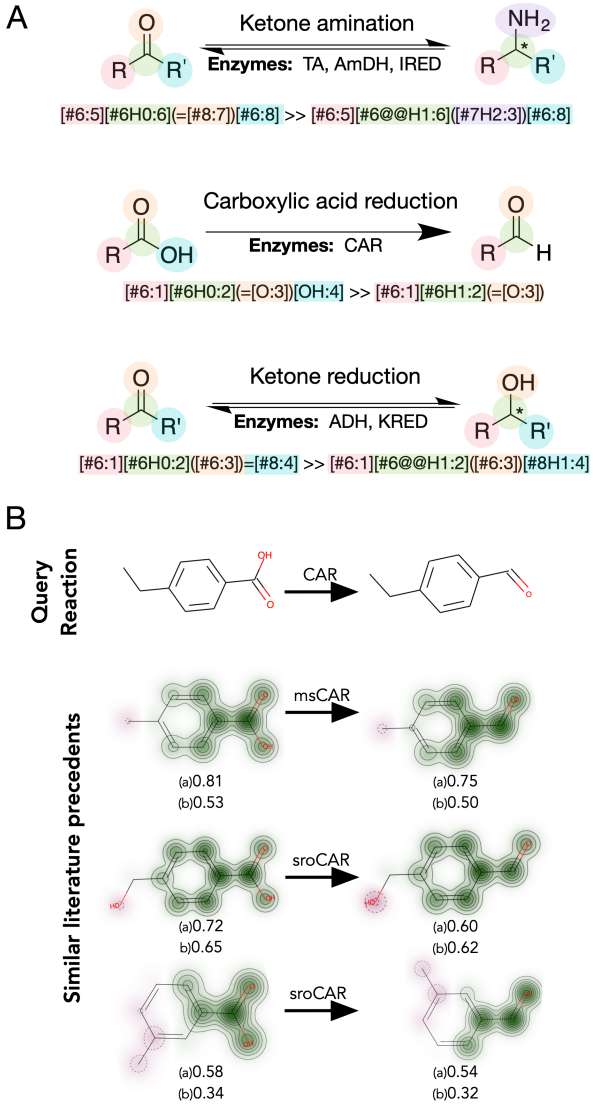

Figure 2. Critical components of RetroBioCat. A. A selection of exemplar reaction rules for industrially relevant enzymes, written as reaction SMARTS. **B.** An example query for similar reactions present in a database of literature precedents. For each molecule, visualization of the atomic contributions to the morgan fingerprint similarity is shown. Below each molecule, the Tanimoto similarity [27] is calculated for (a) RDKit fingerprints and (b) Morgan fingerprints, each using the default settings in RDKit. The best CAR enzyme identified for each reaction is shown.

Finally, many chemistry CASP tools feature a metric for molecular complexity to help guide the retrosynthetic search towards a simpler starting material. RetroBioCat uses the recently described SC-Score, which utilizes a neural network trained on a large number of synthetic chemistry reactions to score the complexity of each molecule between 1 and 5 [28]. Ap-

plied to biocatalysis, this score appears to function well in guiding pathway suggestions towards synthetically useful routes (**Supplementary Figure 1**).

Having established a set of rules describing important reactions in biocatalysis (**Figure 2A**), a method for searching for similar reaction literature precedents (**Figure 2B**), and a complexity metric by which to guide retrosynthetic searches (**Supplementary Figure 1**), we developed two complementary approaches for exploring potential biocatalytic pathways. Firstly, a network exploration mode for 'human-led' CASP, in which the user can explore different routes to a target molecule by expanding a network of biocatalytic disconnections (**Figure 3**). Alternatively, a pathway exploration mode, in which pathways are automatically generated before being ranked according to a user-defined weighted score (**Figure 4**). Importantly, both approaches are primarily available through an interactive web-app, but also as an open-source python package for expert users.

In particular, the network exploration mode can be useful for scientists who may not be familiar with biocatalysis, allowing them to visualize potential biocatalytic disconnections to their target molecule. Furthermore, the ability to add custom reactions onto the graph allows chemical steps to be included by the user in the retrosynthetic search, for the creation of powerful chemoenzymatic cascades. Integrated is the enzyme identification module, which colors reaction nodes green where a similar literature precedent is identified, or red where only negative data has been reported. Further data on substrate specificity, 'buy-ability' or molecular complexity is also available through the interactive graph. For example, hovering over a green node displays further data on the activity and literature source for that enzyme (**Figure 3**)

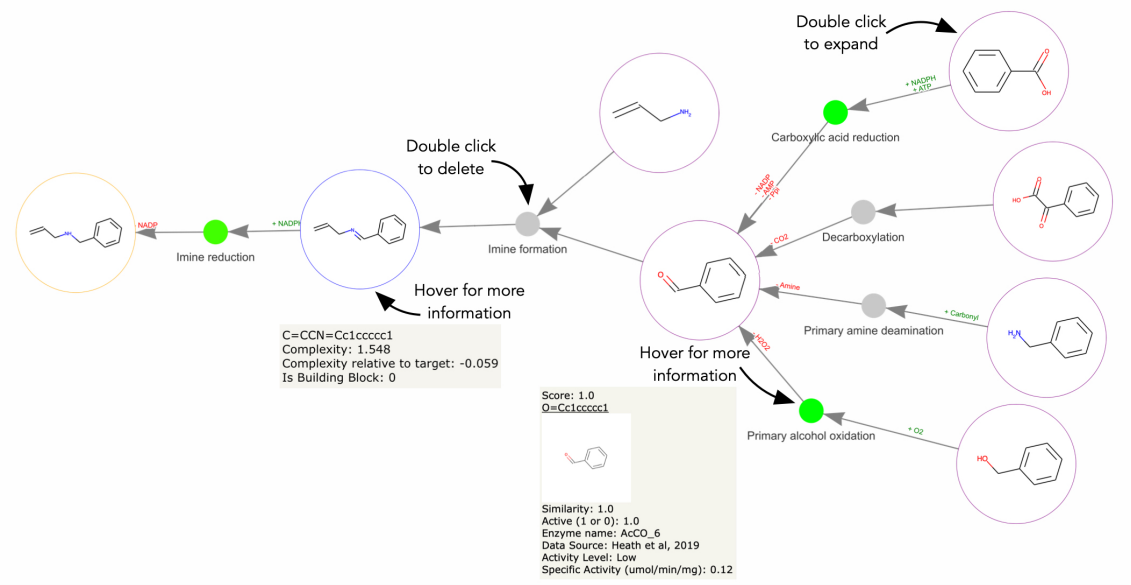

Figure 3. 'Human-led' exploration of a network of potential biotransformations using 'Network explorer'. Each substrate node can be iteratively expanded to reveal further possible biotransformations. Reaction nodes in green indicate high similarity to a literature reported reaction (currently a proof-of-principle dataset). The target molecule is outlined in orange, and 'buyable' compounds outlined in purple.

Alternatively, the pathway exploration mode seeks to automatically generate useful suggestions for possible pathways to a target molecule. To do this, a network is first generated by applying reaction rules iteratively up to a user-defined maximum length. Networks which reach a user-defined limit to the number of nodes are reduced in size by removing the outer-most 'worst' reactions, as scored by the change in molecular complexity. Pathways are then generated by a best first search approach, which prioritizes steps with higher changes in molecular complexity until all possible pathways have been generated, or a limit to number of pathways is reached. A weighted score is used to evaluate and rank each pathway, taking into account the change in molecular complexity, the number of steps, whether the starting material appears in a catalogue of buyable building blocks, and the number of steps with a similar literature precedent.

To test both approaches, we carried out a thorough review of the biocatalytic cascades reported in the literature, generating a test-set of 52 pathways (**Figure 4, Supplementary Figure 2**). Except for C-H oxidation by P450 enzymes, all of the reactions in the test-set were correctly predicted by RetroBioCat. Importantly, the majority of pathways were suggested within the top few suggestions using pathway explorer with only the default settings for the weighted score, validating this as a useful approach for the automated design of biocatalytic cascades (**Supplementary Figure 2**). Details of the approach used by RetroBioCat for both network and pathway explorer are available in the supporting information.

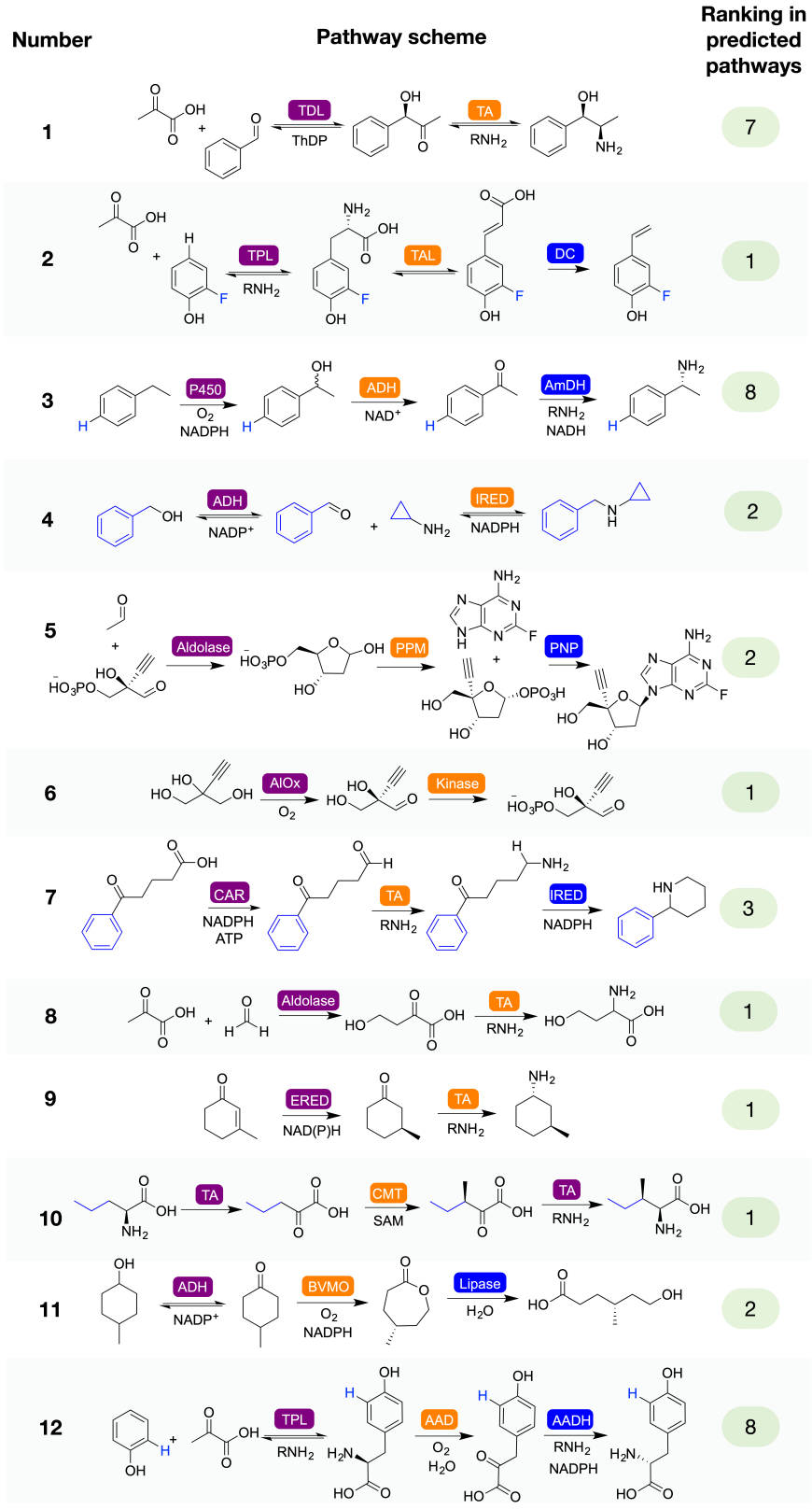

Figure 4 An example selection of some of the biocatalytic cascades identified in the literature and used as a test-set. The complete set of 52 cascades is available in the supporting information.[5,29–70] In some cases, a number of cascades were demonstrated with different R groups, for which we have chosen a single example, highlighted in blue. Pathway rankings by RetroBioCat using a maximum of 4 steps and the default weights for molecular complexity, the number of steps, the number of enzymatic steps similar to literature precedents, and the availability of the starting material, are shown. Pathways are marked as identified even where RetroBioCat suggests additional steps.

CASP tools should strive to augment the abilities of scientists seeking to design new routes to a target molecule. An intuitive and easy to use user-interface, as we have developed for RetroBioCat, is therefore crucial. Furthermore, the manually curated reaction rules utilized by RetroBioCat strike a balance between being general enough so that the potential for enzyme engineering or discovery is captured, whilst providing context where necessary so as to be realistic.

Suggestions for potential biocatalytic transformations even without literature precedent are themselves a valuable resource, as in many cases enzyme screening panels can be employed to find the right enzyme for a specific reaction. However, where there is literature precedent for a reaction, suggestions are more robust and easier to implement if these are automatically identified. Here, we have demonstrated the use of molecular similarity to automate this process and have begun the construction of a database of synthetic biotransformations described in the literature, with further contributions to be reported in future work.

Pathway explorer offers automated ranking of suggested pathways using a selection of metrics. We show that this functions well in suggesting previously reported pathways early in the ranking system. Future improvements could seek to provide further information on the suitability of each suggested pathway. For example, thermodynamics, cofactor usage, substrate and product solubility, toxicity, reaction conditions, starting material price and predicted pathway kinetics could all offer more insight into which pathway is the most promising for experimental characterization.

Several challenges still remain for the refinement of RetroBioCat. For example, at present enzymatic C-H activations such as hydroxylations and halogenations are currently not fully included, as the context in these reactions rules requires more careful consideration. Additionally, larger, more complex target molecules are sometimes handled inadequately by RetroBioCat, possibly due to a need for more bond forming enzymes in biocatalysis. Future integration of CASP tools for organic chemistry will allow better solutions to be suggested in cases where some chemical steps may be necessary. Additionally, incorporating the reaction rules developed for metabolic engineering could further blur the lines between biocatalysis and biosynthesis.

In summary, RetroBioCat offers an accessible set of tools for computer-aided design of biocatalytic cascades. These tools should be useful in highlighting the potential of enzymes for organic synthesis, and for the design of de novo biocatalytic pathways.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supporting Information (.docx) – contains details of the approach adopted by RetroBioCat and the supporting figures

AUTHOR INFORMATION

Corresponding Authors

* Nicholas J. Turner: Nicholas.turner@manchester.ac.uk
* Sabine L. Flitsch: sabine.flitsch@manchester.ac.uk

Author Contributions

Code for RetroBioCat written by WF. Pathway test-set generated by LJH. Initial draft of the manuscript written by WF, with subsequent contributions from all authors. All authors have given approval to the final version of the manuscript.

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