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Retrosynthetic design of metabolic pathways to chemicals not found in nature

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Abstract

Biology produces a universe of chemicals whose precision and complexity is the envy of chemists. Over the last 30 years, the expansive field of metabolic engineering has many successes in optimizing the overproduction of metabolites of industrial interest, including moving natural product pathways to production hosts (*e.g.*, plants to yeast). However, there are stunningly few examples where enzymes are artificially combined to make a chemical that is not found somewhere in nature. Here, we review these efforts and discuss the challenges limiting the construction of such pathways. An analogy is made to the retrosynthesis problem solved in chemistry using algorithmic approaches, recently harnessing artificial intelligence, noting key differences in the needs of the optimization problem. When these issues are addressed, we see a future where chemistry and biology are intertwined in reaction networks that draw on the power of both to build currently unobtainable molecules across consumer, industrial, and defense applications.

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Synthetic biology, Systems biology, Artificial intelligence, Machine learning.

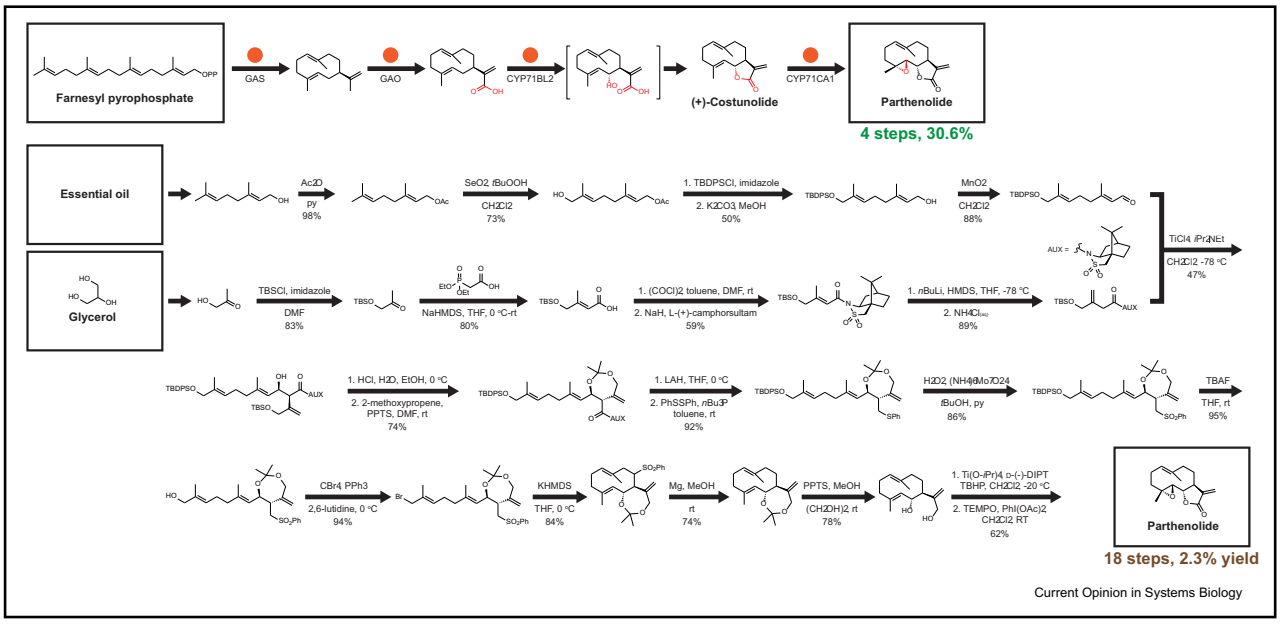
Introduction

When posed with a new molecule to build, organic chemists have to work backwards. Known as 'retrosynthesis,' they identify the series of chemical transformations that can construct the target from simpler chemical building blocks derived from petroleum or other sources [1]. Chemicals that are large and complex with many functional groups and stereocenters have required Herculean efforts to build; for example, halichondrin B has 32 stereocenters (4 billion isomers) and requires a sprawling total synthesis whose longest linear path is 47 reactions [2]. Solutions have been found to incredibly challenging retrosynthesis problems, many of which are recorded in the tomes Classics in Total Synthesis I, II, and III [3–5]. Human creativity has been the power behind these solutions, but as the literature size has grown to an unmanageable size, computer algorithms have been developed to guide the identification of reaction paths [**6].

Cells are the envy of chemists as they are able to build complex chemicals at high yields under ambient conditions. They excel in dictating patterns of stereochemistry, and their products are impossibly functionalized. In fact, many of the most lauded retrosynthesis efforts are to rebuild the chemicals made by biology, for example, hard-to-source pharmaceuticals, which can be built with fewer steps biosynthetically (Figure 1). With a view of the complex chemicals built by the natural world, it is clear that it would be revolutionary to be able to harness these processes to build unnatural molecules of such complexity by design.

Reaching this vision has been inhibited by several challenges. Only a fraction of the enzymes sequenced have been characterized. Their high specificity makes it more difficult to mix and match them between pathways as part of retrosynthetic effort. Before the emergence of DNA synthesis, it was difficult to come by the genetic material for enzymes described in the literature [7]. Physically combining enzyme-encoding genes to build a pathway has only recently been made possible with genetic part libraries to control expression, DNA assembly to put the enzymes together, 'Foundries' to build and debug designs, and methods to work with nonmodel organisms [8–16]. Directed evolution has been able to expand enzyme specificity to more substrates and catalyze new reactions, including those not known to occur in nature [17,18]. Artificial intelligence is increasingly being applied to the design process.

Figure 1



Comparison of metabolic and chemical routes to parthenolide [339,340]. The pathway has been identified and transferred from its native organism (*Tanacetum parthenium*) to yeast, and the theoretical yield of the biosynthetic route is shown (0.306 g/g glucose).

The rise of the bioeconomy will see biochemistry merge with organic chemistry as the means by which novel chemicals are discovered and produced. This manuscript reviews the first steps of this process towards the automation of retrosynthetic design in biochemistry. Like its counterpart in chemistry, the goal of metabolic retrosynthetic design is to start with a desired chemical and then determine the enzymatic transformations required to build it from metabolites made by the cell. First, we review the metabolic retrosynthesis projects completed to date. As in chemistry, they have relied on human expertise and creativity, and the largest projects have been to rebuild natural products in a production strain (*e.g.*, move a plant pathway to yeast). Second, we will describe efforts to mine enzymes and evolve their specificity and ability to perform reactions not found in nature. Finally, we will review recent efforts to develop algorithms where a user can specify a chemical structure and the software identifies the combination of enzymes required to build it in a cell.

Classics in total retrobiosynthesis

Moving natural product pathways from plants to production hosts, such as yeast, makes up the most complex metabolic engineering projects to date. All the biosynthetic enzymes are present in the plant, and in some cases, they can all be moved into the new host together [19–22]. This is not always possible as an enzyme may be unknown or it is nonfunctional in the new host due to misfolding, localization, missing cofactors, precursor supply, feedback inhibition, or differences in the intracellular conditions. This is solved by replacing portions of the pathway sourced from different organisms. Figure 2a shows published pathways that are not entirely sourced from a single organism and require more than two enzymatic steps from the nearest cellular metabolite [**23–56].

The route to noscapine, an anticancer alkaloid derived from the opium poppy, is the most complex example to date [**23]. A pathway was constructed in yeast to make noscapine from tyrosine, requiring 18 enzymatic steps, of which only 13 involve enzymes from the opium poppy (*Papever somniferum*). Of the remaining five, one is from the brown rat (*Rattus novegicus*), one from bacteria (*Pseudomonas putida*), and three from other plants (*Coptis japonica* and *Eschscholzia californica*) [**23,*57–59]. Another example is the reconstruction of the tetrahydrocannabinol (THC) pathway from *Cannabis sativa* in yeast [39,60–64]. Only 5 enzymes were from the native plant, with the remaining 6 sourced from four diverse bacterial species. Both the noscapine and THC projects required protein engineering to alter the enzymes to eliminate feedback inhibition and alter localization tags. Broad enzyme substrate specificity was recognized as being critical in combining enzymes from diverse sources to form a new pathway.

Xenobiotic molecules are those not known to occur in nature. Retrobiosynthetic design has been applied to build these compounds by combining a series of enzymes that synthesize it from a cellular metabolite

[**24,26,**28,41–47,65–71]. To our knowledge, only a few such pathways have been constructed that combine three or more enzymatic steps (Figure 2b) [**28,**44,**45]. The construction of these pathways is challenging because, by definition, enzymes have to be found to act on chemicals that are different than their native substrates. As a result, the molecules produced to date are much simpler than those obtained by rebuilding natural pathways and do not approach the complexity known to be possible with biology. The longest published retrobiosynthetic pathway is from the metabolite *a*-ketoglutarate to 6-aminoacprotic acid (6-ACA), which is a precursor for nylon-6 [*72]. Constructed in *Escherichia coli*, it combines enzymes from archea (*Methanococcus aeolicus*) and bacteria (*Azobacter vinlandii*, *Lactococcus lactis*, and *Vibrio fluvialis*) [26,*72–76]. To find these, a panel of 3–15 enzymes had to be screened for each catalytic step, and the top enzymes were noted to have broad substrate specificity [*72,73].

Metabolic pathways can also be introduced into cells to modify a chemical added to the media that is not a natural metabolite. Retrosynthetic design can be applied to identify enzymes from different sources to convert the chemical to a desired product (Figure 2c) [54–56]. Both azelaic acid and carvolactone are found in nature but are not part of the *E. coli* metabolome. An example of a xenobiotic compound is *p*-bromophenylglycine, which was produced as part of an effort to create green routes to α-amino acids that avoid cyanide-based chemistry [56]. This pathway combines four heterogenous enzymes from diverse bacteria (*Pseudomonas* sp. VLB120, *Sphingomonas* sp. HXN-200, *Pseudomonas putida*, and *Streptomyces coelicolor*) with a step performed by a native *E. coli* enzyme.

Mining the world's enzymes

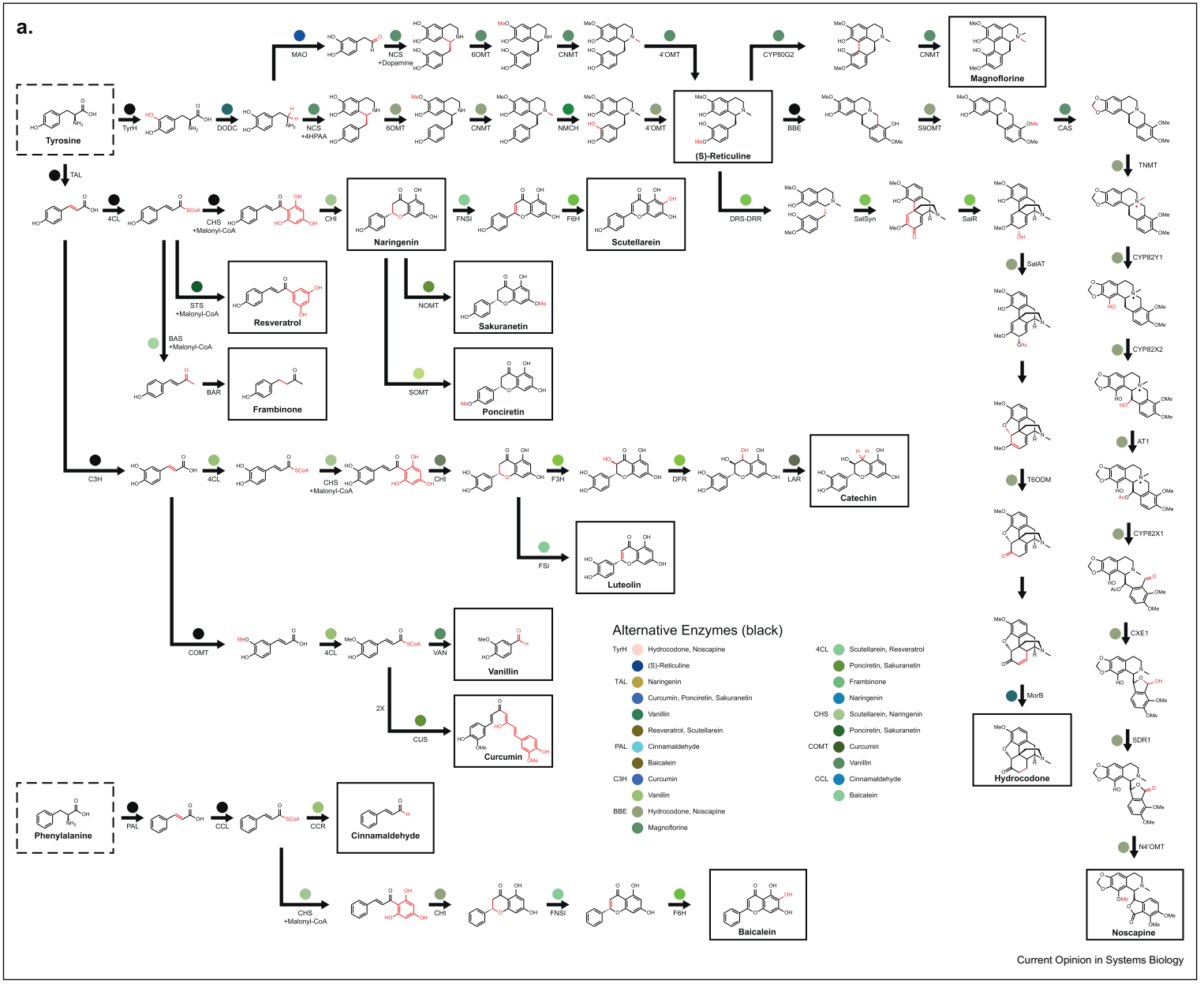
Metabolic retrosynthesis projects require having access to enzymes that will perform the necessary catalytic steps to convert a metabolite into the desired product. An enzyme's specificity is defined as the range of substrates on which it will perform its function. Some enzymes, especially those in central metabolism, are highly specific whereas others are promiscuous. When building a pathway, the specificity profile is crucial information to select an enzyme to function on an intermediate that is not its natural substrate. Making this choice is complicated by the relatively few well-characterized enzymes, particularly with respect to specificity information. However, the convergence of new technologies suggests that we are reaching an inflection point and a deluge of enzyme data is forthcoming.

The number of enzymes represented in earth's biodiversity is extraordinary, and only a tiny fraction has been experimentally characterized. DNA sequencing and environmental metagenomics are starting to illustrate this potential (Figure 3a). Of the estimated 10^{31} nucleotides on earth, 10^{12} have been sequenced and are available in the NCBI database (as of 2019) [77]. It is estimated that the database holds 40 million unique enzymes, a number that doubles every 24 months [78–*82]. Experimentally keeping up with this seems overwhelming, but the convergence of informatics, DNA construction, automation, and screening technologies are increasingly exploiting the information in databases as a resource.

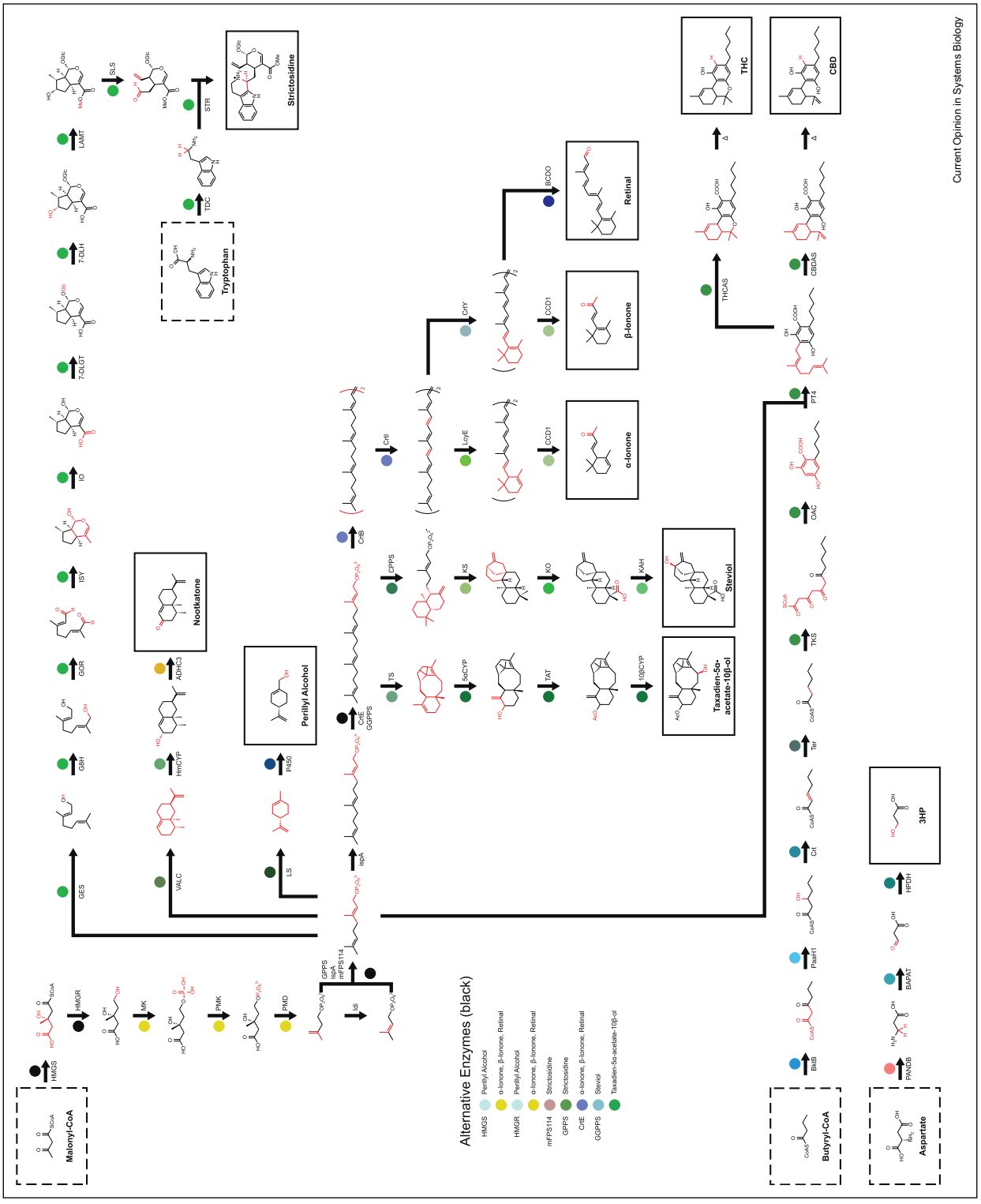
Part mining refers to the now common practice of converting sequence information in a database to physical genes and then screening for a desired function. Figure 3b shows enzyme mining efforts described in the literature, and many more such screens have been performed at companies to populate internal nonpublic databases. Bioinformatic tools to select genes have been getting more sophisticated. Originally, only sequence similarity to a target enzyme guided the search using algorithms such as the Basic Local Alignment Search Tool (BLAST) [78,83–95]. Metabolic databases have compiled reaction and pathway information (Kyoto Encyclopedia of Genes and Genomes (KEGG) and MetaCyc) [96–98]. BRENDA contains manually curated biochemical data for enzymes, including mutants, and substrate profiles [99]. It is increasingly common for genes to be sequenced in the context of genomes (Figure 1a), and local genetic context can be used to help predict its substrate [100–**102]. If one is trying to mine enzymes with diverse activities or substrate profiles, sequence similarity networks can help guide diversity into unexplored regions [103–106]. Homology modeling and computed ligand docking, while notoriously inaccurate in making specific predictions, are still useful to downselect sequences for screening [**104–106].

Once a set of genes is identified, physically building the DNA is straightforward, given the cost, accuracy, and throughput improvements achieved by synthesis companies. Even a low-budget project can now build 100s of enzymes, and while building all 255,463 sequenced P450 enzymes in NCBI is currently cost limited (~$30 million), the continuous decline in price may soon even make that possible [78,107,108]. Screening even a small set of genes can be time consuming, particularly when the chemistry is not conducive to a high-throughput assay. This has been addressed recently through robotic automation, miniaturization of the reaction vesicle using droplets (10^8 reactions per day), and microcapillaries (10^6 per chip) [*105,**109–114]. It is more challenging to obtain the substrate profiles for a large number of enzymes, as opposed to their activity against a single

Figure 2



Classics in total retrobiosynthesis are shown for the production of **(a)** natural chemicals in a heterologous host, **(b)** xenobiotic compounds, and **(c)** the conversion of exogenous chemicals to a product [**23–40,42,**44,46,48–56,68–71]. Pathways are shown for 42 products made from 15 precursor metabolites or exogenous chemicals (black boxes). The pathways are based on enzymes from 80 species (colored dots above reaction arrows and legend). The pathways are compiled from multiple projects, and if different enzymes were used to perform the same reaction across these projects, these are shown as a black dot (insets expand to show all enzyme sources). Enzymes native to the chassis organism are named but without a colored dot. Spontaneous reactions have no labels or dots. This figure is intended to be comprehensive for all the pathways we could identify in the literature. Some famous metabolic engineering examples are excluded because they involve the production of native metabolites (e.g., isobutanol) or require less than two steps from a native metabolite (e.g., propanediol) or all the enzymatic steps from a native metabolite are sourced from a single organism (e.g., artemisinin) [65,86,341–344].



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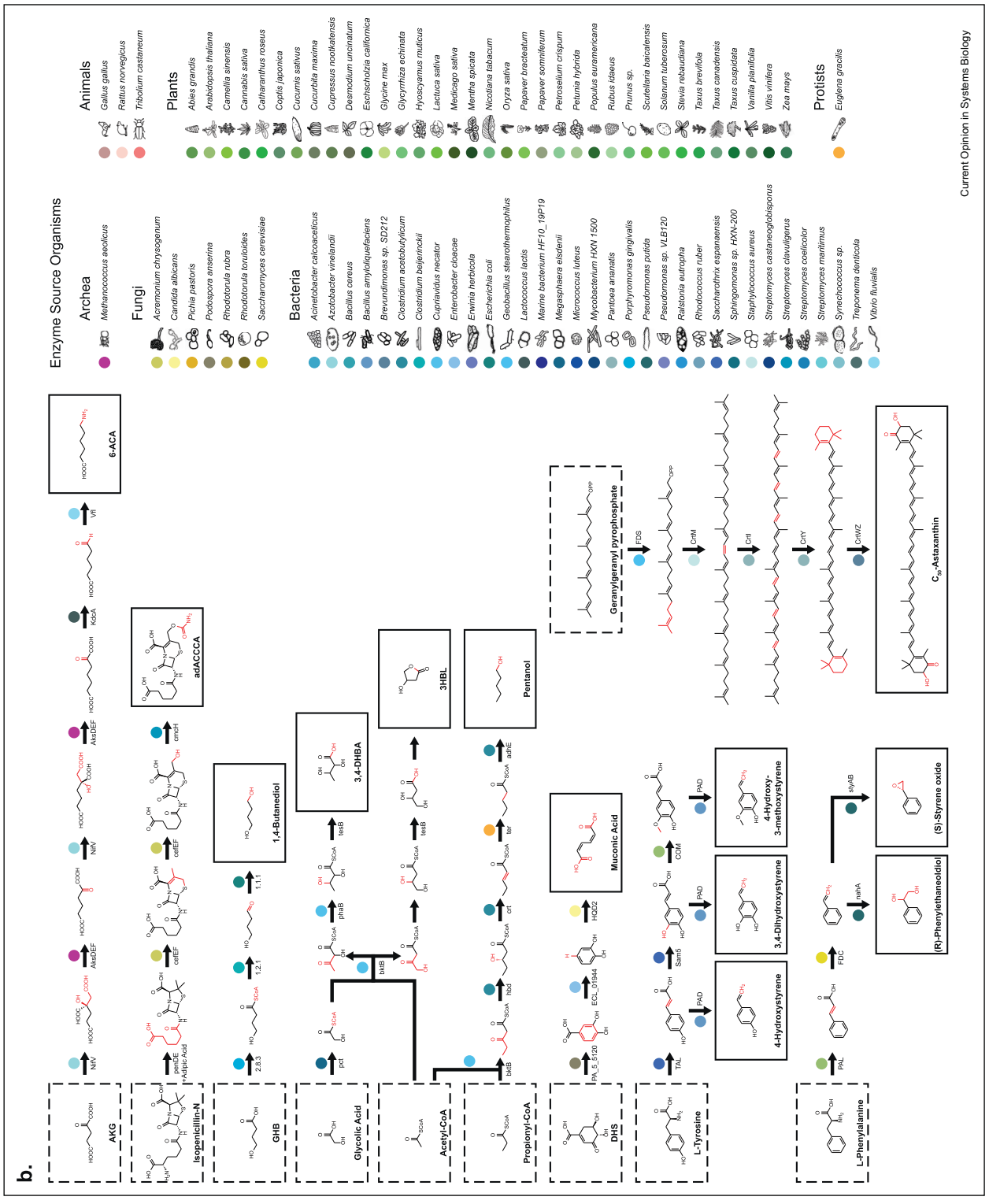

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Figure 2

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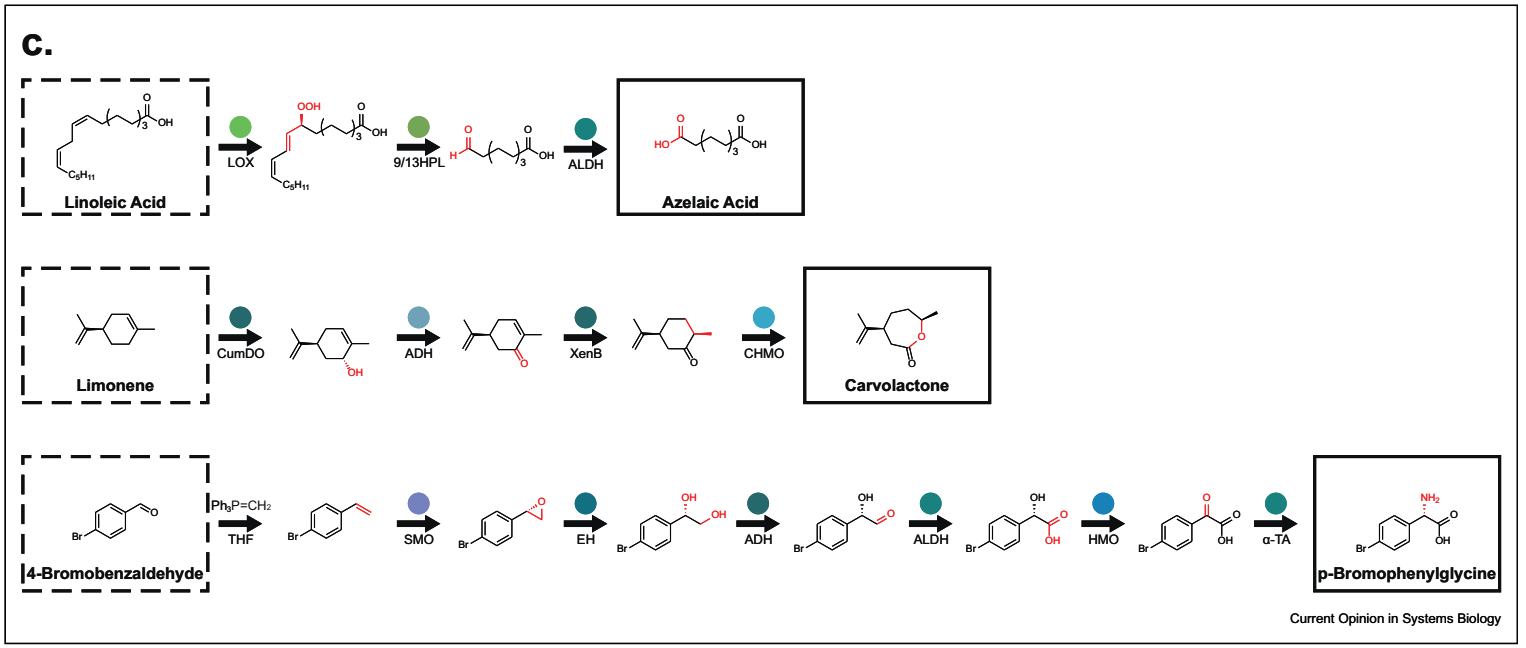

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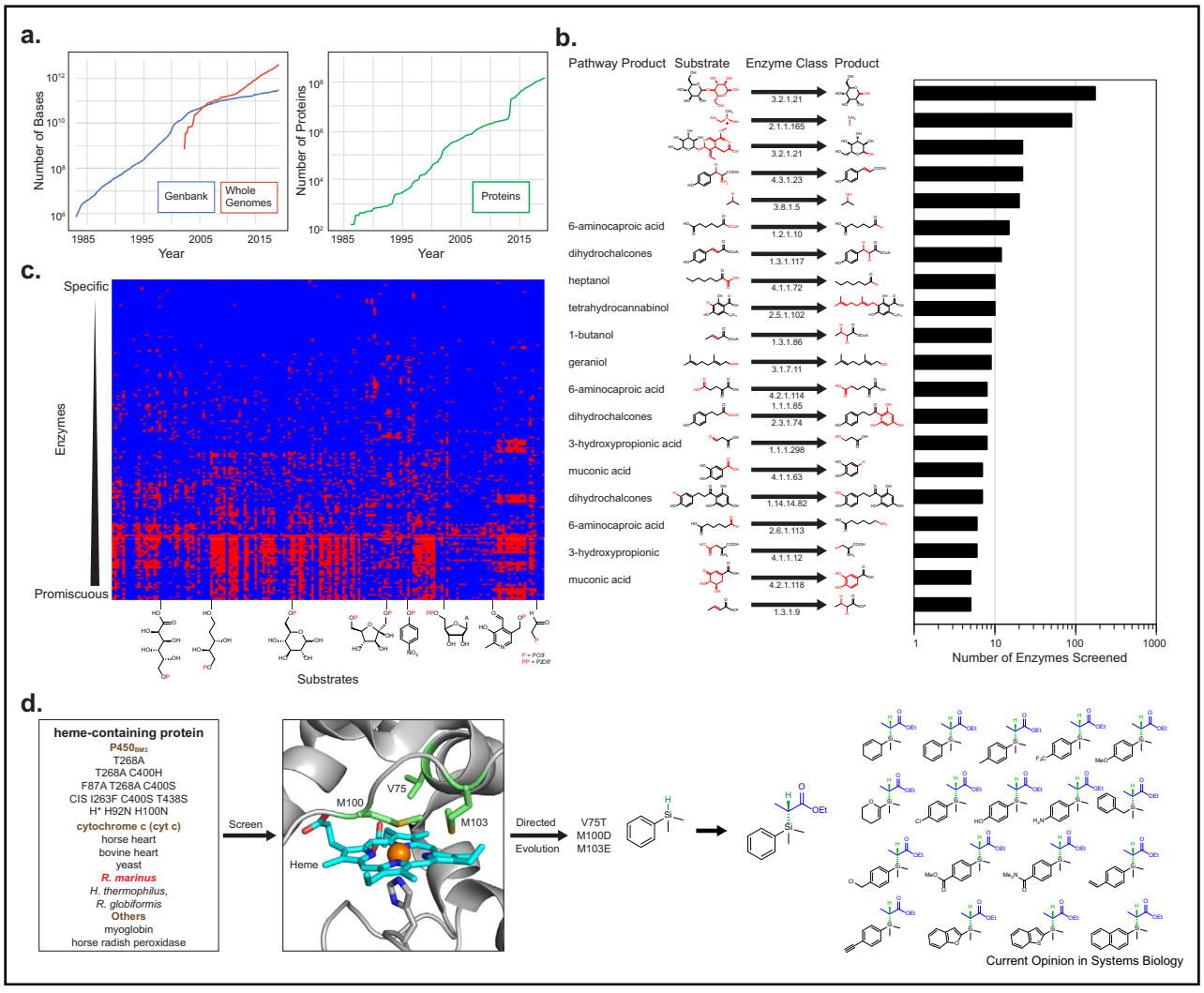
Figure 3



Enzyme mining efforts. **(a)** Number of bases in NCBI Genbank database and proteins in NCBI RefSeq database [81,345] **(b)** Enzyme mining efforts from the literature that involve five or more candidates obtained from a sequence database using DNA synthesis [27,**28,39,*72,87–89,92–95,**104,*105,346]. If the mining effort was part of a metabolic engineering project, the target molecule is listed. The EC number of each enzyme is shown along with the reaction for which it was screened. **(c)** A massive enzyme specificity screen is shown for 217 haloalkanoate dehalogenases against 167 substrates (of which 8 representative examples are shown on the x-axis) [**126]. Red dots show the substrates that each enzyme will catalyze. **(d)** A combined enzyme mining–directed evolution experiment is shown to make an enzyme that catalyzes the enantioselective carbene insertion into Si-H bond [**122]. The resulting enzyme is promiscuous, and the varied products are shown.

substrate. When the reaction is coupled (e.g., NAD^+ is produced), then this can form the basis for a high-throughput screen that is substrate agnostic [115–118]. Substrates can also be spotted onto a microarray and a specificity profile obtained in a single experiment, although this will not be compatible with all chemistries [119]. A more generalizable approach is to use metabolomics to multiplex many substrates (10^3) in a single reaction [115,**120].

Enzyme promiscuity

Promiscuous enzymes are often preferred when building a new biosynthetic pathway or when initiating an enzyme engineering project for a new substrate target [26,**28,121,**122]. Even a small amount of activity can provide the 'hook' that can then be optimized via directed evolution [112,123–125]. The degree of promiscuity can widely vary even within the same enzyme class. In one extreme case, a dehalogenase was active against 143 of 169 substrates tested, whereas 53/217 of the enzymes were active on <5 substrates (Figure 3c) [**126]. When evolving an enzyme to a new target, it is often observed that intermediate mutants increase in promiscuity before increasing activity and specificity in later rounds [123,127–131]. Even a single mutation can switch an enzyme from being specific to promiscuous. A

goal could be to create a 'universal' set of enzyme functions with representatives of each possible reaction type. For part mining, bioinformatic tools may be able to make mutations prior to synthesis that are predicted to increase the promiscuity of the target, for example, by creating a more spacious ligand-binding pocket [80,**104,*105,130–144].

Enzymes also exhibit catalytic promiscuity, where the catalytic residues perform alternative reaction chemistries [18,136,145–149]. Enzymes can be evolved to perform functions not found in nature and difficult to perform chemically, such as stereospecific carbene insertion into carbon–carbon [**150], silicon–hydrogen [**122], or boron–hydrogen [**151] bonds. The parent protein can be surprising: an enzyme that produced enantiopure organosilicon chemicals was found by making three mutations to cytochrome *c*, an electron transfer protein that does not perform a catalytic function in nature (Figure 3d) [**122]. This suggests that organizing part mining efforts by cofactor (e.g., heme-containing) or potential chemistries may aid screening efforts. Computational protein design has also been used to initiate new catalytic functions into a scaffold, including a retro-aldolase, Kemp eliminase, and Diels-Alderase [152–155]. Even though the initial activities are usually low, the computational methods can provide fodder for directed evolution optimization [**109].

Promiscuity is not a problem when purified enzymes are used as catalysts for a chemical process; in fact, it is an advantage as the same enzyme could be integrated into the processing of multiple chemical reactions [121,**122,138]. However, it can pose problems when trying to combine enzymes to build a biosynthetic pathway. A promiscuous enzyme is more likely to act unintentionally on natural cellular metabolites. The resulting products draw resources away from the cell, could be toxic, and these reactions could use up energy and redox resources (*e.g.*, ATP and NADPH) [156]. They can also generate multiple products, complicating purification [**104]. This creates a tension in the discovery process, where promiscuity makes it easier to find an enzyme that acts on a new target but harder to incorporate into an *in vivo* biosynthetic pathway. Computational tools and screening strategies have been developed to aid this process [**104,157].

Chemistry: towards retrosynthesis by artificial intelligence

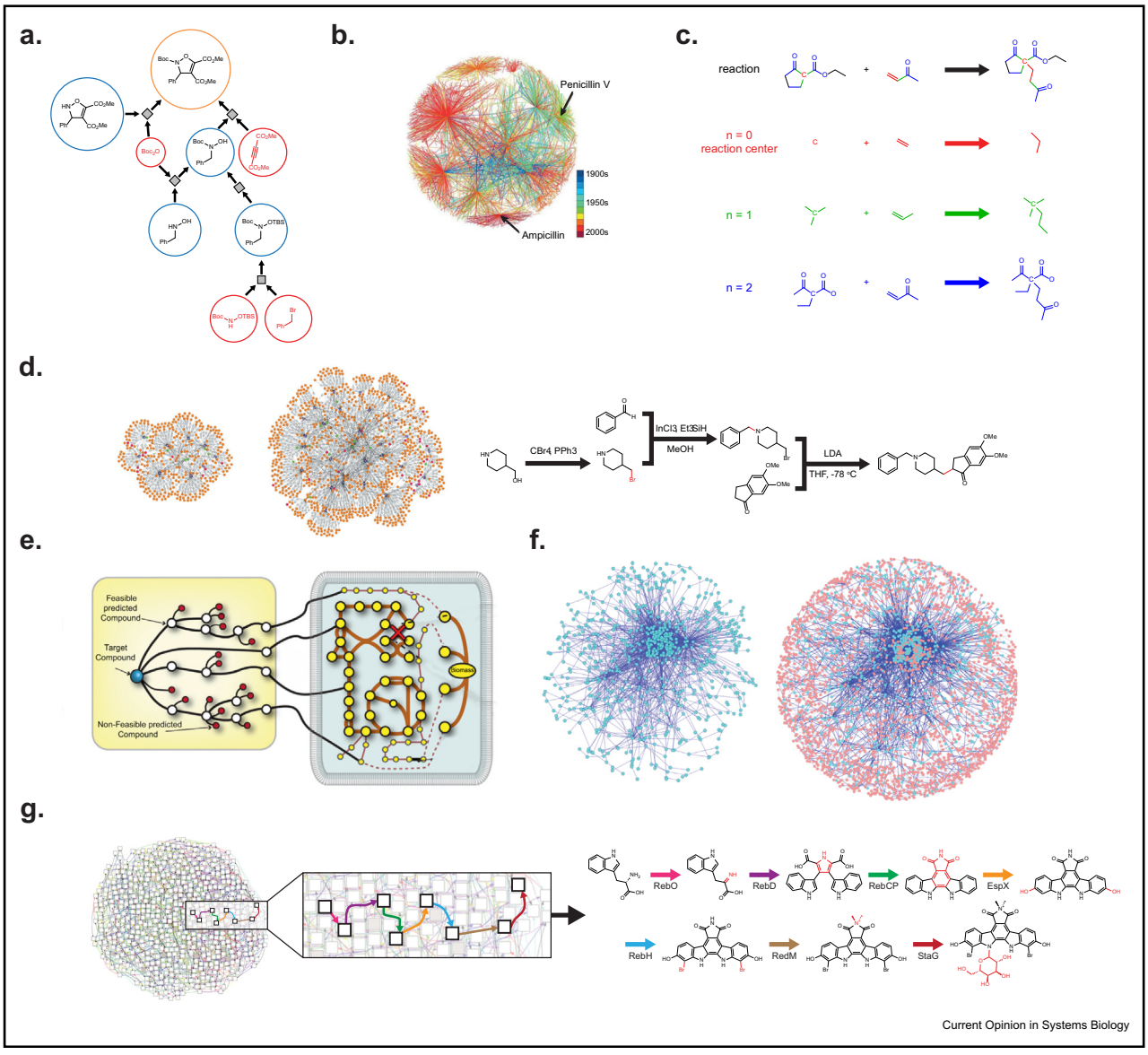
Since the 1960s, the potential for computer algorithms to augment the retrosynthetic decision-making process has been recognized [158,159]. The fundamental challenge of retrosynthesis, both for a human and a computer, is that there is a combinatorial explosion of possible reactions as one iteratively steps backwards towards simpler compounds. One can imagine this as a graph, where each node is a chemical and the edges are reactions (Figure 4a). The goal of the algorithm is to identify a path from the target molecule to precursors that are commercially available [**6]. This search is nontrivial: for the halichondrin B example involving 47 steps, assuming an average of 100 potential reactions per intermediate [**160], there are $\sim 10^{94}$ paths. Retrosynthetic software packages differ in how they build this space using reaction rules, search the space, and weigh individual steps and complete pathways (Table 1). Artificial intelligence is increasingly being applied to problems in organic chemistry, including all aspects of retrosynthesis [**161–170].

As the chemical literature has grown, it has become increasingly challenging to manage the decision-making process and make researchers aware of the possibilities. To address this, a 'Network of Organic Chemistry' has been constructed on the basis of every reaction published in the literature (maintained in the Reaxys database) [163,164,171–175]. As of 2019, the network has 35 million chemicals (1000-fold more than the human metabolome) [**6]. This graph can be used for retrosynthesis by finding the node corresponding to the target molecule and then using a routing algorithm to identify a path to commercially available substrates (Figure 4b) [**6,176–**178]. Each path is feasible because it corresponds to a series of experimentally validated reactions. However, this approach is limited by only being able to predict how to build molecules present in the network and the reactions are sparse, thus limiting the possibility of discovering creative or overlooked possibilities.

The alternative is to condense the reaction information into 'rules' that represent moves that can be made in the networks. Rules represent generic reactions that can be applied to obtain the target chemical when certain functional groups are present, to generate precursor(s) one-step back. The rules may include the atoms surrounding the reaction centers (chemical environment, Figure 4c) and incompatible functional groups that should be avoided. They represent an informed prediction that allows them to be applied to chemicals for which there is not an exact match in the literature. There is a trade-off where too little detail in the rule leads to unrealistic chemistry but too much reverts to the specific reactions (those described by the Network of Organic Chemistry) [163,175]. Each algorithm has a different approach to deal with this trade-off in an attempt to build accurate yet broadly applicable rules.

Early retrosynthesis algorithms were based on databases of rules that required meticulous data entry by experts [**6,163,168,169]. For example, the Chematica software uses a set of ~60,000 rules [**6]. The exponential growth of the literature has made human curation no longer feasible. ARChem Route Designer automatically extracts rules from reaction databases, but its focus on atoms close to the reaction center leads to errors because

Figure 4



Retrosynthetic design algorithms for organic chemistry and metabolic engineering. **(a)** A simple retrosynthetic search is shown from a starting compound (orange) to the available compounds (red). Adapted from Ref [**161]. **(b)** A small subnetwork (~0.1%) of Network of Organic Chemistry is shown, where the nodes are chemicals and edges are reactions [**6]. Edge colors correspond to the year when the reaction was reported, showing the growth of chemical knowledge. **(c)** An example of a generic reaction rule based on the distance (n) to the reaction center. **(d)** A demonstration of a retrosynthetic search is shown by Chematica after 8 (left) and 35 (right) iterations after defining donepezil as the target compound. A top-ranked pathway is shown. Adapted from Ref [**6]. **(e)** A scheme is shown of the retrosynthetic search for a path from a target compound to the nearest metabolite produced by the cell. The image is taken from a description of GEM-Path [**45]. **(f)** The *E. coli* metabolic network (blue, left) is shown embedded in a greater network of all hypothetic metabolic reactions on the basis of reaction rules (right) [208]. **(g)** A directed graph describing potential paths from L-tryptophan to 540 bisindoles. An example of retrobiosynthetic plan for 1,11-dibromo-2,10-dihydroxy-6,6-dimethyl-12-glucosyl-indolo[2,3-a]pyrrolo[3,4-c]carbazole-6-ium-5,7-dione is highlighted. Adapted from Ref. [10].

of long-distance effects (*e.g.*, the acceleration of a leaving group by an allosteric functional group) or conflicts because of multiple reactive groups [175,179]. Increasing the number of atoms and functional groups further from the reaction center leads to more realistic reaction paths [180]. Alternative approaches have been used to predict the transformation of products to reactants that do not require explicit rules. The reaction database can be searched to determine whether structurally similar molecules have been synthesized in order to suggest a retrosynthetic step [**181]. A neural network has been trained on the USPTO database to map a product (encoded as a SMILES (Simplified Molecular Input Line Entry System) string) to a reactant (SMILES string) [*166].

Table 1

Software for retrosynthesis.

Name	Reactions	Reaction source	Ranking factors	Website	Ref
Organic chemistry					
ChemPlanner (ARChem Route Designer)	Reaction signatures (rules)[a]	MOS [347] Beilstein Crossfire[b] [173]	Scoring function	https://www.cas.org/products/scifinder-n/chemplanner	[179]
IC*SYNTH*	Reaction signatures (rules)[c]	SPRESI[web] [348]	Scoring function	https://www.infochem.de/synthesis/ic-synth	[180]
Synthia™ (Chematica)	Bipartite graph/hand-curated rules[d]	Reaxys [173]	Cost/scoring function	https://www.sigmaaldrich.com/chemistry/chemical-synthesis/synthesis-software.html	[**6,**160,**184]
Metabolism					
BNICE	Bond-electron matrices (rules)[e]	KEGG [96]	Reaction ΔGs	http://lcsb-databases.epfl.ch/pathways/atlas/	[195]
enviPath	SMIRKS [349] (rules)[f]	EAWAG-BBD [350]	Reaction 'likelihood'	https://envipath.org/	[214]
NovoStoic	Reaction signatures (rules)[a]	MetRxn [351]	Reaction ΔGs Profit margin	https://github.com/maranasgroup/rePrime_novoStoic	[**215]
PathPred	RDM patterns[g]	KEGG [96], BRENDA [99]	Structural similarity	https://www.genome.jp/tools/pathpred/	[212]
RetroPath2.0	SMIRKS (rules)[f]	MetaNetX [352]	Sequence availability Flux	https://www.myexperiment.org/workflows/4987.html	[**213]
XTMS	Reaction signatures (rules)[a]	MetaCyc [97], KEGG [96]	Promiscuity Path length Unknown steps Toxicity reaction ΔG	http://xtms.issb.genopole.fr/	[209]

[a] Reaction center and relevant neighboring functional groups.

[b] MOS (Methods in Organic Synthesis) is now Synthetic Reaction Updates, and Beilstein Crossfire is now Reaxys.

[c] Reaction center, first-degree, and second-degree neighboring atoms.

[d] Generic reaction description, compatible functional groups, required protecting groups, and reaction condition.

[e] Matrices recording bond breaking and formation around the reaction center.

[f] Text-based reaction pattern matching around the reaction center.

[g] Vectors encoding atom types gained and lost in a reaction.

Once the rule set is defined, branching algorithms start from the target chemical and search the space [**6,182,183]. The search ends when it finds chemicals that are commercially available (*e.g.*, the ~200,000 compounds available in the Sigma-Aldrich catalog) [**160]. Enumerating all possible paths is computationally impossible, so the algorithms use a metric to determine which rules should be pursued at each step, thus stopping the algorithm from pursuing unpromising branches (Figure 4d). All software packages (Table 1) use metrics designed to avoid reactions involving strained intermediates, unlikely structures, or those that are nonselective [**6,**160,179,180]. Rules can also be eliminated where chemicals have functional groups that are sensitive to the reaction conditions [182]. Recently, an AI approach was developed that combines a Monte Carlo tree search with three neural networks to select the best candidate reactions for expansion [**161]. These neural networks are trained to evaluate only the reaction center atoms, the reaction center and first-degree neighboring atoms, and predict feasibility on the basis of augmented negative reaction data from the literature. In a provocative study, Molga et al. showed that Chematica could be applied to identify reaction paths that circumvent patent portfolios by locking not-to-be-altered bonds or motifs during the search [**184].

The algorithms typically find many putative paths, which are then ranked by an objective function. This can bias the solution towards paths that are low cost, reduced labor, environmentally friendly, have the fewest steps, go through the most 'popular' intermediates, avoid regulated compounds and toxic intermediates, or whatever a user defines as important [**6,185].

Despite development over decades, there have only recently been published success stories regarding the automated discovery of new and elegant reaction paths to difficult-to-synthesize compounds. *ICS*SYNTH was shown to be able to suggest an unusual path to the pharmaceutical oxaspiroketone that was experimentally verified to improve yield [180]. In a stunning effort, retrosynthetic paths were predicted by Chematica and experimentally validated for eight high-value and biologically active compounds (BRD7/9 inhibitor, α-hydroxyetizolem, ATR kinase inhibitor, an inhibitor of acute-myeloid-leukemia cell proliferation, *(S)*-4-hydroxyoxyduloxetine, 5β/6β-hydroxylurasidone, dronedarone, and the natural product engelheptanoxide C) [**160,186]. These solutions reduced the number of steps, generated significant yield improvements, identified unique and simpler routes, used alternative source materials, lowered costs, and reduced synthesis time.

Biochemistry: finding a retrosynthetic route through metabolism

In metabolic engineering, the goal of retrosynthesis is to identify a path of enzymatic reactions linking a desired molecule to a cellular metabolite precursor [187,188]. While articulated similarly to the organic chemistry retrosynthesis problem, there are some important differences. Foremost, all the biochemical reactions in a cell occur concurrently as a system, rather than as a series of discrete steps, and a heterologous pathway operates in this context [189]. It is the ultimate '1-pot' reaction where all the reaction conditions (solvent, temperature, and so on) are essentially identical. Enzyme promiscuity is common; for example, 37% of native *E. coli* enzymes have more than one substrate [190]. This leads to a highly interconnected network where all the reactions may not be known. The carbon from the feedstock (*e.g.*, sugar) can be diverted down different paths and away from the desired product, thus reducing the yield (mass product/mass feedstock), a key metric for economic success [187]. There are also many alternative paths by which one metabolite can be converted into another, each of which could be targeted by an engineer to improve flux.

Software has been developed to facilitate metabolic retrosynthesis (Table 1) [187,188,191–194]. When a user defines a target molecule, the algorithms search for a reaction path connecting it to a list of acceptable starting metabolites (Figure 4e). This search is performed on a metabolic network, analogous to the Network of Organic Chemistry, where each node is defined as a metabolite and the edges are enzyme-catalyzed reactions (Figure 4f) [195]. The maps are constructed using databases of metabolic reactions (see Table 1 for references). One of the most common is KEGG, which is metabolite centric and describes reactions generically by their EC number (a classification system organizing chemical reactions) [196].

Different networks can be used, depending on the engineering problem being addressed. For example, if the goal is to identify alternate routes through metabolism to increase flux through genetic engineering, then only the network associated with the chassis organism needs to be searched (*e.g.*, *E. coli* has 3755 metabolites) [197–203]. The goal could also be to define metabolic pathways from other organisms that could be moved into the target host [202,204–**206]. Then, all 18,505 metabolites and 11,146 reactions listed in KEGG irrespective of the source organism can be used to build a 'Universal Reaction Network' [207–*210]. When the target molecule is defined by the user, a search can then be performed from it to metabolites associated with the host organism [**45,211]. These approaches limit the user to select metabolites made either by the chassis

organism or known in biology, which are often the targets of metabolic engineering projects.

Rule-based approaches enable the expansion of the metabolic networks beyond empirically characterized reactions. Reaction rules are derived following approaches that are similar to those described for chemical retrosynthesis and may be manually curated or built automatically. Rules typically specify the reaction center and match the chemical structure a defined number of bonds away from that atom, by tallying the changes in bonds, or by changes in chemical features (roughly corresponding to the third digit of an EC number) [**44,**45,179,195,208,209,212–225]. To make more accurate predictions, the rules can be reduced by additional enzyme data, structural similarity between substrates, and molecular properties [**45,207,**213,226]. The molecular diversity of metabolites is less than the universe of organic chemistry, and this is reflected in the number of rules required. For example, only 250 rules capture most of the reactions in the KEGG database [195,227,228] and algorithms use as few as 50 [229], far smaller than the 60,000 rules in Chematica [**6].

The 'ATLAS of Biochemistry' and 'Metabolic *in silico* Network Expansion' databases were built using the complete set of hypothetical metabolites calculated on the basis of reaction rules [**220,**230]. Even these networks, at up to 571,000 nodes, are significantly smaller than the 35 million chemicals in the Network of Organic Chemistry. The connectivity is also less, with an average of $\sim$3 reactions per node, as opposed to $>$200 [231]. Thus, there is less need to develop advanced search algorithms. Small networks and short path searches can be performed through enumeration [198,229,232]. For example, 107,272 paths were found to isobutanol in *E. coli* [229]. Larger networks require branching algorithms to enumerate the pathways. The pruning steps in the network search can be decided on the basis of metrics that include the conservation of atoms between the substrate and product [200,201,205,233–235]. The organization of metabolic networks is different as compared to chemistry networks, particularly with the presence of hub compounds (ATP, NADPH, and so on) [199,200,203,231,236]. This can complicate a search, and several approaches have been taken to efficiently prune paths, for example, conserving functional groups to avoid short circuiting the search and creating unusable paths [234–237].

Objective functions are used to prioritize pathways for experiments [**44,**45,187,191,193,208,209,212,**215,238]. Most algorithms consider the shortest path as being advantageous as it requires the fewest heterologous enzymes and potentially toxic intermediates [187,211,**213,232,239,240]. Some paths have higher ATP requirements, thus reducing yield or lead to redox imbalances that can slow growth [**215,232]. The thermodynamic feasibility of pathways can be calculated on the basis of the predicted ΔG of each reaction [195,*210,238,**241–*243]. Toxic intermediates can also be avoided using predicted or empirical toxicity data [208,232,244–246]. Flux balance analysis is used to calculate the yield achievable with each path [211,**213,**215,232,247]. GEM-Path is able to calculate the yield under different oxygenation and growth conditions [**45].

The production of 1,4-butanediol (BDO) in *E. coli* is the only example of a retrosynthesis algorithm being used to design a *de novo* pathway to a xenobiotic compound [**44]. Genomatica used their in-house SimPheny Biopathway Predictor and a set of 50 reaction rules to identify 10,000 pathways between 4 and 6 genes to produce BDO from *E. coli* metabolites. These pathways were ranked on the basis of the number of steps, thermodynamic feasibility, and predicted yield. This guided the design of the pathway shown in Figure 2b and led to a strain that entered industrial production. While this software is not publicly available, several alternative algorithms have been able to reproduce this result and even suggest potential for improvement (Table 1) [**45,**215].

Most software packages only provide a set of reactions, for example, as EC numbers, and do not identify specific enzymes. GEM-Path supplies the user with the first enzyme homolog in its database that matches each reaction [**45]. RetroPath 2.0 considers the number of available enzyme sequences during pathway selection and has a metric for enzyme promiscuity as a measure of likelihood that an enzyme will catalyze a particular reaction [**206,**213]. This type of approach could be adapted for enzyme mining, where algorithms guide the sets of enzymes to be screened at each step. Taken a step further, data from enzyme mining efforts could be used to map enzymes to specific reactions and substrates. This approach has been taken within the chemical space of xenobiotic bisindoles in order to identify combinations of 21 enzymes obtained via part mining and used to create a barcoded DNA assembly scheme that allows a user to rapidly build a pathway to one of 540 potential target molecules (Figure 4g) [10].

Combining chemical and biochemical routes

The integration of chemical and biochemical steps will expand the molecule space accessible with either suite of techniques alone. Molecules that are trivial to chemistry, such as tetrahydrofuran, are difficult to make with biology because of toxicity or a lack of enzymes that perform the required reaction [10]. Conversely, chemistry struggles with regioselectivity and making modifications to large and highly functionalized molecules. Retrosynthesis algorithms could be developed that

divide a complex synthesis between chemical and biological steps, including those requiring multiple living cells, resting cells, and/or purified enzymes.

Increasingly, enzymatic steps are being introduced into chemical processes at an industrial scale [248–252]. As an example, nitrile hydratase is used to make 600,000 tons per year of acrylamide [253–256]. Merck developed a process to the pharmaceutical sitagliptin using an enzyme obtained by directed evolution to perform a challenging transamination (Figure 5a) [257,258]. Enzymes can be combined with each other and even metal catalysts to perform 1-pot reactions [257,259–261]. Similarly, there are techniques that treat cells as 'bags of enzyme' by pelleting cultures and resuspending them as resting cells or taking the extra disruptive steps to create freeze-dried cells or cell-free lysate. These approaches are cheap ways to implement a single or few enzymatic steps, rather than building a molecule from a sugar feedstock through central metabolism. These formulations are effectively treated as catalysts and have been combined with chemical and enzymatic steps, including in 1-pot reactions (Figure 5b) [54,262,263].

There are many ways to divide up chemical and biological steps in a retrosynthetic design. Chemical steps are often put before or after cellular processing. This can be to deal with a final step that produces a toxic product (e.g., to make artemisinin) or to feed cells substrates with chemical modifications difficult to produce enzymatically (e.g., to make halogenated benzylisoquinoline alkaloids) [**23,264]. Mixing and matching these approaches can lead to complex process schemes. For example, a patented route to caprilactam (a nylon-6 precursor) is to take lysine purified from cells, chemically convert it to aminohex-2-enoic acid, enzymatically convert that to 6-ACA, with a final chemical conversion to caprilactam [265]. There are many considerations in designing such processes, including the ability for substrates to cross the cell membrane, toxicity, and the balance between purification costs and decreased effectiveness in combining steps. Multiple species of living cells can be cocultured that perform different stages of the reaction scheme, for example, culturing engineered *E. coli* with yeast to make oxygenated taxanes or with *P. putida* to make carvolactone (Figure 2b and c) [*53,55]. The catechin pathway takes this to the extreme, with each of the four modules being contained in its own bacterial strain and using a coculture technique for production of the final product (Figure 2a) [25]. Substrates can be fed to cells that facilitate post-fermentation processing. For example, THC and bicyclobutanes were diversified by feeding cells substrates that can perform click chemistry (Figure 5c) [39,**122]. Currently, all these processes are put together in an *ad hoc* manner using expert knowledge. Algorithms will have to be developed that explore the potential paths and balance the numerous constraints in order to full harness the potential [266].

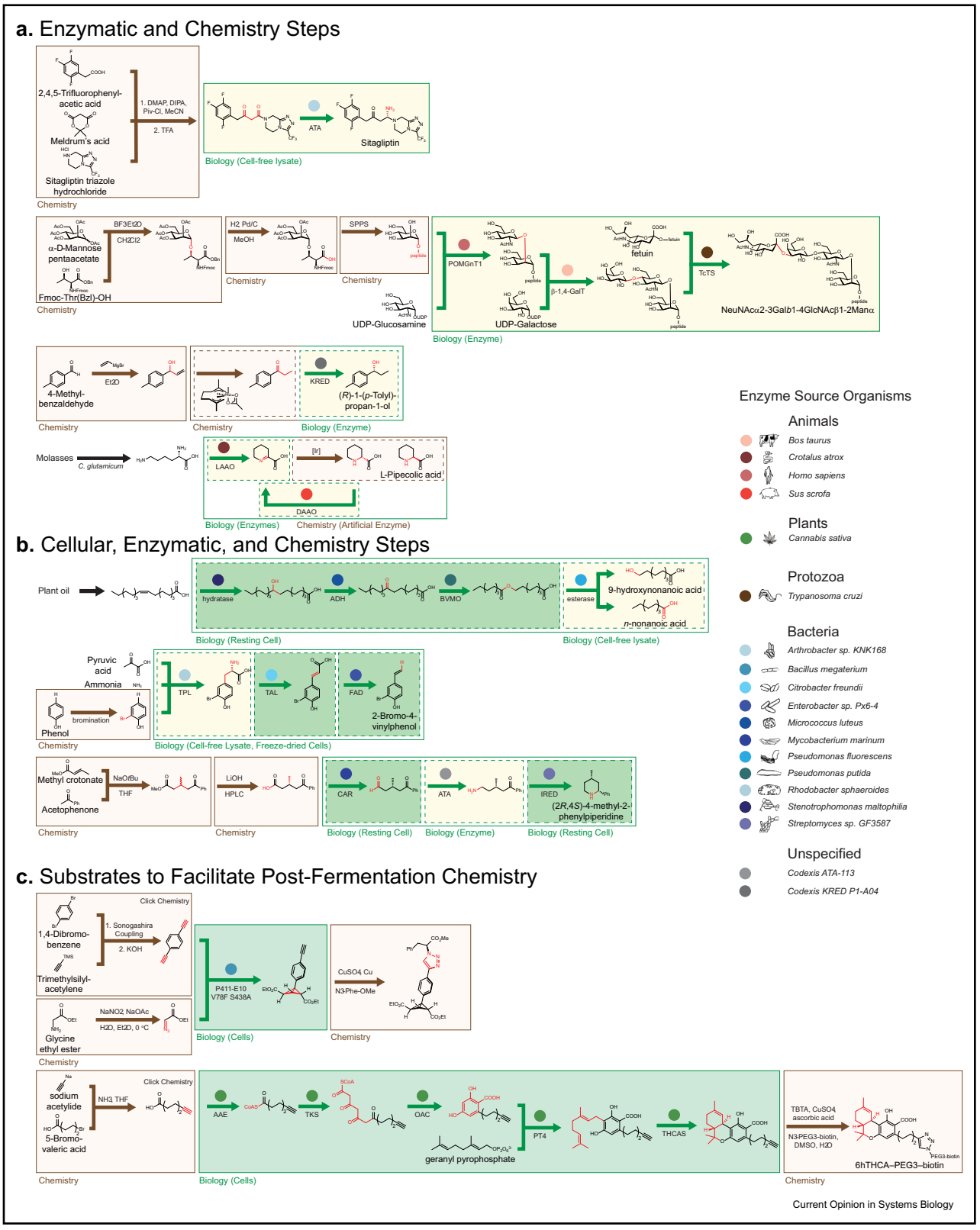
Advanced synthetic biology to facilitate retrosynthesis

This review has focused on the problem of retrosynthesis, that is, the identification of a set of enzymes that will convert a cellular metabolite into a xenobiotic product. Increasing the complexity of the molecules that can be produced will require additional tools. These include methods to increase the flux to the precursor metabolites and the control of when enzymes are expressed and where they are localized. This section focuses on anticipating those techniques required to build 40+ enzyme pathways to make a target molecule and not the additional work required to make the strain and process economically viable.

Strain development with the goal of enabling long retrosynthesis pathways may be subtly different compared to classical optimization for metabolic engineering projects. For example, both require increased flux to the precursor metabolite, but with differing goals. As a retrosynthetic pathway gets longer, each added enzyme is likely to decrease the amount of product made from the precursor. Thus, it is required to have the highest possible concentration of precursor metabolite available to the first enzyme. To enable a retrosynthesis project, this could come at the cost of decreasing yield from glucose or reducing the growth rate. This is a different objective than is typical in metabolic engineering projects, where optimizing yield and growth are critical [15,267–277]. For example, considering that many of the targets shown in Figure 2a are derived from tyrosine, one could imagine building strains of a platform chassis (*e.g.*, *Saccharomyces cerevisiae*) that produce the highest concentration of intracellular tyrosine possible without concern for its yield from glucose. This could be done for other commonly needed precursors [**45] to create a series of retrosynthesis-ready strains. Similarly, flux models and bioinformatics could be applied to identify and knockout native enzymes that are likely to modify chemicals derived from these precursors (as opposed to a specific pathway), thus reducing the likelihood of inadvertently siphoning intermediates away. For these efforts, selecting the right chassis is critical to avoid toxicity, express folded enzymes, or enhance precursor availability. Efforts in the field to create simplified genomes, develop fast-growing hosts, and tame diverse environmental organisms will provide more options in the future [15,278–286].

Central to organic chemistry is the concept of order of operations, where each reaction is performed as an independent step, and the product is purified before proceeding to the next reaction. While it is possible to combine steps into '1-pot' reactions, it is not possible to combine all those required for a complex retrosynthesis

Figure 5



Examples are shown of retrosynthetic efforts involving combinations of chemical and biological steps. The biological steps can involve enzymes in a cell-free context **(a)** or implemented by resting or living cells **(b)** [54,257,259–263]. Solid boxes indicate 1-pot reactions consisting of multiple simultaneous operations (dashed boxes). Enzyme colors indicate the source organisms of the enzymes (legend); 'unspecified' refers to enzymes that are part of kits provided by Codexis where the source information is not provided. **(c)** Two examples are shown where substrates are fed to cells that enable click chemistry to introduce further chemical modifications after isolating the product from fermentation [39,**122].

scheme (e.g., Figure 1b). Order of operations is unused in metabolic engineering: all enzymes are coexpressed all the time. In systems biology, there has been some characterization of the relationship between the timing of enzyme expression and metabolic cost for amino acid biosynthesis [287]. Order of operations is also encoded in megasynthases, but the ability to manipulate these systems remains elusive (much less harness for full retrosynthesis) [288–291]. Synthetic regulatory networks (gene circuits) have been used to switch enzyme expression on when the fermentation changes from the growth to production phase, reduce the accumulation of toxic intermediates, change the regulatory response to alternative feedstocks, and express enzymes under conditions when they are active [292–303]. A more advanced function would be to use them to time the expression of enzymes to build a molecule in steps, where the order of enzyme expression is required to build the target molecule. This could enable more complex pathway designs, for example, the biochemical equivalent of protection–deprotection as a means to avoid certain modifications by promiscuous enzymes.

Far from being bags of homogeneous biomolecules, cells offer means to spatially organize metabolic pathways. Naturally, localization is used to channel the molecule effectively between enzyme reaction sites, stop toxic intermediates from imparting their effect, or localize enzymes where the substrates are at their highest concentration [*304]. Megasynthases, for example polyketide synthases, are enzyme 'assembly lines' that progressively transform the product as it is passed between domains [288–290,305,306]. The modular nature of the enzyme sparked early efforts to implement retrosynthesis schemes by mixing-and-matching domains; while the understanding of this process has improved, this capability remains elusive [307–311]. Many copies of these enzymes can self-assemble into large (0.4 μm) organelle-like complexes localized at the cell membrane [312,313]. Various approaches have been developed to artificially scaffold enzymes by fusing them to binding domains that then assemble onto a protein, DNA, RNA, or lipid shell, that can then form larger structures [314–320]. These impart their effect by increasing the local concentration of intermediates and reducing the residence time of toxic intermediates [314,321].

Subcellular compartments, or organelles, can be used to separate enzymatic reaction steps. In eukaryotes, it is common practice to direct some enzymes in a pathway to organelles, such as mitochondria or the vacuole, to take advantage of higher concentrations of some metabolites or conditions that favor the activity of the participating enzymes (*e.g.*, salt or pH) [9,87,322]. Artificial organelles with a unique chemical signature have also been built in yeast by locally building up the synthesis of unused phospholipid species [323]. Membraneless organelles that form because of phase separation have been used to create an orthogonal translation system that can build proteins with noncanonical amino acids [324]. Prokaryotes are able to build phage-like microcompartments, into which enzymes are targeted, that have pores that selectively allow substrates in and sequester toxic intermediates [325–327]. Typically, the microcompartment genes occur together with metabolic genes as a cluster in the genome. The rules by which signal sequences can direct heterologous enzymes to the microcompartment and mutations can be made to the pore are being elucidated [325,328–335]. The prokaryotic microcompartment has been moved to yeast and incorporated into a biosynthetic pathway from tyrosine to norcoclaurine [336]. There are many ways that a target pathway could be sequestered via scaffolding proteins or divided up among organelles and microcompartments. Mathematical models have been developed in order to guide this decision-making [327,**337,338].

Conclusion

What is required to tame biology's ability to build molecules such that complex xenobiotic chemicals can be made by design? Even for simple targets, there have been only a tiny number of success stories (Figure 2b)—especially when compared to the enormous body of work from metabolic engineering efforts over the last few decades. We propose that this emerges from a tension between enzyme discovery favoring promiscuity but use in a pathway requiring specificity. Combining enzymes from different pathways, therefore, requires intensive efforts at each step to screen enzymes for those that perform the function required out-of-context with follow-up engineering efforts to deal with their promiscuity (either directly or indirectly). However, the field is at an inflection point of possibilities: enzyme mining, engineering, and evolution when combined with high-throughput metabolomics are going to lead to a deluge of specificity data. Algorithms for retrosynthetic design will integrate these data, to guide pathway construct to branch from a cellular metabolite to target molecule. New methods in artificial intelligence offer the possibility to integrate chemistry and biology into complex reaction schemes to provide access to complex molecules not achievable with either alone. Core capabilities in engineering cells need to be improved, including genome engineering, synthetic regulatory networks, and the construction of physical structures in cells. Achieving large retrosynthetic designs to build a xenobiotic molecule of a scale of a natural product is such a complex process that it will require integrated computer-aided design packages that combine retrosynthesis, metabolic flux analysis, protein engineering, and genetic circuit design automation.

Conflict of interest statement

Nothing declared.

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