ChemBioChem

Minireviews
doi.org/10.1002/cbic.202000452

Chemistry Europe
European Chemical Societies Publishing

Cell-Free Exploration of the Natural Product Chemical Space

Jonathan W. Bogart,[a, b, c] Maria D. Cabezas,[a, b, c] Bastian Vögeli,[a, b, c] Derek A. Wong,[a, b, c] Ashty S. Karim,[a, b, c] and Michael C. Jewett*[a, b, c, d, e]

Natural products and secondary metabolites comprise an indispensable resource from living organisms that have transformed areas of medicine, agriculture, and biotechnology. Recent advances in high-throughput DNA sequencing and computational analysis suggest that the vast majority of natural products remain undiscovered. To accelerate the natural product discovery pipeline, cell-free metabolic engineering approaches used to develop robust catalytic networks are being repurposed to access new chemical scaffolds, and new enzymes capable of performing diverse chemistries. Such enzymes could serve as flexible biocatalytic tools to further expand the unique chemical space of natural products and secondary metabolites, and provide a more sustainable route to manufacture these molecules. Herein, we highlight select examples of natural product biosynthesis using cell-free systems and propose how cell-free technologies could facilitate our ability to access and modify these structures to transform synthetic and chemical biology.

Introduction

Natural products have inspired the development of numerous pharmaceuticals,[1,2] food preservatives,[3,4] crop protectants,[5] cosmetics,[6,7] biological probes,[8] and more.[9–11] Given their importance to society, the ability to access, manipulate, and engineer the natural product chemical space is highly sought after.[12] For example, new compounds are constantly needed to combat the ongoing battle against antibiotic resistance.[13] Traditional antibiotic discovery approaches have been hampered by rediscovery and have failed to bring new antibiotic scaffolds to market over the last 30 years.[14] Despite this challenge, evidence suggests that the majority of natural products remain undiscovered and this critical natural resource has far from dried up.[15] In fact, high-throughput DNA sequencing and a growing collection of powerful bioinformatic tools have uncovered unique "cryptic" natural product gene clusters that may serve as next-generation pharmaceuticals or tools.[16–22] Thus, high-throughput "bottom-up" approaches (e.g., metagenomics) can access the silent genetic potential of microorganisms and lead to a renaissance in the field of natural products.[23–30] Unfortunately, methods to express biosynthetic gene clusters lag behind the strategies used to identify them.[18] Alleviating this biosynthetic bottleneck would provide two major resources: i) new scaffolds with unique functions and ii) new enzymes with biomanufacturing utility. Importantly, these natural product tailoring enzymes may serve as valuable tools to produce natural product-like compounds by combinatorial biosynthesis.[31–33]

In this minireview, we discuss select advances in cell-free natural product biosynthesis and the exciting opportunity of combining cutting edge cell-free technologies to facilitate natural product pathway assembly, enzyme discovery, and pathway evolution towards combinatorial biosynthesis. First, we highlight recent works as useful blueprints for the assembly of native natural product pathways using cell-free systems. Then, we present high-throughput, cell-free strategies for enzyme characterization and evaluation of their substrate promiscuity, followed by a discussion about leveraging these natural promiscuities to assemble novel pathways and compounds. Finally, we touch on how merging bioorthogonal chemistry with cell-free synthesized natural products can provide chemical diversity not accessible through enzymatic or chemical synthesis alone. Collectively, cell-free systems provide tools with the potential to create and diversify the natural product chemical space at an unprecedented rate, helping to bridge the gap between the pace of biosynthetic gene cluster identification and characterization.

[a] Dr. J. W. Bogart, Dr. M. D. Cabezas, Dr. B. Vögeli, D. A. Wong, Dr. A. S. Karim, Prof. Dr. M. C. Jewett
Department of Chemical and Biological Engineering
Northwestern University
Evanston, IL 60208 (USA)
E-mail: m-jewett@northwestern.edu
[b] Dr. J. W. Bogart, Dr. M. D. Cabezas, Dr. B. Vögeli, D. A. Wong, Dr. A. S. Karim, Prof. Dr. M. C. Jewett
Chemistry of Life Processes Institute
Northwestern University
Evanston, IL 60208 (USA)
[c] Dr. J. W. Bogart, Dr. M. D. Cabezas, Dr. B. Vögeli, D. A. Wong, Dr. A. S. Karim, Prof. Dr. M. C. Jewett
Center for Synthetic Biology, Northwestern University
Evanston, IL 60208 (USA)
[d] Prof. Dr. M. C. Jewett
Robert H. Lurie Comprehensive Cancer Center
Northwestern University
Chicago, IL 60611 (USA)
[e] Prof. Dr. M. C. Jewett
Simpson Querrey Institute, Northwestern University
Chicago, IL 60611 (USA)

This article is part of a Special Collection on Microbial Biosynthesis and Interactions. To view the complete collection, visit our homepage

Advantages of Cell-Free Pathway Engineering

Extract-based cell-free systems offer several unique advantages over complementary *in vivo* and purified enzyme-based approaches for enzyme characterization and pathway assembly. The foundational principle is that discrete biosynthetic pathways can be constructed through modular assembly of cell-free lysates pre-enriched with enzyme(s) produced either by overexpression in a lysate chassis strain (*in vivo* production) or by direct cell-free gene expression (Figure 1).[34,35] Cell-free gene expression is not constrained by sustaining life, therefore, much of the cellular resources can be directed towards producing a desired product. High concentrations of desired enzyme homologues or entire pathways can be produced in a few hours from linear DNA templates. Typically performed at small scales (microliter), cell-free gene expression (CFE) allows for 100 s of enzyme homologues (or variants) to be expressed in parallel without specialized equipment. In addition, cell-free systems offer a controllable and open environment for precise substrate, intermediate, and product manipulation and monitoring (Figure 1). This enables stepwise reconstruction of complex natural pathways with the ability to easily observe and characterize intermediates and products, rapidly identify biosynthetic bottlenecks, troubleshoot individual steps, and deliver high yields without the need for enzyme purification.[36,37] Cell-free systems can bolster *in vitro*, purified approaches by providing a native-like metabolism and precursor pools. For example, a dehydratase from the nisin biosynthetic pathway eluded reconstitution *in vitro* for 20 years until the addition of bacterial cell extract allowed the desired dehydration activity.[38] These advantages provide a basis for exploring biosynthetic enzymes and engineering natural product pathways with CFE.

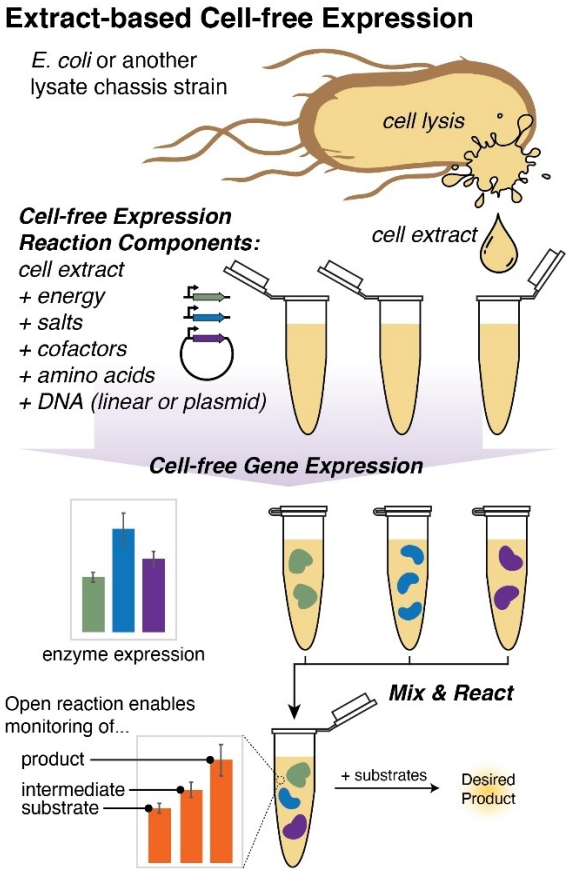

Figure 1. General scheme of cell-free gene expression and i*n vitro* reconstitution of a biosynthetic pathway.

High-Throughput Pathway Assembly in the Cell-Free Environment

Using CFE, natural product pathways can be rapidly assembled by combining multiple enzymes at user-defined concentrations. For example, a diketopiperazine was recently produced in the cell-free environment by combining two enzymes in the gramicidin-S biosynthetic gene cluster (Figure 2).[39] The modular and customizable nature of cell-free systems enables combinatorial biosynthesis of complete pathways where individual steps in a given pathway can be replaced with enzyme homologues from different strains, organisms, or even engineered enzymes.[36] One early example used pre-enriched extracts to reconstruct a flexible route to aminoglycosides in a stepwise manner.[40] These results informed the design of custom aminoglycoside biosyntheses, using enzymes from multiple natural pathways, to produce superior analogues by *in vivo* production. Similarly, a five enzyme pathway was assembled by CFE for the production of *n*-butanol.[41] This pathway was recently expanded to combinatorially screen hundreds of pathway designs for the production of 3-hydroxybutyrate and *n*-butanol.[42] Top-performing pathways were then implemented in the non-model organism *Clostridium autoethanogenum*, ultimately improving *in vivo* production by more than 20- and fourfold, respectively. This is a key example of screening a desired pathway in a high-throughput fashion that would otherwise be slow, or potentially not feasible, *in vivo*.

Extract-based cell-free systems have now expanded to produce full natural product pathways spanning many classes of compounds (Figure 2). Optimal pathways for two monoterpenes (limonene and pinene) and a sesquiterpene (bisabolene) were developed by screening over 150 unique sets of enzymes in 580 discrete pathway conditions.[43–45] Three non-ribosomal peptide natural products (NRPs) have been produced using cell-free systems. A CFE system was used to express the entire valinomycin gene cluster (>19kb) and produce valinomycin in a one-pot reaction in just a few hours.[46] Importantly, the two enzymes within the pathway, Vlm1 (370 kDa) and Vlm2 (284 kDa), represent two of the largest enzymes ever reported using cell-free protein synthesis. Additionally, lysates pre-enriched with these enzymes produce high titers (~30 mg/L) that rival yields from heterologous production in *E. coli*.[47] A separate study described similar syntheses of NRPs indigoidine and rhabdopeptide using the commercially available PURExpress system.[48] The authors were also able to use CFE to synthesize several other megasynthases from additional NRPs,

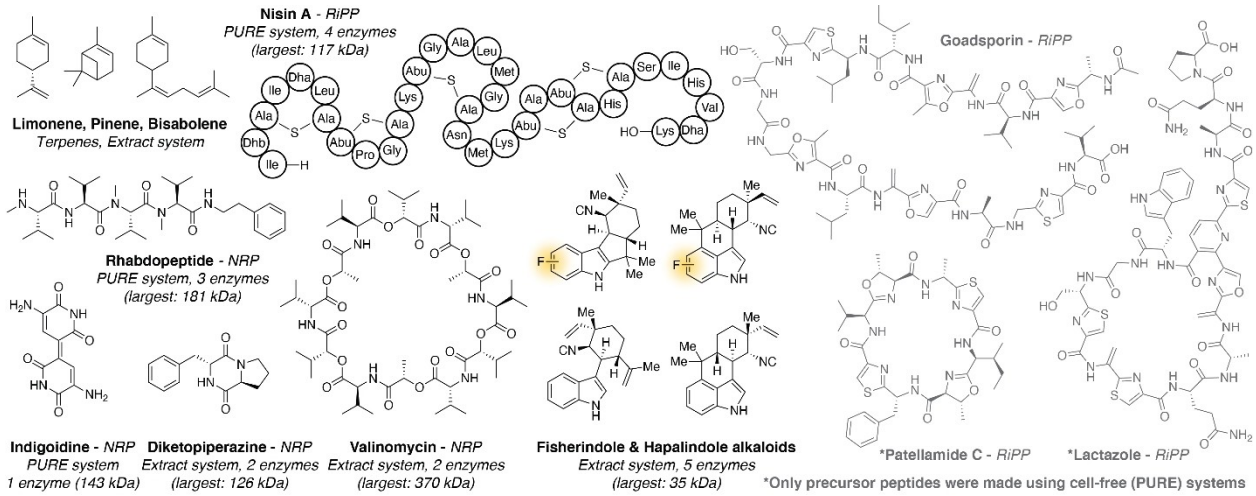

Figure 2. Natural products made using various cell-free systems (PURE or extract-based). The total number of enzymes and the largest enzyme made in each system are noted. Unnatural variants that were made using cell-free technologies are highlighted in yellow. Natural products in gray are examples for which only part of the biosynthesis was performed in a cell-free system. Only the precursor peptides were synthesized using the PURE system, and all enzymes were expressed in heterologous hosts and purified.

fatty acid, and polyketide pathways although their native activity was not demonstrated. A series of indole alkaloids was produced using cell-free technology.[49] Several unnatural halogenated indole compounds were produced by simply feeding reactions with chemically synthesized precursors. Lastly, the pathway for the prototypical lanthipeptide, nisin, was recently rebuilt using extract-based CFE.[50] The authors coupled cell-free nisin biosynthesis with an antibiotic activity assay which allowed them to screen over 3000 analogues and quickly identify two variants that were more active than the parent compound. Cell-free systems have been used to synthesize other ribosomally synthesized and post-translationally modified peptide (RiPP) natural products, however only precursor peptides (or analogues) were synthesized by CFE.[51–56] These initial examples of natural product pathways established in cell-free systems mark the starting point of a field that will improve metabolic engineering efforts for a wide variety of natural product classes.

Cell-Free Platforms Facilitate Enzyme Characterization

With an ultimate goal of (combinatorial) natural product pathway assembly, an important first step is the evaluation of the constituent biosynthetic enzymes.[31] Sequence similarity networks are often used to organize enzyme homologues into families based on protein sequences, and ultimately, their presumed function.[57] Typically, the number of homologues tested in a given study is low because traditional biochemical assays rely on heterologous expression and require time-consuming and labor-intensive enzyme purification steps. High-throughput enzyme characterization using cell-free systems might enable a large percentage of enzyme families to be examined in parallel (Figure 3), as has been the case with other metabolic pathways like limonene as described above. This allows for the rapid identification of enzymes with desired qualities, evaluation of substrate promiscuity, and enables one to make broad conclusions about enzymes families rather than single family members.

To study enzyme families in depth, the throughput of enzyme expression must be matched with rapid and quantitative biochemical assessment. In this regard, cell-free platforms have already been integrated with high-throughput enzyme screening assays; including, self-assembled monolayers for matrix-assisted desorption/ionization mass spectrometry (SAMDI-MS), mRNA display, in-droplet reaction microfluidics, and next generation sequencing (Figure 4). By combining cell-free protein synthesis with SAMDI-MS, reactive metabolites are captured on monolayers and analyzed by MALDI-TOF MS (Figure 4a). This approach was used to rapidly screen over 800 unique reaction conditions to optimize the synthesis of hydroxymethylglutaryl-CoA, a biosynthetic precursor of mevalonate and isoprenoid metabolites.[58] Using a capture technique similar to native chemical ligation, all acyl intermediates on CoA could be simultaneously analyzed, revealing a full picture of the biocatalytic system. Similarly, SAMDI has also been used to investigate the promiscuity of N-glycosyltransferases (NGTs). More than 3000 peptide substrates were rapidly screened in 13 903 unique reaction conditions to develop precise peptide acceptor sequences that showed robust glycosylation both *in vitro* and *in vivo*.[59]

mRNA display has been repurposed to intensively investigate the promiscuity of RiPP tailoring enzyme PaaA, in the biosynthesis of pantocin A (Figure 4b).[60] Over 34 million substrates were screened to reveal PaaA's broad substrate tolerance. While this current approach was designed specifically around the chemistry performed by PaaA and is not broadly applicable to other RiPP enzymes, it represents a first major

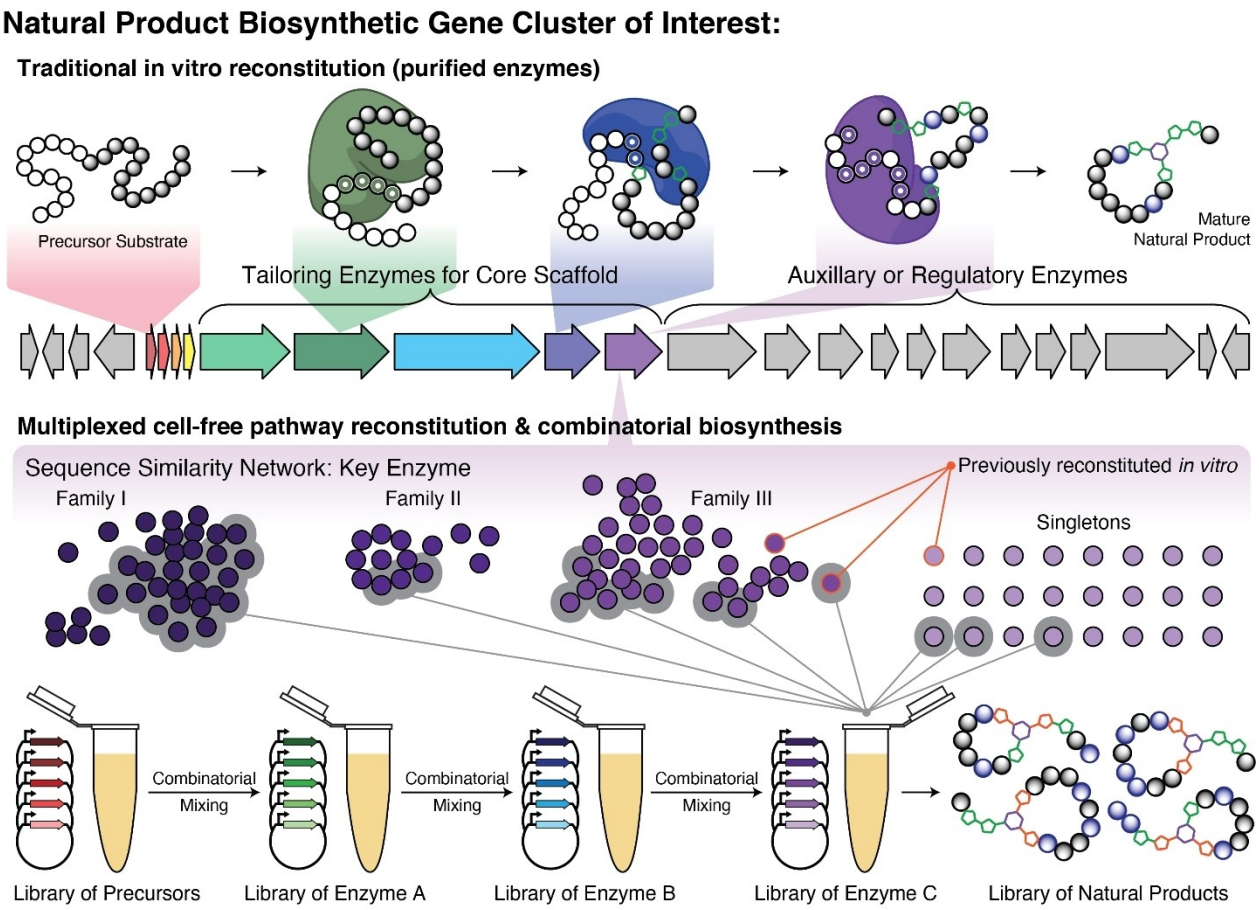

Figure 3. Cell-free reconstitution and combinatorial biosynthesis of a natural product. The pathway depicted is loosely based on general RiPP biosynthesis. Large numbers of key enzyme homologues (purple), visualized by sequence similarity networks, can be expressed on small scales in parallel to investigate their function in a high-throughput manner. This strategy can be multiplexed to the entire pathway and enable combinatorial biosynthesis of natural product libraries.

step in using massive display libraries to inform broadly on substrate promiscuity of natural product tailoring enzymes. Importantly, it provides another intriguing access point for generating libraries of natural products for drug discovery.

In a recent study, droplet-based microfluidic sorting together with next-generation sequencing enabled functional screening of a 1 million-membered metagenomic library, which revealed previously undiscovered hydrolases and profiled their substrate promiscuity.[61] While this study was performed *in vivo*, this methodology relied on lysing cells within droplets to release enzymes and modify their substrates. Enzyme transformations are sampled and analyzed at the *in vitro* stage, therefore a cell-free systems screening approach could be implemented to accelerate the discovery pipeline (Figure 4c).

While some of the examples mentioned above describe strategies to determine enzyme specificity/promiscuity outside the context of natural products, we anticipate that these technologies will be useful to investigate natural product tailoring enzymes that modify peptides (RiPPs, NRPs, antimicrobial peptides) and CoA substrates (isoprenoids, polyketides) as well as natural product glycosyltransferases (glycorandomization).

Towards Cell-Free Combinatorial Natural Product Biosynthesis

One of the exciting features of cell-free gene expression is the ability to easily mix-and-match enzymes in a combinatorial fashion to rapidly prototype and optimize a given pathway. Screening such pathways *in vivo* is challenging because troubleshooting variants that produce truncated side-products or nonfunctional products is time-consuming and can be convoluted. Cell-free extract-based screening methods are well positioned to accelerate testing of hybrid pathways and advance the field of natural product synthetic biology towards the long-standing goal of producing custom molecules by rationally designing biosynthetic pathways. Analogous to the natural product workflow, combinatorial biosynthesis of glycan motifs was demonstrated using a small series of glycosyltransferases. In total, 37 putative pathways yielded 23 unique glycans, 18 of which had never been synthesized previously.[62] The ability to exploit the innate promiscuity of natural product tailoring enzymes and assemble hybrid pathways by combining enzymes from different natural product biosynthetic clusters could yield new-to-nature natural products.

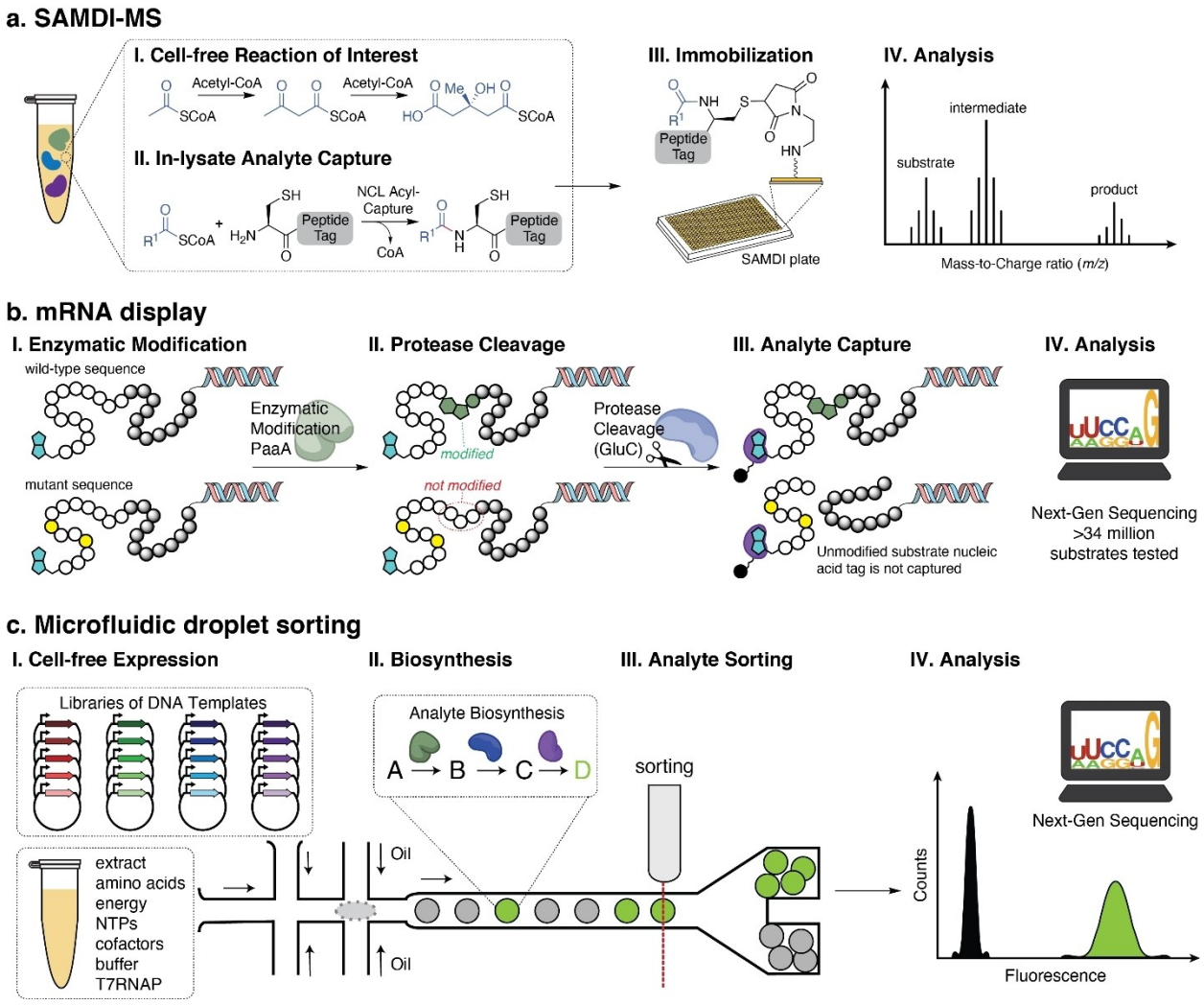

Figure 4. High-throughput methods to rigorously evaluate enzyme function and substrate promiscuity.

RiPPs represent a suitable entry point for the synthesis of hybrid natural products, as libraries of precursor peptides can be synthesized directly in the cell-free environment and then combined with a library of tailoring enzymes also synthesized via CFE (Figure 3). RiPPs have been shown to be amenable to hybridization by the successful production of functional thiazoline-lanthipeptide, thiazoline-sactipeptide and thiazoline-lanthionine hybrids *in vivo*.[63] Similarly, enzymes from disparate RiPP pathways were used to synthesize natural and unnatural thiopeptides based on thiocillin and lactazole scaffolds *in vitro* with peptide substrates synthesized using the PURE system.[55] Furthermore, many RiPP precursor peptides engage structurally similar enzyme domains, termed RiPP recognition elements (RREs), that allow tailoring enzymes to recognize and modify their substrates.[64] These unique recognition elements may be exploited or engineered to develop superior, more versatile catalysts. For instance, the cyclodehydratase LynD, from a cyanobactin pathway, was fused to a fragment of its native precursor peptide that engaged this RRE. This fusion made the enzyme constitutively active and able to modify precursor peptides that lack this RRE-binding sequence.[65] Similar strategies could enhance the already broad substrate promiscuity and enable further substrate sampling.

Integrating Biological and Chemical Synthesis of Natural Products

In developing new-to-nature pathways, some target transformations may be inaccessible enzymatically or difficult to evolve. In these cases, the open environment of a cell-free reaction offers the opportunity to incorporate synthetic chemistry through hybrid chemical reactions.[66] Chemical transformations may be applied as final tailoring steps or as intermediate steps to allow further enzymatic derivatization. In a recent *in vivo* study, a hybrid pathway generated several dozen analogues of the natural product violacein, including those that bore aryl-bromides. These unnatural brominated analogues were further derivatized in crude lysates using

Table 1. Advantages and Limitations of Cell-free Gene Expression Systems.

Advantages	Limitations
+ Produces enzymes directly from a DNA template (linear or plasmid)	– Lacks a diverse set of host extract systems (plant & fungi extracts are not well-explored)
+ Produces enzymes in hours	– Suffers from low expression yields in some systems (e.g., plants)
+ Enables stepwise & complete pathway reconstitution & can be integrated with synthetic chemical transformations	– Behaves unpredictably in complex conditions (e.g., oxygen sensitivity)
+ Enables rapid, combinatorial pathway assembly	– Struggles to express large proteins (>200 kDa), small peptides (<10 kDa), and install post-translational modifications
+ Exhibits tolerance to toxic metabolites	– Exhibits degradation of some enzymes and natural products
+ Allows easy metabolite monitoring due to the open reaction environment	– Lacks scale-up design rules
+ Provides access to nano- and micro-liter scales	

Suzuki-Miyaura cross couplings to afford an additional 20 analogues that are inaccessible by purely enzymatic means.[67] In a separate study, oxidative elimination of *Se*-phenylselenocysteine using peroxide allowed site selective installation of multiple dehydroalanines (Dhas; a common modification in natural products[68]) on peptides generated with the PURE system.[55] These Dha-bearing peptides served as substrates for pyridine synthases that catalyze unique aza-[4 + 2] cycloadditions to afford two classes of thiopeptide natural products. Combining both chemical and enzymatic transformations is a useful tactic to generate new-to-nature natural products that may be challenging to access using either method independently.

Challenges and Opportunities for Cell-Free Natural Product Biosynthesis

Several aspects of the natural product biosynthesis must be considered when applying cell-free technology (Table 1). For example, polyketides and nonribosomal peptide natural products are typically synthesized by particularly large enzymes (> 150 kDa). While such enzymes have been synthesized in CFE systems, there are still relatively few examples.[46,48] Several strategies could be used to address this. First, pre-enriched lysates from traditional heterologous expression might provide one current alternative strategy to access larger constructs.[46] Second, specialized extracts, rich in cold shock proteins or chaperones may provide an attractive solution.[69] Third, the expression of each individual enzyme could be separately optimized using lysates from different strains or organisms. Indeed, the idea of organismal amalgams not limited to a single host-derived extract is another alluring feature of cell-free systems.

Enzymes that require special or unpredictable cofactors, metabolites, or post-translational modifications are ongoing challenges in natural product biosynthesis in general and represent opportunities for cell-free systems. While some known cofactors and metabolites may be added to cell-free premixes, these scenarios may be best approached by using host derived extracts. Indeed, *Streptomyces* extracts have been prepared, and although these systems are less productive than many *E. coli* counterparts, improvements are being made.[70–72] Many interesting natural products are produced by hosts that inhabit anaerobic environments. Creating cell-free systems that function anaerobically to produce oxygen-sensitive enzymes are just starting to be explored.[73] Access to these enzymes could open a range of unique and useful chemistries.[74,75] Cell-free systems are primed to rapidly explore the natural product chemical space and the field is rich with opportunity to expand its access to new biocatalysts and natural products.

Conclusion

The combinatorial biosynthesis of natural products has been a long-standing and evolving goal in the natural product and synthetic biology communities. Key to realizing this goal is generating versatile sets of diverse biocatalysts capable of constructing building blocks, assembling them into core scaffolds, and further diversifying those scaffolds. While tremendous progress has been made using both *in vivo* and purified *in vitro* systems, knowledge gaps still exist in how individual biosynthetic enzymes function and how multiple enzymes can be assembled to create novel pathways.[76,77] In addition, the number of putative biosynthetic gene clusters greatly outnumbers those that have been characterized.

Cell-free technologies have just begun to demonstrate their potential impact on natural product synthesis through production of terpenes, NRPs, indole alkaloids, saccharides, and RiPPs. The above examples highlight how the user-friendly nature, modularity, and speed at which one can go from gene to natural product can make extract-based cell-free systems an enabling technology to rapidly explore the natural product chemical space. Integration with computer-aided pathway design and machine learning could further accelerate design-build-test cycles.[42,78] Although an extract-based cell-free gene expression system has not yet, to our knowledge, been used to characterize a previously unknown natural product pathway, all the tools are in place to accomplish this. Taken together, we anticipate that cell-free systems will serve as a powerful, high-throughput engine for enzyme and cryptic pathway characterization, expand our ability to access and manipulate complex chemical space, and establish a new natural product renaissance.

Author Contributions

The authors contributed to all aspects of the article.

Acknowledgments

We acknowledge support from the National Institutes of Health grant 1U19AI142780-01, the DARPA 1000 Molecules Program HR0011-15-C-0084, the Department of Energy grant DE-SC0018249, the Office of Energy Efficiency and Renewable Energy grant DE-EE0008343, the David and Lucile Packard Foundation, and the Camille Dreyfus Teacher-Scholar Program. The U.S. Government is authorized to reproduce and distribute reprints for Governmental purposes notwithstanding any copyright notation thereon. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of DARPA or the U.S. Government. B.V. acknowledges support from the SNSF Early Postdoc.Mobility fellowship P2SKP3_184036. D.A.W. acknowledges support from the National Science Foundation Graduate Research Fellowship under grant no. DGE-1842165.

Conflict of Interest

M.C.J. has a financial interest in Design Pharmaceuticals Inc. and SwiftScale Biologics. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict of interest policies. All other authors declare no conflicts of interest.

[1] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2015**, *79*, 629–661.
[2] A. L. Harvey, R. Edrada-Ebel, R. J. Quinn, *Nat. Rev. Drug Discovery* **2015**, *14*, 111–129.
[3] G. D. Mogoşanu, A. M. Grumezescu, C. Bejenaru, L. E. Bejenaru, *Academic Press*, **2017**, 365–411.
[4] T. Thery, K. M. Lynch, E. K. Arendt, *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 1327–1360.
[5] F. E. Dayan, C. L. Cantrell, S. O. Duke, *Bioorg. Med. Chem.* **2009**, *17*, 4022–4034.
[6] A.-D. Fortineau, *J. Chem. Educ.* **2004**, *81*, 45.
[7] M. Séquin, *Encyclopedia of Applied Plant Sciences* **2017**, 393–398.
[8] E. E. Carlson, *ACS Chem. Biol.* **2010**, *5*, 639–653.
[9] M. P. Narsing Rao, M. Xiao, W.-J. Li, *Front. Microbiol.* **2017**, *8*, 71.
[10] D. D. Soejarto, E. M. Addo, A. D. Kinghorn, *J. Ethnopharmacol.* **2019**, *243*, 112056.
[11] A. Kang, T. S. Lee, *Biotechnology for Biofuel Production and Optimization* **2016**, 35–71.
[12] G. Karageorgis, D. J. Foley, L. Laraia, H. Waldmann, *Nat. Chem.* **2020**, *12*, 227–235.
[13] E. D. Brown, G. D. Wright, *Nature* **2016**, *529*, 336–343.
[14] Scientific Roadmap for Antibiotic Discovery. *Pew Charitable Trusts* (11 May **2016**); http://www.pewtrusts.org/antibiotic-discovery.
[15] C. R. Pye, M. J. Bertin, R. S. Lokey, W. H. Gerwick, R. G. Linington, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 5601–5606.
[16] M. S. Donia, P. Cimermancic, C. J. Schulze, L. C. W. Brown, J. Martin, M. Mitreva, J. Clardy, R. G. Linington, M. A. Fischbach, *Cell* **2014**, *158*, 1402–1414.
[17] J. R. Doroghazi, J. C. Albright, A. W. Goering, K.-S. Ju, R. R. Haines, K. A. Tchalukov, D. P. Labeda, N. L. Kelleher, W. W. Metcalf, *Nat. Chem. Biol.* **2014**, *10*, 963–968.
[18] J. C. Navarro-Muñoz, N. Selem-Mojica, M. W. Mullowney, S. A. Kautsar, J. H. Tryon, E. I. Parkinson, E. L. C. de los Santos, M. Yeong, P. Cruz-Morales, S. Abubucker, A. Roeters, W. Lokhorst, A. Fernandez-Guerra, L. T. D. Cappelini, A. W. Goering, R. J. Thomson, W. W. Metcalf, N. L. Kelleher, F. Barona-Gomez, M. H. Medema, *Nat. Chem. Biol.* **2020**, *16*, 60–68.
[19] C. Lok, *Nature News* **2015**, *522*, 270–273.
[20] K. Blin, S. Shaw, K. Steinke, R. Villebro, N. Ziemert, S. Y. Lee, M. H. Medema, T. Weber, *Nucleic Acids Res.* **2019**, *47*, W81–W87.
[21] J. I. Tietz, C. J. Schwalen, P. S. Patel, T. Maxson, P. M. Blair, H.-C. Tai, U. I. Zakai, D. A. Mitchell, *Nat. Chem. Biol.* **2017**, *13*, 470–478.
[22] N. J. Merwin, W. K. Mousa, C. A. Dejong, M. A. Skinnider, M. J. Cannon, H. Li, K. Dial, M. Gunabalasingam, C. Johnston, N. A. Magarvey, *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 371–380.
[23] R. D. Kersten, J.-K. Weng, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E10961–E10969.
[24] K. Yamanaka, K. A. Reynolds, R. D. Kersten, K. S. Ryan, D. J. Gonzalez, V. Nizet, P. C. Dorrestein, B. S. Moore, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 1957–1962.
[25] B. O. Bachmann, S. G. Van Lanen, R. H. Baltz, *J. Ind. Microbiol. Biotechnol.* **2013**, *41*, 175–184.
[26] M. H. Medema, M. A. Fischbach, *Nat. Chem. Biol.* **2015**, *11*, 639–648.
[27] G. D. Wright, *Microb. Biotechnol.* **2018**, *12*, 55–57.
[28] T. A. Scott, J. Piel, *Nat. Rev. Chem.* **2019**, *3*, 404–425.
[29] M. F. Freeman, C. Gurgui, M. J. Helf, B. I. Morinaka, A. R. Uria, N. J. Oldham, H.-G. Sahl, S. Matsunaga, J. Piel, *Science* **2012**, *338*, 387–390.
[30] A. Milshteyn, J. S. Schneider, S. F. Brady, *Chem. Biol.* **2014**, *21*, 1211–1223.
[31] E. Kim, B. S. Moore, Y. J. Yoon, *Nat. Chem. Biol.* **2015**, *11*, 649–659.
[32] T. Rodrigues, D. Reker, P. Schneider, G. Schneider, *Nat. Chem.* **2016**, *8*, 531–541.
[33] C. T. Walsh, *ChemBioChem* **2002**, *3*, 124–134.
[34] A. D. Silverman, A. S. Karim, M. C. Jewett, *Nat. Rev. Genet.* **2020**, *21*, 151–170.
[35] N. E. Gregorio, M. Z. Levine, J. P. Oza, *Methods and Protocols* **2019**, *2*, 24.
[36] A. S. Karim, M. C. Jewett, *Methods Enzymol.* **2018**, *608*, 31–57.
[37] Q. M. Dudley, A. S. Karim, M. C. Jewett, *Biotechnol. J.* **2015**, *10*, 69–82.
[38] N. Garg, L. M. A. Salazar-Ocampo, W. A. van der Donk, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 7258–7263.
[39] A. W. Goering, J. Li, R. A. McClure, R. J. Thomson, M. C. Jewett, N. L. Kelleher, *ACS Synth. Biol.* **2017**, *6*, 39–44.
[40] J. W. Park, S. R. Park, K. K. Nepal, A. R. Han, Y. H. Ban, Y. J. Yoo, E. J. Kim, E. M. Kim, D. Kim, J. K. Sohng, Y. J. Yoon, *Nat. Chem. Biol.* **2011**, *7*, 843–852.
[41] A. S. Karim, M. C. Jewett, *Metab. Eng.* **2016**, *36*, 116–126.
[42] A. S. Karim, Q. M. Dudley, A. Juminaga, Y. Yuan, S. A. Crowe, J. T. Heggestad, S. Garg, T. Abdalla, W. S. Grubbe, B. J. Rasor, D. Coar, M. Torculas, M. Krein, F. E. Liew, A. Quattlebaum, R. O. Jensen, J. A. Stuart, S. D. Simpson, M. Köpke, M. C. Jewett, *Nat. Chem. Biol.* **2019**, *10*, 319.
[43] Q. M. Dudley, A. S. Karim, C. J. Nash, M. C. Jewett, *Metab. Eng.* **2020**, *61*, 251–260.
[44] Q. M. Dudley, C. J. Nash, M. C. Jewett, *Synth. Biol.* **2019**, *4*, 656.
[45] F.-X. Niu, Y.-B. Huang, Y.-P. Shen, L.-N. Ji, J.-Z. Liu, *J. Agric. Food Chem.* **2020**, *68*, 2139–2145.
[46] L. Zhuang, S. Huang, W.-Q. Liu, A. S. Karim, M. C. Jewett, J. Li, *Metab. Eng.* **2020**, *60*, 37–44.
[47] J. Li, J. Jaitzig, L. Theuer, O. E. Legala, R. D. Süssmuth, P. Neubauer, *J. Biotechnol.* **2015**, *193*, 16–22.
[48] I. Siebels, S. Nowak, C. S. Heil, P. Tufar, N. S. Cortina, H. B. Bode, M. Grininger, *bioRxiv* preprint **2020**, *33*, https://doi.org/10.1101/2020.04.04.025353.
[49] Y. Khatri, R. M. Hohlman, J. Mendoza, S. Li, A. N. Lowell, H. Asahara, D. H. Sherman, *ACS Synth. Biol.* **2020**, *9*, 1349–1360.
[50] R. Liu, Y. Zhang, G. Zhai, S. Fu, Y. Xia, H. Ben, X. Cai, Y. Zhang, Y. Li, Z. Deng, Liu, T. *bioRxiv* preprint **2019**, https://doi.org/10.1101/757591.
[51] Y. Goto, Y. Ito, Y. Kato, S. Tsunoda, H. Suga, *Chem. Biol.* **2014**, *21*, 766–774.
[52] T. Ozaki, K. Yamashita, Y. Goto, M. Shimomura, S. Hayashi, S. Asamizu, Y. Sugai, H. Ikeda, H. Suga, H. Onaka, *Nat. Commun.* **2017**, *8*, 1–13.
[53] Y. Goto, H. Suga, *Bull. Chem. Soc. Jpn.* **2018**, *91*, 410–419.
[54] Y. Goto, H. Suga, *ChemBioChem* **2020**, *21*, 84–87.
[55] S. R. Fleming, T. E. Bartges, A. A. Vinogradov, C. L. Kirkpatrick, Y. Goto, H. Suga, L. M. Hicks, A. A. Bowers, *J. Am. Chem. Soc.* **2019**, *141*, 758–762.
[56] A. A. Vinogradov, M. Shimomura, Y. Goto, T. Ozaki, S. Asamizu, Y. Sugai, H. Suga, H. Onaka, *Nat. Commun.* **2020**, *11*, 1–13.
[57] P. Shannon, A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, *Genome Res.* **2003**, *13*, 2498–2504.
[58] P. T. O'Kane, Q. M. Dudley, A. K. McMillan, M. C. Jewett, M. Mrksich, *Sci. Adv.* **2019**, *5*, eaaw9180.
[59] W. Kightlinger, L. Lin, M. Rosztoczy, W. Li, M. P. DeLisa, M. Mrksich, M. C. Jewett, *Nat. Chem. Biol.* **2018**, *14*, 627–635.

[60] S. R. Fleming, P. M. Himes, S. V. Ghodge, Y. Goto, H. Suga, A. A. Bowers, *J. Am. Chem. Soc.* **2020**, *142*, 5024–5028.
[61] P.-Y. Colin, B. Kintses, F. Gielen, C. M. Miton, G. Fischer, M. F. Mohamed, M. Hyvönen, D. P. Morgavi, D. B. Janssen, F. Hollfelder, *Nat. Commun.* **2015**, *6*, 1–12.
[62] W. Kightlinger, K. E. Duncker, A. Ramesh, A. H. Thames, A. Natarajan, J. C. Stark, A. Yang, L. Lin, M. Mrksich, M. P. DeLisa, M. C. Jewett, *Nat. Commun.* **2019**, *10*, 2364–13.
[63] B. J. Burkhart, N. Kakkar, G. A. Hudson, W. A. van der Donk, D. A. Mitchell, *ACS Cent. Sci.* **2017**, *3*, 629–638.
[64] B. J. Burkhart, G. A. Hudson, K. L. Dunbar, D. A. Mitchell, *Nat. Chem. Biol.* **2015**, *11*, 564–570.
[65] J. Koehnke, G. Mann, A. F. Bent, H. Ludewig, S. Shirran, C. Botting, T. Lebl, W. E. Houssen, M. Jaspars, J. H. Naismith, *Nat. Chem. Biol.* **2015**, *11*, 558–563.
[66] J. R. Swartz, *AIChE J.* **2012**, *58*, 5–13.
[67] H.-E. Lai, A. M. C. Obled, S. M. Chee, R. M. Morgan, S. V. Sharma, S. J. Moore, K. M. Polizzi, R. J. M. Goss, P. S. Freemont, *bioRxiv* preprint **2019**, *51*, https://doi.org/10.1101/202523.
[68] J. W. Bogart, A. A. Bowers, *Org. Biomol. Chem.* **2019**, *17*, 3653–3669.
[69] K. Higuchi, T. Yabuki, M. Ito, T. Kigawa, *Biotechnol. Bioeng.* **2020**, *117*, 1628–1639.
[70] J. Li, H. Wang, Y.-C. Kwon, M. C. Jewett, *Biotechnol. Bioeng.* **2017**, *114*, 1343–1353.
[71] J. Li, H. Wang, M. C. Jewett, *Biochem. Eng. J.* **2018**, *130*, 29–33.
[72] H. Xu, W.-Q. Liu, J. Li, *ACS Synth. Biol.* **2020**, *9*, 1221–1224.
[73] A. Krüger, A. P. Mueller, G. A. Rybnicky, N. L. Engle, Z. K. Yang, T. J. Tschaplinski, S. D. Simpson, M. Köpke, M. C. Jewett, *Metab. Eng.* **2020**, *62*, 95–105.
[74] W. Buckel, B. T. Golding, *Annu. Rev. Microbiol.* **2006**, *60*, 27–49.
[75] Y. Nicolet, *Nat. Can.* **2020**, *3*, 337–350.
[76] J. Li, P. Neubauer, *New Biotechnol.* **2014**, *31*, 579–585.
[77] J. Li, L. Zhang, W. Liu, *Synth. Syst. Biotechnol.* **2018**, *3*, 83–89.
[78] W. Finnigan, L. J. Hepworth, N. J. Turner, S. Flitsch, *ChemRxiv.* preprint **2020**, DOI: https://doi.org/10.26434/chemrxiv.12571235.v1.

Manuscript received: July 9, 2020
Revised manuscript received: August 7, 2020
Accepted manuscript online: August 11, 2020
Version of record online: September 22, 2020