Versatile and Facile One-Pot Biosynthesis for Amides and Carboxylic Acids in E. coli by Engineering Auxin Pathways of Plant Microbiomes

Navya Menon, Daniel Richmond, Mohammad Rejaur Rahman, and Binuraj R. K. Menon*

ABSTRACT: The development of enzymatic routes toward amide and carboxylic acid bond formation in bioactive molecular scaffolds using aqueous conditions is a major challenge for biopharmaceutical and fine chemical industrial sectors. We report biocatalytic and kinetic characterization of two indole-3-acetamide (IAM) pathway enzymes, tryptophan-2-monooxygenase (iaaM) and indole-3-acetamide hydrolase (iaaH), present in plant microbiomes that produce indole-3-acetic acid (IAA). In this pathway, tryptophan is converted to indole-3-acetamide by the monooxygenase activity of iaaM, followed by its hydrolysis to form carboxylic acid by iaaH enzyme. Since IAA or auxin is an essential natural plant hormone and an important synthon for natural plant hormones and phytoregulators, with a profound role in plant growth and development functioning as auxin pathways, plant microbiome, halogenases, synthetic biology, one-pot enzyme cascades, biocatalysis

1. INTRODUCTION

There is renewed scientific interest to develop biocatalytic routes that modify inexpensive proteogenic amino acids toward creating bioactive synthons with amide and carboxyl groups, which could be exploited for pharmaceutical, agrochemical, and other industrial applications.1−5 Enzymes that can directly catalyze the formation of amide or carboxyl group can offer attractive alternatives to many currently adopted synthetic and abiotic reactions. Once rationally engineered into microorganisms, these could lead to the sustainable and continuous synthesis of divergent molecules and functionalities, such as α-keto and α-hydroxy acids, alcohols, stereoisomers of natural and non-natural amino acids, amines, peptides, etc.6−8

The current enzymatic routes to amide bond formation are mainly via aminolysis of esters in organic solvents using lipases.9,10 Biocatalytic conversion of carboxylic acid to amides is possible in aqueous media via carboxylic acid reductases (CARs), which form reactive acyl intermediates utilizing expensive cofactors, adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADH).11−14 The activated acyl-AMP intermediate could be intercepted by amine nucleophiles in the CAR reaction, leading to amide bond formation. Similar strategies with ATP-dependent amide bond synthetase enzymes are also known.15,16 By mimicking natural amide bond formation reactions using a combination of different N-acyltransferases and ATP-dependent CoA ligases, syntheses of structurally diverse secondary and tertiary amides were reported.17 Recently, a synthetically challenging one-pot reaction with a bio-chemo-bio catalytic route was reported to address the lack of biosynthetic protocols for indolic amides and carboxylic acids (Figure 1B).3

Though monooxygenase-based unique oxidative decarboxylation of amino acids to form amides has been reported in plants and associated microbiomes, these pathways have not been explored further for their synthetic utilities to produce amide and carboxyl acid from aromatic amino acid precursors. For example, auxin biosynthetic pathways primarily act on L-tryptophan by tryptophan-2-monooxygenase (iaaM) to form indole-3-acetamide (IAM), followed by indole-3-acetamide hydrolase (iaaH) activity to form indole-3-acetic acid (IAA) (Figure 2).22 IAA and other auxins are essential natural plant hormones and phyto regulators,23 with a profound role in plant growth and development functioning as auxin pathways, plant microbiome, halogenases, synthetic biology, one-pot enzyme cascades, biocatalysis.
physiological chemical code.24−26 In addition to the known IAA analogues that are used as herbicides, indole amine, IAM, and IAA molecular scaffolds are present in many therapeutical drugs such as indomethacin, apyramide, bunodosine, etc., indicating their biological importance and potential applications (Figure S1).20,27

The chemical routes to IAA and its derivatives are mainly via Fischer indole synthesis that forms an indole heterocycle from phenylhydrazine and a carbonyl compound (Figure 1A).23 Several modifications to Fischer indolization were reported, including hydrohydrazination of terminal alkynes, transition-metal-catalyzed Tsuji−Trost, Heck or Buchwald modifications, etc.18−20 These synthetic methods are inherently toxic and environmentally unfriendly and use deleterious reagents, catalysts, and solvents, which has led to great interest in exploiting different IAA biosynthetic pathways. The major routes to IAA in plants are via indole-3-acetamide (IAM) and indole-3-pyruvic acid (IPA) pathways, though IAA could also be formed via tryptamine (TAM) and indole-3-acetaldoxime (IAOx) routes.28 The incompatibility of IAA pathway enzymes to functionally express in other organisms is often regarded as a major issue that has led to adopting approaches such as using plant-based expression systems or immobilizing native cells to improve the low titer.29,30 Previously, an IAA pathway has been engineered in Escherichia coli via a modified IPA route that requires indole-3-pyruvic acid decarboxylase along with IAA dehydrogenase and E. coli transaminase.31 Alternative metabolic engineering in E. coli with aldehyde dehydrogenase that replaces IAA dehydrogenase was also reported.32 However, none of these pathways were evaluated for substituted IAA molecules, and a major issue was the formation of tryptophol as a byproduct due to reduction of indole-3-acetylaldehyde by endogenous E. coli aldehyde reductases. Though the possibility of using either YUC flavin monooxygenases or indolepyruvate ferredoxin oxidoreductases that directly forms IAA from IPA was suggested to circumvent this, these plant-derived enzymes currently have no soluble and active expression in industrial hosts.33,34 Recently, Nicotiana benthamiana has been used as a transient and plant-based expression system for YUC flavin monooxygenase to produce IAA and halogenated derivatives in detectable amounts (Figure 1C).21

Herein, a detailed kinetic characterization and substrate scope evaluation of two indole-3-acetamide (IAM) pathway enzymes, tryptophan-2-monoxygenase (iaaM) and indole-3-acetamide hydrolase (iaaH), were conducted. We present the first report of an efficient de novo biosynthesis of IAA and its derivative via the IAM pathway in engineered E. coli. Three-enzyme one-pot biotransformation approaches to IAA derivatives for large-scale synthesis are also shown.

Figure 1. Chemical and biological approaches to IAA. (A) Chemical synthesis via Fisher indole synthesis and Heck or Buchwald modifications.18−20 (B) Chemo-biocatalytic routes to indole amides and carboxylic acids via halogenase-based bromination followed by cyanation with Pd catalysts. The cyano groups are converted to amide or carboxyl acid via nitrile hydratase (NHase) or nitrilase (NITR).1 (C) Metabolic engineering of IAA pathway genes in a plant-based compatible host organism.21 (D) First report of an efficient de novo biosynthesis of IAA and its derivative via the IAM pathway in engineered E. coli. Three-enzyme one-pot biotransformation approaches to IAA derivatives for large-scale synthesis are also shown.
study demonstrates how to reconstruct and utilize challenging plant-based natural product pathways to turn on novel chemistries in industrial hosts for large-scale synthesis (Figure 1D).

2. RESULTS AND DISCUSSION

2.1. Identifying Potential IAM Pathway Enzymes and Further Optimization. Our studies began by identifying iaaM and iaaH candidate genes from plants and plant-associated bacterial species from the plant and microbial genome database repository (Figures S2 and S3). The plant-EST blast search with known bacterial IAA enzymes to select plant-expressed sequence tag proteins resulted in shorter nucleotide sequences, which are often less than 16 and 40% sequence coverage with bacterial iaaM and iaaH, making them unsuitable for functional studies.35 This led us to revisit the reported plant-associated bacterial candidates for iaaM and iaaH from *Pseudomonas savastanoi* (accession no: QBM20357.1) and *Alcaligenes faecalis* (accession no: WP_052363035.1), respectively.36,37 The initial aim was to investigate overexpression, solubility, and monooxygenase (iaaM) and hydrolase (iaaH) activity so that IAM pathways could be reconstructed in *E. coli* cells for further synthetic approaches (Figure 2).

When the codon-optimized genes of iaaM and iaaH from the above sources in normal pET vectors were analyzed for protein expression using SDS-PAGE, it was found that the overexpressed proteins were mostly insoluble in *E. coli* BL21(DE3) cells (Figures S4 and S5). An initial HPLC analysis of iaaM and iaaH lysed cells showed negligible activity on tryptophan and IAM, respectively, indicating that a large proportion of the highly expressed iaaM fraction in *E. coli* cells is still in an unfolded state. The previously reported studies on iaaM and iaaH via *E. coli* cells have used tedious protocols that involve several chromatographic purification steps.38,39 Though iaaM was highly overexpressed, only a few milligrams of active purified protein were obtained for these studies.38,40 To improve the solubility and to address the low activity shown by cell lysates, several different approaches were carried out by adjusting growth conditions, using different *E. coli* cell lines and solubility tags such as GST and SUMO, which are summarized in Tables S3 and S4. By creating an expression construct with additional DNA sequences for an N-terminal SUMO tag and overexpressing this new construct in Lemo21(DE3) Competent *E. coli* cells at 24 °C, we attained a highly soluble and active version of iaaM and iaaH proteins (Figures S4 and S5). The SUMO fusion proteins were subsequently purified to homogeneity mainly using single-step Ni²⁺ affinity chromatographic purification, thereby allowing for further kinetic and mutagenesis studies of these enzymes.

2.2. Biochemical Characterization of IAM Enzymes and Site-Directed Mutagenesis. To examine the kinetic parameters of the purified SUMO-iaaM fusion protein, *in vitro* assays were performed with tryptophan and tyrosine. SUMO-iaaM exhibits an apparent $k_{cat}$ of 8.4 s⁻¹ for tryptophan and 0.3 ± 0.05 s⁻¹ for tyrosine (Table 1). The enzyme prefers tryptophan over tyrosine with an ∼2.6-fold improved binding affinity, which is reflected in the $K_M$ values. Consequently, the catalytic efficiency of iaaM for tyrosine (2.7 mM⁻¹ s⁻¹) is 72.7 times lower than that of tryptophan. Though iaaM was reported to show very low oxidase (oxidative deamination) activity toward a few nonaromatic amino acids, no acetamide formation was observed when SUMO-iaaM in *in vitro* assay samples with alanine, methionine, and glycine were analyzed by LC-MS, indicating that the iaaM enzyme predominantly acts on amino acids with indolic and phenyl groups (Figures S6 and S7). Based on the available crystal structure, Arg98, Phe244, Lys365, Trp466, Phe476, Leu478, and Trp519 are the
important active site residues in iaaM, which are present within a 5 Å distance of the substrate and the FAD-binding pocket (Figures 3, S6, and S7). Among these residues, Arg98 interacts with the substrate carboxylic group, whereas Phe244, Lys365, Trp466, and Trp519 are part of a hydrophobic core around the substrate’s indolic binding pocket. Except for Leu478, Lys365, and Trp466, all other residues were conserved in monoamine oxidase family enzymes. In lysine-2-monoxygenase, additional residues (e.g., Asp238) are present at the end of the hydrophobic pocket creating charge interactions preferred for polar and charged side chain amino acid binding, which are totally absent in iaaM.

To identify the role of these residues in substrate-binding and recognition, alanine-substituted mutant enzymes were initially created for Arg98, Phe244, Lys365, Trp466, Phe476, Leu478, and Trp519 residues, and their activity was tested against tryptophan and tyrosine. The results showed that alanine-substituted Arg98, Phe244, Lys365, Trp466, and Phe476 single mutants had a significantly reduced activity toward both tryptophan and tyrosine, indicating the role of these residues in substrate-binding. Replacing Arg98 residue with lysine was then attempted to evaluate whether activity loss was due to the absence of the side chain amino or basic groups in Arg98Ala. The Arg98Lys variant thus created retained 58% of wild-type activity for tryptophan with catalytic turnover ($k_{cat}$) of $0.15 \pm 0.03$ s$^{-1}$ for tryptophan) and $K_{M}$ values (140.8 ± 1.8 μM) significantly compromised (Table 1 and Figure 3). This further confirmed the relative position and role of Arg98 residue, which interacts with the substrate carboxyl group as observed in the crystal structure. Previous mechanistic studies of iaaM from P. savastanoi also indicated that Arg98 is critical for the correct positioning of the substrate in the active site. The double mutants with Arg98Ala mutation were also inactive, supporting the fact that Arg98 is an important residue in the oxidative catalytic mechanism. The kinetic parameters for Trp466Ala and Phe476Ala mutants were not determined due to very low activities. However, the Phe244Ala mutation was found to have a very weak binding affinity for tryptophan (>200 μM), indicating that these residues at the aromatic core are important in substrate recognition as expected. Interestingly, Phe244Ala mutant showed improved reactivity toward tyrosine than tryptophan, though the precise nature of this improved activity is not known; it could be the tyrosine hydroxyl group that may differentially interact within the active site. On the other hand, alanine substitutions at Leu478 and Trp519 were found to have a lower impact on the relative activity of the iaaM enzyme, indicating that these residues have a minor role in catalysis.

Kinetic studies with the purified SUMO-iaaH enzyme showed that the enzyme was active with the natural substrate IAM and benzamide with $k_{cat}$ values of 2.8 ± 0.4 and 0.15 ± 0.02 min$^{-1}$, respectively (Table 1). To identify iaaH active site residues, a homology model via the phyre2 webserver was generated based on amidase signature (AS) enzyme sequences and the crystal structure of glutamyl-tRNA(gln) amidotransferase enzyme (PDB id: 2F2A). The model predicted several catalytic residues of iaaH, including the serine–serine–lysine (Ser176, Ser151, and Lys77) catalytic triad of amide hydrolyase.
enzymes (Figures S8–S10). From the model, it was found that two residues, Ser152 and Thr173, were within the substrate-binding pocket with a possible role in catalysis. To validate this, all five residues were selected for single alanine substitution. Ser151Thr, Ser152Thr, and Ser176Thr single mutants were also generated. The alanine substitution for catalytic triad residues Lys77, Ser151, and Ser176 created mutant enzymes with significantly reduced conversion, retaining only below 10% of wild-type activity. Threonine substitution at Ser151 and Ser176 positions retained most of the wild-type activity, which indicated the involvement of these residues in the proposed reaction mechanism. The Ser152Ala and Thr173Ala mutants were found to be catalytically less affected, indicating their minor role in the reaction mechanism. Though Ser152Ala only exhibited 56% of relative wild-type activity toward IAM, the enzymatic activity was improved to 65% in the Ser152Thr mutant (Table 1 and Figure 3). This could imply that Ser152 possibly assists in the proposed proton transfer mechanism; however, further experiments are needed to confirm this. The steady-state kinetic measurements also revealed that Ser151Thr and Ser152Ala single mutations reduced the wild-type catalytic turnover by fivefold and 12-fold, respectively, and $K_M$ values by fivefold (Table 1).

### 2.3. IAM De Novo Biosynthesis and Substrate Scope via Cascade Reactions

To further explore the scope of developing an in vivo reaction with active IAM pathway enzymes in E. coli cells, we carried out whole-cell assays with SUMO-iaaM and SUMO-iaaH proteins (Figure 4). Initial mass-spectrometry analysis of Lemo21(DE3) cultures that carry pET-DUET-SUMO-iaaM and pCDF-SUMO-iaaH plasmids showed the production of IAM (<2 mg/L) and indole-3-acetic acid (IAA) (MS-detectable) from the endogenous tryptophan. As the tryptophan biosynthesis is energetically unfavorable, endogenous production via chorismite or phosphoenol pyruvate pathways is tightly regulated with different feedback inhibition, attenuation, and repression mechanisms.43,44 As the limited cell supply of tryptophan and indole could be the bottleneck in IAA, we incorporated the tryptophan synthase (Trp_syn) plasmid that carries Salmonella typhimurium tryptophan synthase (α chain and beta2 subunit) into whole-cell reactions to improve IAA titers. Our initial approach to co-express Trp_syn along with pET-SUMO-iaaM and pCDF-SUMO-iaaH constructs indicated that this could improve the IAM and IAA titer; however, best results were obtained when the Trp_syn-overexpressed cell supernatant was used for whole-cell biosynthesis, and up to 5.2 mg/L IAM and 1.2 mg/L IAA were quantified (Table 2).
The major advantage of Trp_syn is its adaptability for one-pot biocatalytic cascades from inexpensive indole molecules. This was previously exploited for the synthesis of noncanonical amino acids and other value-added molecular syntheses.\textsuperscript{42,43} The wild-type and engineered Trp_syn enzyme variants that accept various indolic and serine analogous molecules are currently available.\textsuperscript{45−47} Due to the hydrophobic nature of the indolic substrate and limited cell permeability, Trp_syn reactions were found to perform well in cell lysate assays. To exploit this possibility, cell lysate assays with Trp_syn, iaaM, and iaaH were conducted by feeding 20 mg of indole and serine to this one-pot cascade reaction and leaving it for 2 days. The results showed that up to 19.8 mg of IAM (for Trp_syn-iaaM cascade) and 17.2 mg of IAA (for Trp_syn-iaaM-iaaH cascade) were formed, which is equivalent to 66.6 and 57.5% overall conversion from indole (Table 2). With the success of this approach, we investigated it further to study the substrate scope of both IAM pathway enzymes via cell lysate cascade reactions. The cell lysate cascade reactions were set up with several indole substrates with substitutions at 7, 6, 5, 4, 2, and 1 positions. Chloro- and bromosubstituted indoles with the halogen atom at 7, 6, 5, and 4 positions were accepted by Trp_syn forming the corresponding substituted tryptophan compounds, which were converted to IAM and IAH derivatives by the downstream activity of iaaM and IaaH enzymes (Table 3).

The HPLC conversion from substituted indole showed that for the iaaM enzyme reaction 7-halogenated tryptophan and for iaaH enzyme reaction 7-halogenated indole-acetamides were the best substrates, with 83.9% overall conversion of the 7-halo indole molecule to the final IAA product. The relative conversion of chlorosubstituted indoles was more than that of bromoindoles in the cascade, and this could be possibly attributed to differences in solubility of these molecules and other reasons including different substrate preferences of pathway enzymes. Bromoindoles are oily liquids and less soluble than chloroindoles in polar solvents. Toward other substitutions, iaaM and iaaH enzymes were found to be active for substituted methyl groups. The overall conversion of 7-methyl indoles was 40.7% with 95% conversion to 7-methyl indole-3-acetamide by iaaM and 43% conversion to 7-methyl indole-3-acetic acid by iaaH. Though 5-hydroxy indole and 4-hydroxy indoles were accepted by Trp_syn forming the corresponding hydroxy tryptophan molecules, they were not catalyzed by iaaM enzymes. Similarly, 5-nitro tryptophan, 6-nitro tryptophan, and 5-methoxy tryptophan were not accepted as a substrate by iaaM, indicating that tryptophan with electron-withdrawing groups at these positions is a poor substrate for iaaM monoxygenation reactions. Though 1-methyl indole was converted to 1-methyl indole-3-acetic acid in good yields, 2-methyl tryptophan was not converted to the corresponding IAM and IAA derivatives, indicating strict

| Carbon source and feeding chemical | Assay Type | Enzymes | Amide conversion in milligrams | Carboxylic acid conversion in milligrams |
|-----------------------------------|------------|---------|---------------------------------|-----------------------------------------|
| D-Glucose                         | Whole cell reactions | Trp_syn, iaaM, iaaH | < 2 mg/litre of culture | *detected by MS |
| Indole + L-Serine (total amount of indole added = 20 mg) | Cell Lysate reaction | Trp_syn, iaaM, iaaH | 5.2 ± 1.2 mg/litre of E. coli culture | 1.2 ± 0.5 mg/litre of E. coli culture |
| Tryptophan (total amount of Tryp added = 20 mg) | Cell Lysate reaction | Trp_syn, iaaM, iaaH | 19.8 ± 0.5 | 17.2 ± 0.8 |
|                                  | CLEA       | iaaM, iaaH | PnA, iaaM, iaaH | 0.73 ± 0.1 | 0.33 ± 0.04 |
|                                  |            | iaaM, iaaH | PmA, iaaM, iaaH | 9.60 ± 0.67 | 3.66 ± 0.10 |
|                                  |            | iaaM, iaaH | StH, iaaM, iaaH | 0.73 ± 0.1 | 0.22 ± 0.03 |
|                                  |            | iaaM, iaaH | StH, iaaM, iaaH | 9.43 ± 0.52 | 3.45 ± 0.07 |
|                                  |            | iaaM, iaaH | PyrH, iaaM | 0.76 ± 0.09 | 0.39 ± 0.05 |
|                                  |            | iaaM, iaaH | PyrH, iaaM | 10.03 ± 0.31 | 4.21 ± 0.10 |

*No external glucose source was used in the media. The reaction conditions are detailed in Section 1.5 in the Supporting Information.*

ACS Catal. 2022, 12, 2309−2319
regiopreferences for IAM reaction (Table 3). All of the cascade reaction products of iaaM reactions were scaled up and further characterized using NMR, UV, and mass-spectrometry analyses, and the isolated yields were determined (Figures S11−S21 and S27−S66 and Table S5).

2.4. Halogenase-Based Pathway Generation and Recyclable One-Pot Reactions. Flavin-dependent halogenase (Fl-Hal)-based selective activation of aromatic C−H positions has transformed integrated chemo−bio-synthesis in recent years.48−50 Fl-Hal enzymes PrnA, SttH, and PyrH for regiospecifically halogenated tryptophan 7, 6, and 5 positions, respectively, were studied extensively by different groups, providing an effective catalyst control for the followed Pd-based cross-coupling chemistries.50−56 As we identified that iaaM and iaaH enzymes could accept halogenated compounds at different positions, the possibility of utilizing Fl-Hals (PrnA, PyrH, and SttH) to install halogen bonds in tryptophan was explored. The halogenated tryptophan could be further processed via IAM pathway enzymes generating site-specific halogenated IAMs and IAAs. Our initial aim was to attempt this via generating in vivo cascades in whole-cell systems. Due to the low reactivity of tryptophan Fl-Hals and the low concentration of free tryptophan in whole-cell systems, preparing a detectable amount of in vivo halogenated tryptophan in E. coli cell cultures was found to be challenging. We looked for alternative ways to address this by conducting a partially lysed whole-cell reaction with BL2(DE3) cells that carry an Fl-Hal plasmid (either pET-PrnA, pET-PyrH, or pET-STTH) and feeding tryptophan to the cell cultures (Figure 4).

Table 3. Three-Enzyme One-Pot Cell Lysate Cascade Reaction with Tryptophan Synthase (Trp_syn), Tryptophan-2-Monoxygenase (iaaM), and Indole-3-Acetamide Hydrolase (iaaH) by Feeding Substituted Indole and Serine Molecules

| Position | X | Conversion (%) | Overall conversion (in molar %) |
|----------|---|----------------|-------------------------------|
| 7        | Cl   | 99 ± 1%         | 83.9%                         |
| 6        | Cl   | 99 ± 1%         | 83.9%                         |
| 5        | Cl   | 92 ± 3%         | 80 ± 2%                       |
| 4        | Cl   | 96 ± 4%         | 86 ± 3%                       |
| 1        | Cl   | 69 ± 2%         | 60 ± 2%                       |
| 2        | Cl   | 63 ± 2%         | 60 ± 2%                       |
| 4.5      | Cl   | 61 ± 3%         | 64 ± 1%                       |

Each column represents HPLC conversion values of the final substrate molecule by terminal enzymes in Trp_syn, Trp_syn + SUMO-iaaM, and Trp_syn + SUMO-iaaM + SUMO-iaaH enzyme combinations. The detailed experimental protocol is provided in the Materials and Methods section (Section 1.6 in the Supporting Information). The distribution of products in the final reaction as a sum of substrate, products, and intermediates is shown in Figure S6. NC = no conversion.
cultures allowed us to detect site-specific monohalogenated IAM and IAA molecules with all of the tryptophan Fl-Hal enzymes used in this study. To improve the product yield, we further used completely lysed cell assays using overexpressed and nonpurified Fl-Hal protein solutions. This led to 0.73–0.76 mg of either 5, 6, or 7 monohalogenated IAA and 0.2–0.4 mg of the corresponding IAM derivatives with PyrH, SttH, and PrnA enzymes, respectively (Table 2). The higher titers of nonhalogenated IAM and IAA molecules in the cell cultures suggested that the limiting step of these reactions is the halogenation reactions.

To address this, we conducted cascade reactions using cross-linked enzyme aggregate (CLEA) of Fl-Hals and IAM enzymes in a one-pot reaction setup with an increased enzyme loading. The use of CLEA improved the 7-chlorotryptophan conversion of PrnA to 61% compared to 9% conversion observed in cell lysate assays (Table 2 and Figure 5). Similarly, 6-chlorotryptophan conversion in SttH CLEA was 58%, and that for 5-chlorotryptophan via PyrH CLEA was 58%. The increased chlorotryptophan also contributed to the improved yield of regiospecific halogenated IAM and IAA when reactions were continued with iaaM or combined iaaM/iaaH CLEAs. The CLEA reaction using PyrH Fl-Hals produced the highest monohalogenated IAM and IAA titers, quantified as 10 mg of 5-chloro indole-3-acetamide and 4.2 mg of 5-chloro indole-3-acetic acid (Table 2). A similar level of substituted products was also quantified from PrnA and SttH Fl-Hal CLEA reactions. Though we have not performed further optimizations for large-scale production of halogenated acetamide and acetic acid products, these processes were found to be easily scalable. It is equally important to consider that Fl-Hal CLEA catalysts were made from 1.5 L of cell culture with the provision to feed on higher amounts of tryptophan (instead of the 20 mg used). Encapsulation of Fl-Hals and IAM pathway enzymes in CLEA also allowed us to reuse the catalysts without losing much of the activity when used multiple times. The results showed that conversion of 7-chloro indole-3-acetamide drops only by 13–15% for PrnA-IaaM and PyrH-IaaM CLEA cascade after three rounds of catalysis (Figure S22). We anticipate that by further optimizing scale-up conditions and CLEA constituents via standard bioprocess engineering techniques, our developed method will be widely applicable for direct industrial-scale synthesis.

3. CONCLUSIONS AND PERSPECTIVE

In summary, we have reconstructed and developed a plant-associated biosynthetic pathway in E. coli to produce many substituted IAM and IAA molecules, which was not previously possible. Along with finding a suitable solution to produce a highly overexpressed and soluble E. coli version for IAM pathway enzymes and carrying out detailed mutagenesis analysis, we have opened future venues to engineer these enzymes to produce many other value-added molecules from different amino acid precursors. Toward this direction, we are currently carrying out site-directed and random mutagenesis approaches to evolve the enzyme to accept natural and non-natural amino acids. Compared to other known methods for amide and carboxylic acid bond formation, both enzymes in the IAM pathway use inexpensive cofactors (such as ATP or NADH) and could perform reactions in aqueous conditions. By combining the pathway with tryptophan synthase and flavin-dependent regiospecific halogenases, we have displayed different routes to convert easily available chemicals such as indole and tryptophan to synthesize various IAM and IAA analogues. We have also displayed the compatibility of these enzymes to carry out in vivo reactions and for converting sugar into amide and carboxylic acid-containing bioactive molecules and non-natural analogues. Most importantly, the cell lysate and CLEA-based approaches further expanded the current scope of IAM pathway enzymes. The CLEA-based cascade systems that we have developed here are highly versatile, scalable, quantifiable, and reusable for large-scale synthesis and industrial uses, which will attract wider interest from the biocatalysis community and plant biotechnology researchers to investigate other similar plant-based flavin-dependent enzymes and pathways.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c04901.

https://doi.org/10.1021/acscatal.1c04901
ACS Catal. 2022, 12, 2309–2319
Materials and methods; details of plasmid and protein sequences; HR-MS and LC-MS analysis; reaction kinetics characterization; and details of product characterization using NMR, UV, LR-MS, and HR-MS techniques (PDF)

■ AUTHOR INFORMATION

Corresponding Author
Binuraj R. K. Menon – The Warwick Integrative Synthetic Biology Centre, The University of Warwick, Coventry CV4 7AL, U.K.; School of Biological Sciences, The University of Portsmouth, Portsmouth PO1 2DY, U.K.; Email: Binuraj.menon@port.ac.uk

Authors
Navya Menon – The Warwick Integrative Synthetic Biology Centre, The University of Warwick, Coventry CV4 7AL, U.K.; Collaborative Teaching Laboratory, The University of Birmingham, Birmingham B15 2TT, U.K.
Daniel Richmond – The Warwick Integrative Synthetic Biology Centre, The University of Warwick, Coventry CV4 7AL, U.K.
Mohammad Rejaur Rahman – The Warwick Integrative Synthetic Biology Centre, The University of Warwick, Coventry CV4 7AL, U.K.

Complete contact information is available at: https://pubs.acs.org/10.1021/acscatal.1c04901

Author Contributions
B.R.K.M. conceived and designed the project. N.M. and B.R.K.M. performed the experiments, collected the data, and analyzed the results. D.R. performed experiments, analyzed bioinformatic data, and contributed to data collection. M.R.R. performed experiments and assisted in carrying out specific tasks related to the project. All authors discussed and coordinated the experiments. N.M. wrote the initial draft and together with B.R.K.M. prepared the final version. All authors discussed and agreed to the final draft.

Funding
This work was supported by the UKRI Synthetic Biology for Growth Programme (SBfG) under Grant BB/M017982/1 and the Royal Society under Grant RGS\R2\180317.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

B.R.K.M. is grateful to Warwick Integrative Synthetic Biology Centre (WISB) for their generous funding for Gene synthesis. D.R. received Ph.D. funding support from Midlands Integrative Biosciences Training Partnership (MBTP) and M.R.R. received Ph.D. funding from Commonwealth Scholarship Scheme. The authors are thankful to Professor Greg Challis and CBRF for access to instruments and chemicals.

■ ABBREVIATIONS

IAM, indole-3-acetamide; IAA, indole-3-acetic acid; Trp$_{\text{syn}}$, tryptophan synthase; CLEA, cross-linked enzyme aggregates; FI-Hal, flavin-dependent halogenases; iaaM, tryptophan-2-monoxygenase; iaaH, indole-3-acetamide hydrolase

■ REFERENCES

(1) Craven, E. J.; Latham, J.; Shepherd, S. A.; Khan, I.; Diaz-Rodriguez, A.; Greaney, M. F.; Micklefield, J. Programmable late-stage C–H bond functionalization enabled by integration of enzymes with chemocatalysis. Nat. Catal. 2021, 4, 385–394.
(2) Bartsch, S.; Bornscheuer, U. T. Mutational analysis of phenylalanine ammonia lyase to improve reactions rates for various substrates. Protein Eng. Des. Sel. 2010, 23, 929–933.
(3) Weise, N. J.; Ahmed, S. T.; Parmeggiani, F.; Galman, J. L.; Dunstan, M. S.; Charnock, S. J.; Leys, D.; Turner, N. J. Zymophore identification enables the discovery of novel phenylalanine ammonia lyase enzymes. Sci. Rep. 2017, 7, no. 13691.
(4) Cheng, Z.; Xia, Y.; Zhou, Z. Recent Advances and Promises in Nitrile Hydratase: From Mechanism to Industrial Applications. Front. Bioeng. Biotechnol. 2020, 8, no. 352.
(5) Infasian, P.; Prakinee, K.; Phintha, A.; Trisrivirat, D.; Weeranoppanant, N.; Wongnate, T.; Chaiyen, P. Enzymes, In Vivo Biocatalysis, and Metabolic Engineering for Enabling a Circular Economy and Sustainability. Chem. Rev. 2021, 121, 10367–10451.
(6) Song, W.; Chen, X.; Wu, J.; Xu, J.; Zhang, W.; Liu, J.; Chen, J.; Liu, L. Biocatalytic derivatization of proteinogenic amino acids for fine chemicals. Biotechnol. Adv. 2020, 40, no. 107496.
(7) Parmeggiani, F.; Lovelock, S. L.; Weise, N. J.; Ahmed, S. T.; Turner, N. J. Synthesis of D- and L-phenylalanine derivatives by phenylalanine ammonia lyases: a multi-enzymatic cascade process. Angew. Chem., Int. Ed. 2015, 54, 4608–4611.
(8) Boville, C. E.; Scheele, R. A.; Koch, P.; Brinkmann-Chen, S.; Boller, A. R.; Arnold, F. H. Engineered Biosynthesis of beta-Alkyld Tryptophan Analogs. Angew. Chem., Int. Ed. 2018, 57, 14764–14768.
(9) Liljeblad, A.; Kallio, P.; Vainio, M.; Niemi, J.; Kanerva, L. T. Formation and hydrolysis of amide bonds by lipase A from Candida antarctica; exceptional features. Org. Biomol. Chem. 2010, 8, 886–895.
(10) Zeng, S.; Liu, J.; Anankabinl, S.; Chen, M.; Guo, Z.; Adams, J. P.; Snajdrova, R.; Li, Z. Amide Synthesis via Aminolysis of Ester or Acid with an Intracellular Lipase. ACS Catal. 2018, 8, 8856–8865.
(11) Wood, A. J. L.; Weise, N. J.; Frampton, J. D.; Dunstan, M. S.; Hollas, M. A.; Derrington, S. R.; Lloyd, R. C.; Qiaglia, D.; Parmeggiani, F.; Leys, D.; Turner, N. J.; Flitsch, S. L. Adenylation Activity of Carboxylic Acid Reductases Enables the Synthesis of Amides. Angew. Chem., Int. Ed. 2017, 56, 14498–14501.
(12) Ramsden, J. I.; Heath, R. S.; Derrington, S. R.; Montgomery, S. L.; Mangas-Sanchez, J.; Mulholland, K. R.; Turner, N. J. Biocatalytic N-Alkylation of Amines Using Either Primary Alcohols or Carboxylic Acids via Reductive Aminase Cascades. J. Am. Chem. Soc. 2019, 141, 1201–1206.
(13) Finnigan, W.; Thomas, A.; Cromar, H.; Gough, B.; Snajdrova, R.; Adams, J. P.; Littlechild, J. A.; Harmer, N. J. Characterization of Carboxylic Acid Reductases as Enzymes in the Toolbox for Synthetic Chemistry. ChemCatChem 2017, 9, 1005–1017.
(14) Pongpamorn, P.; Kiattisewee, C.; Kittipanukul, N.; Jaroensuk, J.; Trisrivirat, D.; Maenpuen, S.; Chaiyen, P. Carboxylic Acid Reductase Can Catalyze Ester Synthesis in Aqueous Environments. Angew. Chem., Int. Ed. 2021, 60, 5749–5753.
(15) Petchey, M. R.; Rowlinson, B. J.; Lloyd, R. C.; Fairlamb, I. J. S.; Grogan, G. Biocatalytic Synthesis of McbBamide Using the Amide Bond Synthetase McbA Coupled with an ATP Recycling System. ACS Catal. 2020, 10, 4659–4663.
(16) Petchey, M.; Cuetos, A.; Rowlinson, B. J.; Dannevald, S.; Frese, A.; Sutton, P. W.; Lovelock, S.; Lloyd, R. C.; Fairlamb, I. J. S.; Grogan, G. The Broad Aryl Acid Specificity of the Amide Bond Synthetase McbA Suggests Potential for the Biocatalytic Synthesis of Amides. Angew. Chem., Int. Ed. 2018, 57, 11584–11588.
(17) Philipott, H. K.; Thomas, P. J.; Tew, D.; Fuerst, D. E.; Lovelock, S. L. A versatile biosynthetic approach to amide bond formation. Green Chem. 2018, 20, 3426–3431.
(18) Tsui, J.; Takahashi, H. Organic Syntheses by Means of Noble Metal Compounds. XII.1 Reaction of the Cyclooctadiene-Palladium
Chloride Complex with Ethyl Malonate. J. Am. Chem. Soc. 1965, 87, 3275–3276.

(19) Trost, B. M.; Fullerton, T. J. New synthetic reactions. Allylic alkylation. J. Am. Chem. Soc. 1973, 95, 292–294.

(20) Chen, D.; Chen, Y.; Ma, Z.; Zou, L.; Li, J.; Liu, Y. One-Pot Synthesis of Indole-3-acetic Acid Derivatives through the Cascade Tsuji-Trost Reaction and Heck Coupling. J. Org. Chem. 2018, 83, 6805–6814.

(21) Davis, K.; Gkotsi, D. S.; Smith, D. R. M.; Goss, R. J. M.; Caputi, L.; O’Connor, S. E. Nicotiana benthamiana as a Transient Expression Host to Produce Auxin Analogs. Front. Plant Sci. 2020, 11, No. 581675.

(22) Lehmann, T.; Hoffmann, M.; Hentrich, M.; Pollmann, S. Indole-3-acetamide-dependent auxin biosynthesis: a widely distributed way of indole-3-acetic acid production? Eur. J. Cell Biol. 2010, 89, 895–905.

(23) De Rybel, B.; Audenaert, D.; Beeckman, T.; Kepinski, S. The past, present, and future of chemical biology in auxin research. ACS Chem. Biol. 2009, 4, 987–998.

(24) Duca, D.; Lory, J.; Patten, C. L.; Rose, D.; Glick, B. R. Indole-3-acetic acid in plant-microbe interactions. Antonic van Leeuwenhoek 2014, 106, 85–125.

(25) Liu, Y. Y.; Chen, H. W.; Chou, J. Y. Variation in Indole-3-Acetic Acid Production by Wild Saccharomyces cerevisiae and S. paradoxus Strains from Diverse Ecological Sources and Its Effect on Growth. PLoS One 2016, 11, No. e0160524.

(26) Fu, S. F.; Wei, J. Y.; Chen, H. W.; Liu, Y. Y.; Lu, H. Y.; Chou, J. Y. Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms. Plant Signal. Behav. 2015, 10, No. e1048052.

(27) Lewis, J. H.; Stine, J. G. Nonsteroidal Antiinflammatory Drugs and Leukotriene Receptor Antagonists. In Drug-Induced Liver Disease, 3rd ed.; Kaplowitz, N.; DeLeve, L. D., Eds.; Academic Press: Boston, 2013; Chapter 22, pp 369–401.

(28) Bunsangiam, S.; Sakpuntoon, V.; Srisuk, N.; Ohashi, T.; Fujiyama, K.; Limtong, S. Biosynthetic Pathway of Indole-3-Acetic Acid in Basidiomycetous Yeast Rhodotorula flavidus. Mycobiology 2019, 47, 292–300.

(29) Myo, E. M.; Ge, B.; Ma, J.; Cui, H.; Liu, B.; Shi, L.; Jiang, M.; Zhang, M.; Zhang, K. Indole-3-acetic acid production by Streptomyces fradiae NZK-259 and its formulation to enhance plant growth. BMC Microbiol. 2019, 19, No. 155.

(30) Shao, J.; Li, S.; Zhang, N.; Cui, X.; Zhou, X.; Zhang, G.; Shen, Q.; Zhang, R. Analysis and cloning of the synthetic pathway of the indole-3-pyruvate pathway by plant growth promoter Xylophilus paradoxis. Arch. Biochem. Biophys. 2020, 6814, 2591–2613.

(31) B. J.; Rackham, E. J.; Marelli, E.; Hamed, R. B.; Goss, R. F. H. Tailoring Tryptophan Synthase TrpB for Selective Quaternary Bond Formation. J. Am. Chem. Soc. 2019, 141, 19817–19822.

(32) Watkins-Dulaney, E.; Strathof, S.; Arnold, F. Tryptophan Synthase: Biocatalyst Extraordinaire. ChemBioChem 2021, 22, 5–16.

(33) Francis, D.; Winn, M.; Latham, J.; Greeney, M. F.; Micklefield, J. An Engineered Tryptophan Synthase Opens New Enzymatic Pathways to beta-Methyltryptophan and Derivatives. ChemBioChem 2017, 18, 382–386.

(34) Micklefield, J.; Henry, J. M.; Sharif, H. H.; Menon, B. R.; Shepherd, S. A.; Greeney, M. F.; Micklefield, J. Integrated catalysis opens new arylation pathways via regiodivergent enzymatic C-H activation. Nat. Commun. 2016, 7, No. 11873.

(35) Sharma, S. V.; Tong, X.; Pubill-Ulldemolins, C.; Cartmell, C.; Bogosyan, E. J.; Rackham, E. J.; Marelli, E.; Hamed, R. B.; Goss, R. J. M. Living GenoChemetics by hyphenating synthetic biology and synthetic chemistry in vivo. Nat. Commun. 2017, 8, No. 229.

(36) Menon, B. R. B.; Richardon, D.; Menon, N. Halogenases for biosynthetic pathway engineering: Toward new routes to natural and non-naturals. Catal. Rev. 2020, 1–59.

(37) Mondal, D.; Fisher, B. F.; Jiang, Y.; Lewis, J. C. Flavin-dependent halogenases catalyze enantioselective olefin halocyclization. Nat. Commun. 2021, 12, No. 3268.

(38) Shepherd, S. A.; Karthikeyan, C.; Latham, J.; Structed, A. W.; Thompson, M. L.; Menon, B. R. K.; Styles, M. Q.; Levy, C.; Leys, D.; Micklefield, J. Extending the biocatalytic scope of regioselectively flavin-dependent halogenase enzymes. Chem. Sci. 2015, 6, 3454–3460.

(39) Schepel, C.; Sewald, N. Enzymatic Halogenation: A Timely Strategy for Regioselective C-H Activation. Chem. – Eur. J. 2017, 23, 12064–12086.

(40) Shepherd, S. A.; Karthikeyan, C.; Latham, J.; Structed, A. W.; Thompson, M. L.; Menon, B. R. K.; Styles, M. Q.; Levy, C.; Leys, D.; Micklefield, J. A Structure-Guided Switch in the Regioselectivity of a Tryptophan Halogenase. ChemBioChem 2016, 17, 821–824.

(41) Glotsi, D. S.; Ludewig, H.; Sharma, S. V.; Connolly, J. A.; Dhaliwal, J.; Wang, Y.; Unsworth, W. P.; Taylor, R. J. K.; McLachlan, M. M. W.; Shanahan, S.; Naismith, J. H.; Goss, R. J. M. A marine viral
halogenase that iodinates diverse substrates. *Nat. Chem.* 2019, 11, 1091–1097.
(56) Brown, S.; O'Connor, S. E. Halogenase Engineering for the Generation of New Natural Product Analogues. *ChemBioChem* 2015, 16, 2129–2135.