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Scalable biosynthesis of the seaweed neurochemical kainic acid

Jonathan R. Chekan[a], Shaun M. K. McKinnie[a], Malia L. Moore[a], Shane G. Poplawski[b], Todd P. Michael[b], & Bradley S. Moore[a],[c]*

Abstract: *Kainic acid, the flagship member of the kainoid family of natural neurochemicals, is a widely used neuropharmacological agent that helped unravel the key role of ionotropic glutamate receptors, including the kainate receptor, in the central nervous system. Worldwide shortages of this seaweed natural product in 2000 prompted numerous chemical syntheses that now number in excess of 70, including scalable preparations with as few as six-steps. Herein we report the discovery and characterization of the concise two-enzyme biosynthetic pathway to kainic acid from* L*-glutamic acid and dimethylallyl pyrophosphate in red macroalgae and show that the biosynthetic genes are co-clustered in genomes of* Digenea simplex *and the edible* Palmaria palmata*. Moreover, we applied a key biosynthetic α-ketoglutarate-dependent dioxygenase enzyme in a biotransformation methodology to efficiently construct kainic acid on the gram scale. This study establishes both the feasibility of mining seaweed genomes for their biotechnological prowess and the applicability of the genes as biocatalysts in the synthesis of fine chemicals.*

The tropical seaweed *Digenea simplex* has been used for centuries in Asia as an anthelmintic agent to treat parasitic worm infections.[1] In the 1950s, the active compound, kainic acid, was isolated,[2,3] enabling its use as a combination treatment for *Ascaris* infections up until the 1990s.[4–6] While the exact anthelmintic mechanism of action remains unclear, kainic acid functions as an ionotropic glutamate receptor (iGluR) agonist.[7] iGluRs mediate neuronal cell-cell communication by binding to glutamate and facilitating influx of Ca^{2+} into the cell.[8] Structurally similar to glutamic acid, kainic acid can bind more efficiently to iGluRs and thereby stimulate excessive influx of Ca^{2+}, which leads to excitotoxicity and cell death.[9] In fact, kainic acid was important in the initial discovery and characterization of several classes of iGluRs, such as the eponymous kainate receptor,[10] and has been exploited to create mouse model systems to study neurological diseases,[11] particularly temporal lobe epilepsy.[12,13] In the 50 years since kainic acid was isolated, over 70 synthetic routes have been established (Figure 1).[14] These syntheses employ diverse methods to produce the kainoid ring pharmacophore such as ring opening followed by cyclization,[15]

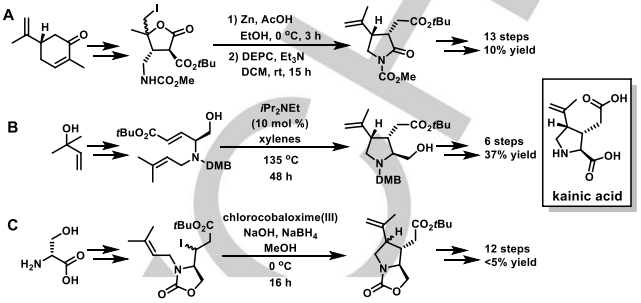

Figure 1. Notable synthetic routes to kainic acid by (A) Fukuyama,[15] (B) Ohshima,[16] and (C) Baldwin[17] and coworkers.

ene-cyclization,[16] or radical formation.[17] Unfortunately, challenges in generating the three contiguous stereocenters often limit these approaches to low yields or many steps. In contrast to the synthetic work, little progress has been made on elucidating how kainic acid is constructed by seaweeds. Recently, we established the biosynthetic logic for domoic acid production in microalgal *Pseudo-nitzschia multiseries* diatoms through the discovery of a four-gene cassette (*dabA-D*) and the confirmation of their in vitro enzymatic functions (Figure 2A).[18] The structural similarity between domoic acid and kainic acid allowed us to propose a conserved route of biosynthesis (Figure 2B). *N*-prenylation of L-glutamate with dimethylallyl pyrophosphate (DMAPP) via a DabA homolog would produce a "prekainic acid" pathway intermediate that could conceivably be cyclized directly with a DabC homolog to generate kainic acid.

To validate our hypothesis, we performed whole genome sequencing of the well-studied kainic acid producer *D. simplex* from Japan to identify the kainic acid biosynthetic genes and determine their genetic context. Red macroalgal genomes have proven challenging due to their close association with a diverse array of marine microbes and crustaceans, which means sequencing efforts result in highly complex metagenomes if they are not cured with antibiotics.[19] Moreover, identifying biosynthetic cassettes in red macroalgae genomes, which are often tandem duplications and nested in repetitive sequence, is almost impossible with current high throughput short read (~ 100 bp) sequencing technologies. However, the new single molecule long read sequencing platforms, such as Oxford Nanopore Technologies (ONT), generate single reads in the hundreds of kilobases (kb), which greatly facilitates resolving tandemly duplicated genes and repeats, assembling metagenomes, and generating highly contiguous reference genomes.[20] We sequenced *D. simplex* on the ONT MinION platform and produced 7 Gb of sequence with a read N50 of 7 kb and the longest read at 235 kb. We were able to find long reads that contained genes suggestive of kainic acid biosynthesis, but due to the amount of microbial sequence we were not able to assemble the *D. simplex* genome. Therefore, we sequenced *D. simplex* on the higher throughput ONT PromethION platform and generated 47 Gb of sequence with a read N50 of 7 kb and the longest read at 1.2 Mb (Figure S1). The resulting correction-less

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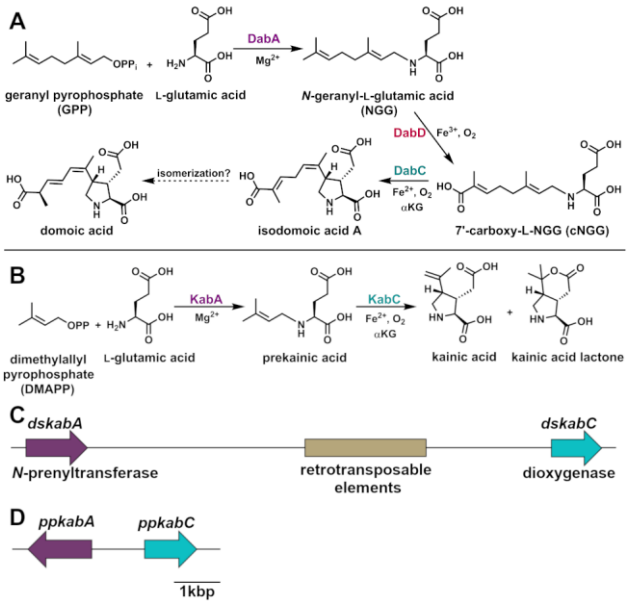

Figure 2. The proposed (A) domoic acid[18] and (B) kainic acid biosynthetic pathways. The kainic acid biosynthesis (*kab*) gene cluster from (C) *D. simplex* and (D) *P. palmata.* Scale bar is representative for both (C) and (D).

genome assembly was 299 Mb with 8,246 contigs. The contig N50 was 57 kb and the longest contig was 3.9 Mb. Based on GC% analysis, we predict 205 Mb of the assembly is *D. simplex*, while 88 Mb is high GC% bacterial contigs. This assembly was subsequently polished with Illumina short-read sequencing.

Upon analyzing the *D. simplex* genome for the presence of homologs to the domoic acid biosynthetic (*dab*) genes, we uncovered a 12 kb kainic acid biosynthesis (*kab*) cluster containing an annotated *N*-prenyltransferase, α-ketoglutarate (αKG) dependent dioxygenase, and several retrotransposable elements (integrase, reverse transcriptase, and RNase H domains) (Figure 2C). Many organisms, including bacteria, fungi, plants, and unicellular algae, are known to cluster genes within the same biosynthetic pathway.[18,21,22] The *kab* gene clustering is consistent with early examples of red algae clustering genes[19] and begins to suggest that it may be a feature in red algal specialized metabolism as well. To evaluate whether these features are conserved within other *kab* clusters, we performed genome sequencing of a second known kainic acid producer, *Palmaria palmata*,[23] to determine the genetic context of its *kab* genes. Using one MinION chip and no genome assembly steps, we were able to identify a single 47 kb read that contained both *ppkabA* and *ppkabC* genes. As with *D. simplex*, the kainic acid genes are tightly clustered (Figure 2D). However, the gene synteny is not conserved with a different gene orientation and a short 1.1 kb intergenic region between *ppkabA* and *ppkabC* that lacks retrotransposable elements.

Analysis of the NCBI transcriptome database identified similar *kab* transcripts from four different red algae, namely *Grateloupia filicina*, *Palmaria hecatensis, Rhodophysema elegans,* and the previously sequenced *P. palmata*.[24,25] These three additional red algae are not known kainic acid producers and demonstrate that the genes responsible for biosynthesis are found throughout distinct red algae lineages (Figure S2). Sequence alignment of the KabA *N*-prenyltransferase and the KabC αKG-dependent dioxygenase proteins revealed high levels of conservation (Figures S3 and S4).

To confirm the proposed kainic acid biosynthetic pathway, *dskabA* and *dskabC* were successfully expressed in *Escherichia coli* and purified to homogeneity (Figure S5). Incubation of DsKabA with the anticipated substrates DMAPP and L-glutamate produced *N*-dimethylallyl-L-glutamic acid (prekainic acid) as confirmed by NMR structural elucidation and LCMS comparison with a synthetic standard (Figure 3). Consistent with other members of the terpene cyclase family fold, Mg^{2+} is a required cofactor in the reaction. We next explored the substrate selectivity of DsKabA. Exchange of L-glutamate for D-glutamate showed diminished turnover while L-glutamine, L-aspartate, L-asparagine, and glycine did not produce appreciable amounts of *N*-prenylated products (Figure S6). In contrast, when we replaced DMAPP with GPP in the L-glutamate reaction, we clearly observed the formation of *N*-geranyl-L-glutamic acid (L-NGG), the first biosynthetic precursor to domoic acid (Figure S6). This high level of amino acid selectivity and modest prenyl donor promiscuity was also observed with DabA from domoic acid biosynthesis.[18] To support the physiological relevance of prekainic acid in the biosynthesis of kainic acid, we examined the aqueous extract of *D. simplex*. High resolution LCMS analysis revealed the presence of a compound with both the exact mass and retention time of prekainic acid (Figure S7).

After reconstituting DsKabA activity, we next aimed to determine if DsKabC could directly convert prekainic acid to kainic acid. Incubation of DsKabC with prekainic acid, αKG, L-ascorbate, and Fe^{2+} resulted in the conversion of prekainic acid to two products with the expected mass of kainic acid (Figure 3). Addition of the iron chelator EDTA nearly abolished activity. The major product had the same HPLC retention time and MS of commercially available kainic acid, and was further confirmed by to be kainic acid following NMR structural elucidation. Examination of the in vitro products of three additional algal KabC

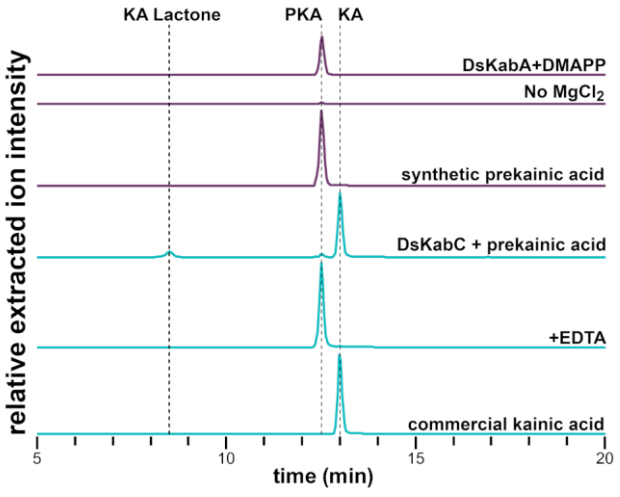

Figure 3. LCMS analysis of DsKabA and DsKabC in vitro assays. Each trace represents the $(M-H)^-$ extracted ion chromatogram for prekainic acid (214.1 ± 0.5 *m/z*) and kainic acid (212.1 ± 0.5 *m/z*). DsKabA converts L-Glu and DMAPP to prekainic acid in a Mg^{2+} dependent manner (purple traces). DsKabC can convert prekainic acid (PKA) to kainic acid (KA) and kainic acid lactone (KA Lactone), but turnover is nearly undetectable when the iron chelator EDTA is added (cyan traces).

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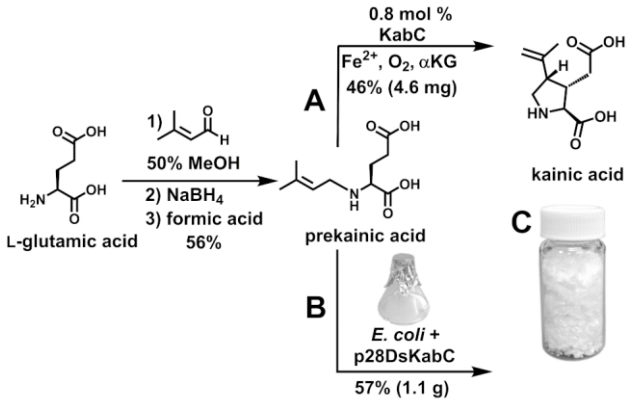

Figure 4. Discovery of the kainic acid biosynthetic genes enabled two new kainic acid production routes: (A) one-enzyme chemoenzymatic synthesis and (B) biotransformation with *E. coli* cells expressing the *dskabC* gene on a pET28 vector. (C) Lyophilized kainic acid in a scintillation vial generated by biotransformation.

enzymes revealed a similar trend, wherein prekainic acid is converted to kainic acid and a second compound (Figure S8). Notably, the KabC ortholog from *G. filicinia*, GfKabC, produced primarily this other kainic acid isomer. By scaling up the GfKabC reaction, we isolated and characterized this product by NMR to reveal kainic acid lactone (Figure 2B). Kainic acid lactone was previously isolated from kainic acid producing red algae[26] and was also found in our *D. simplex* sample (Figure S7). In contrast to the agonistic activity of kainic acid, kainic acid lactone has been shown to be an iGluR antagonist.[27] Curiously, the relative ratio of kainic acid to kainic acid lactone appears to vary depending on the particular KabC ortholog (Figure S8). While the biochemical basis for this differential product distribution is an ongoing focus of study, we suggest a chemical mechanism analogous to that proposed for DabC[18] which diverges following pyrrolidine ring formation to rationalize the observed chemotype (Figure S9).

Once the Kab enzymatic functions were validated, we next focused on developing a scalable kainic acid production system that would take advantage of the strengths of both chemical synthesis and biocatalysis. Instead of purifying recombinant KabA and isolating its product from a large-scale biochemical reaction, we readily synthesized prekainic acid by reductive amination of L-glutamate with 3-methyl-2-butenal (Figure 4). The stereospecific cyclization reaction to form the kainic acid pyrrolidine ring, on the other hand, is synthetically challenging and is reminiscent of the cobalt-mediated radical cyclization strategy employed by Baldwin and coworkers in the 1990s on an *N*-prenylated amino acid substrate (Figure 1C).[17] Rather, we explored using KabC to produce kainic acid. We first evaluated an in vitro system that used prekainic acid, αKG, Fe^{2+}, and purified DsKabC from 1 L of *E. coli* cells. After a 16 h incubation, 10 mg of synthetic prekainic acid was completely converted to produce 4.6 mg kainic acid with a 46% isolated yield and a 26% overall isolated yield over two steps (Figure 4A and S10). Although this chemoenzymatic route was successful, the need to purify DsKabC prevents this method from being applicable for large scale production of kainic acid. Therefore, we attempted a biotransformation strategy that would bypass the need to purify enzymes and directly convert synthetic prekainic acid to kainic acid in *E. coli* cells expressing DsKabC (Figure 4B). We developed a streamlined strategy in which prekainic acid was synthesized and the entire unpurified crude reaction mixture was added to the *E. coli* medium. After 40 h, we observed the nearly complete consumption of an 8 mmol prekainic acid synthetic reaction in a 3 X 1 L *E. coli* biotransformation to kainic acid (Figure S11). Employing a two-step purification procedure using activated carbon followed by preparatory reverse phase HPLC, we purified 1.1 g of kainic acid with a combined overall isolated yield of 32% and > 95% purity as assessed by NMR (Figure 4C and S12).

Although there are already many scalable synthetic processes for kainic acid, the procedures require at least six synthetic transformations with yields < 40% (Figure 1).[14,16] In contrast, biology has evolved an extremely efficient two-step biosynthetic process. The first step, catalyzed by the *N*-prenyltransferase KabA, is readily replicated using established synthetic procedures. In contrast, the second step, in which KabC catalyzes the stereocontrolled formation of the trisubstituted pyrrolidine ring, is not easily accomplished synthetically. By combining the strengths of prekainic acid synthesis and DsKabC-mediated biocatalysis, we developed a simple and scalable kainic acid production method that is also economically attractive and flexible for analog generation.

Overall, combining genomic information with in vitro enzymatic characterization enabled us to not only discover the enzymes responsible for the biosynthesis of a seaweed natural product, but also to develop new methods to produce this important research tool.

Experimental Section

Experimental details including DNA sequencing, protein expression, protein purification, production isolation, chemical synthesis and spectroscopic characterization are provided in the Supporting Information online. All sequence data has been deposited to NCBI databases. The *Digenea simplex* and *Palmaria palmata* bioprojects have been deposited as PRJNA509898 and PRJNA509900, respectively. A complete list of all accession numbers can be found in Supplementary Table 3.

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Conflict of Interest

J.R.C., S.M.K.M, and B.S.M. have submitted a provisional patent on the production of kainic acid using the described methods.

Keywords: kainic acid • biosynthesis • red algae • biotransformation • natural product

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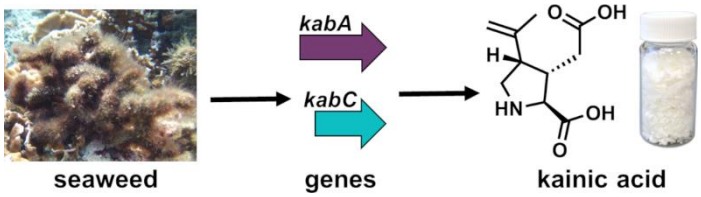

Kainic acid is a marine natural product that has been used as an anthelmintic agent for centuries and, more recently, a neurological tool. Using a genomic sequencing approach, the genes responsible for the biosynthesis of kainic acid were discovered in two different seaweeds. In vitro characterization validated enzymatic activity and enabled gram scale production of kainic acid using a biotransformation approach.

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