Organic LETTERS

Letter

pubs.acs.org/OrgLett

Biosynthesis of the Tetramic Acids Sch210971 and Sch210972

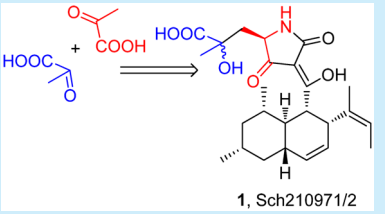
Thomas B. Kakule,[†] Shuwei Zhang,[‡] Jixun Zhan,[‡] and Eric W. Schmidt*,[†]

[†]Department of Medicinal Chemistry, L. S. Skaggs Pharmacy Institute, University of Utah, Salt Lake City, Utah 84112, United States

[‡]Department of Biological Engineering, Utah State University, Logan, Utah 84322, United States

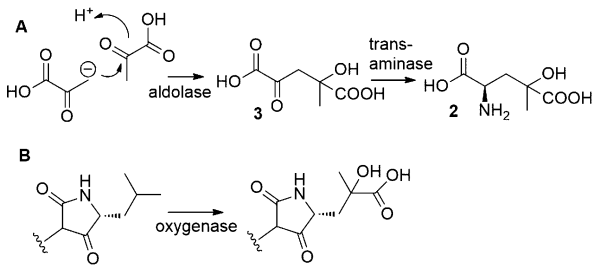
Supporting Information

ABSTRACT: A biosynthetic pathway to fungal polyketide−nonribosomal peptide natural products, Sch210971 (**1a**) and Sch210972 (**1b**) from *Hapsidospora irregularis*, was characterized by reconstitution and heterologous expression in *Fusarium heterosporum*. Using genetic, biochemical, and feeding experiments, we show that the incorporated amino acid 4-hydroxyl-4-methyl glutamate (HMG) is synthesized by an aldolase, probably using pyruvate as the precursor.



Nonproteinogenic amino acids (those not normally found in proteins) are abundant components of secondary metabolites, contributing greatly to natural chemical diversity.[1] Among these, 4-hydroxyl-4-methyl glutamate (HMG, **2**) has long been known because it is concentrated in several plants.[2] In the 1960s, a biosynthetic pathway to plant HMG was proposed,[3] wherein two units of pyruvate would undergo aldol condensation to 4-hydroxyl-4-methyl-2-oxoglutarate (HMOG, **3**) (Scheme 1).

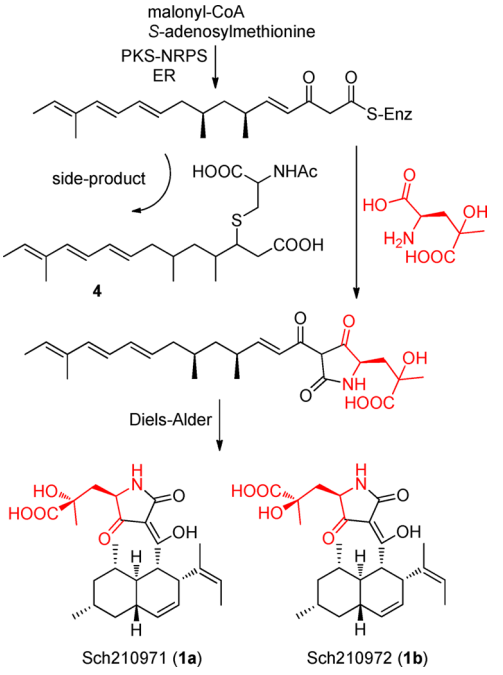
Scheme 1. Hypotheses for HMOG Biosynthesis



Subsequently, transamination would yield HMG. Aldolases were isolated in the late 1960s that were thought to catalyze HMOG synthesis *in vivo*.[3,4] However, *in vitro*, they only catalyzed the reverse reaction, degrading HMOG to pyruvate. Because this reaction also proceeds spontaneously in the presence of magnesium, isotope exchange assays were used to provide indirect evidence that the reaction could proceed in the forward direction.[4]

Later, HMG was also discovered as an amino acid incorporated into co-occurring fungal secondary metabolites, Sch210971 (**1a**), Sch210972 (**1b**) and other close structural relatives.[5,6] Compound **1b** is a decalin-containing tetramic acid derivative that inhibits the cytokine receptor. Only its relative configuration has been reported. Over the past decade, the biosynthesis of several decalin-tetramic acids has been studied, revealing that their core structures are synthesized by dimodular polyketide synthase−nonribosomal peptide synthetase (PKS-NRPS) proteins.[7] In these large hybrid biosynthetic enzymes, the PKS synthesizes the acetate-derived portion, including the decalin, while the NRPS adds the amino acid. An auxiliary enoyl reductase (ER) protein and Diels−Alderase are essential to decalin formation as well (Scheme 2).[8−10] Therefore, it could be predicted that **1** would be synthesized by a PKS-NRPS and auxiliary ER. Two likely possibilities could be envisioned for HMG incorporation (Scheme 1). The amino acid could be derived from leucine, for example, via α-ketoglutarate-dependent oxidation that is well-known in NRPS amino acid biochemistry.[1] Such an oxidase would act on an amino acid either before or after

Scheme 2. Biogenesis of 1, Showing Isolated Shunt Product 4



Received: March 11, 2015

ACS Publications © XXXX American Chemical Society

DOI: 10.1021/acs.orglett.5b00715
Org. Lett. XXXX, XXX, XXX−XXX

incorporation by the PKS-NRPS and would represent a quite novel route to HMG. Alternatively, an aldolase−transaminase route may be possible, as proposed for the plant metabolites but not further investigated in recent decades.

In a discovery program employing novel fungi, we rediscovered the diastereomers **1a** and **1b**, but in a fungus not previously known to produce the compound (*Hapsidospora irregularis*). Because of our interest in tetramic acids and the novel nonproteinogenic HMG subunit, we chose to investigate its biosynthesis.

The gene cluster for Sch210971/2 was identified by sequencing. The genome of *H. irregularis* was sequenced and assembled to provide 28.4 Mbp (1924 contigs, 55.8% GC). Automated annotation predicted 14 573 proteins. BLAST searching for PKS-NRPS and auxiliary ER proteins unveiled 3 PKS-NRPS genes, but only one with an accompanying ER, leading us to propose that this was the correct cluster, *tas* (*tetramic acid Sch210971/2*; Figure 1A and Table 1). The *tas*

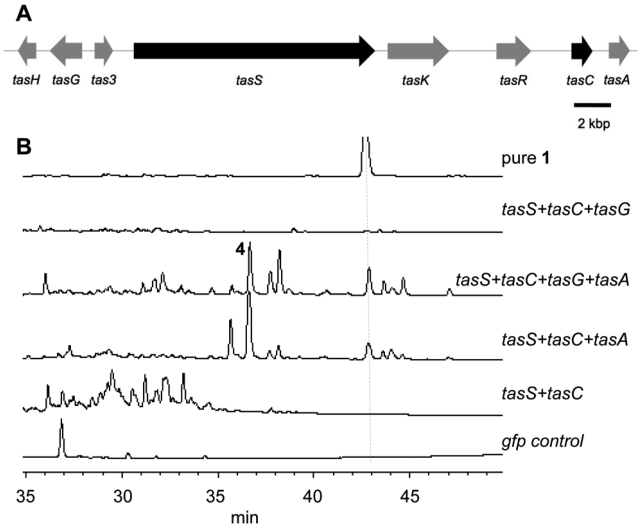


Figure 1. (A) *H. irregularis tas* gene cluster schematic. (B) Coexpression of genes from *H. irregularis tas* cluster in heterologous host *F. heterosporum*, followed by extraction and HPLC-DAD analysis. The blue line indicates presence of Sch210971/2 only when aldolase is coexpressed.

Table 1. Predicted Function of *tas* Genes

gene name	length (aa, nt)	predicted function
tasH	324, 1070	oxidase
tasG	325, 1675	transaminase
tas3	397, 1194	Diels−Alderase
tasS	4061, 12450	PKS-NRPS
tasK	981, 3152	amidohydrolase
tasR	566, 1701	regulator
tasC	385, 1158	ER
tasA	270, 889	aldolase

gene cluster was deposited in GenBank, accession number KP835202. In addition, we identified coclustered genes that were predicted to be involved in forming HMG, including genes encoding an oxidase, an aldolase, and an aminotransferase. Overall, *tas* was very similar to an uncharacterized cluster in the genome sequence of *Acremonium chrysogenum* ATCC 11550.

On the basis of the proposed activities of the cluster genes, it was impossible to eliminate either the aldolase or oxidase biosynthetic hypotheses without further testing. Hence, all the genes were cloned into vectors by yeast homologous recombination, and heterologously expressed in the recently reported *Fusarium heterosporum* expression platform.[11,12] The cluster also contained homologues of two genes universally found in decalin-tetramic acid clusters, including an amidohydrolase and a Diels−Alderase.[10] Close homologues of these genes are found in the *F. heterosporum* genome, and in our experience, these do not need to be transferred to *F. heterosporum* to produce closely related decalin-tetramates.[11,13]

Heterologous expression was used to demonstrate that *tas* was correctly identified as the Sch210971/2 biosynthetic locus (Figure 1B). We used the fungus *F. heterosporum* because it synthesizes sufficient recombinant products for simple chemical characterization.[11] The following combinations of genes were coexpressed: (1) PKS-NRPS *tasS* and ER *tasC*; (2) *tasS*, *tasC*, and aldolase *tasA*; (3) *tasS*, *tasC*, and transaminase *tasG*; (4) all four genes *tasS*, *tasC*, *tasA*, and *tasG*. No compounds were detected in condition 1, when just the decalin-tetramate synthesizing genes were present, nor were compounds detected in condition 3, with the addition of transaminase. However, in conditions 2 and 4, which both included the aldolase, authentic Sch210971 and Sch210972 were obtained, as validated by HPLC, including coinjection with an authentic standard, and by MS experiments. In some conditions, we also obtained a minor amount of compound **4**, which is similar to an intermediate previously isolated in heterologous PKS-NRPS experiments and which represents the pre-Diels−Alder PKS structure of **1** (Scheme 2).[12] Compound **4** was purified and characterized by NMR from a 100 mL culture (see Supporting Information). It is likely that the host aspartate aminotransferase is involved in transforming HMOG into HMG; homologues of this enzyme are known to be promiscuous in other systems.[14,15]

The requirement for aldolase enzyme, and the lack of a requirement for the clustered oxidase *tasH*, strongly implicated the aldolase mechanism rather than leucine oxidation in the synthesis of **1**. To further investigate this possibility, we synthesized HMOG from pyruvate[16] and fed it to *F. heterosporum* expressing just *tasS* and *tasC*, lacking the aldolase (Figure 2). Indeed, feeding with HMOG fully restored production of **1**, while an identical control culture lacking HMOG did not produce any recombinant compounds.

To further verify that HMOG was a true intermediate of the pathway and to rule out alternative explanations, we synthesized HMOG using **1**-^{13}C pyruvate, which was selected because the label on carboxyl was unlikely to wind up in the polyketide portion of the molecule even if HMOG was degraded to pyruvate in the culture. We used five different ratios of labeled/unlabeled pyruvate in chemical synthesis of HMOG, including 100%, 75%, 50%, 25%, and 0% labeled pyruvate. This provided either pure labeled or unlabeled HMOG, containing either zero or two ^{13}C units, or it provided statistical mixtures of zero, one, or two ^{13}C labels. It was envisioned that these mixtures would enable us to rule out confounding factors. Labeled HMOG was fed to *F. heterosporum* containing *tasS* + *tasC* (Figure 3). Cultures were extracted and analyzed by HPLC-MS. In the event, **1** with unlabeled pyruvate provided a major ion at *m*/*z* 446, while that with 100% ^{13}C label provided a major ion at *m*/*z* 448. Fermentations derived from 25%, 50%, or 75% labeled HMOG contained the predicted mixtures of peaks at *m*/*z* 447, 448, and 449. These results revealed that HMOG was incorporated intact into **1** and that metabolism into individual pyruvate subunits within the fungus did not interfere with

DOI: 10.1021/acs.orglett.5b00715
Org. Lett. XXXX, XXX, XXX−XXX

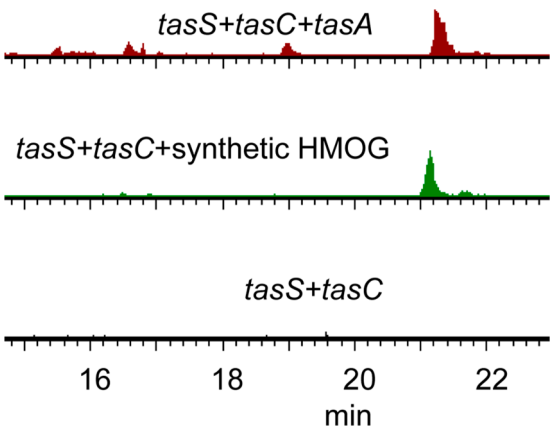


Figure 2. Incorporation of synthetic HMOG. Culture extracts were analyzed by HPLC-MS. Shown are filtered chromatograms (*m/z* 445.7−446.7). When aldolase *tasA* is present (top), compound **1** is produced. Without *tasA* (bottom and middle), **1** is only produced with addition of synthetic HMOG (middle).

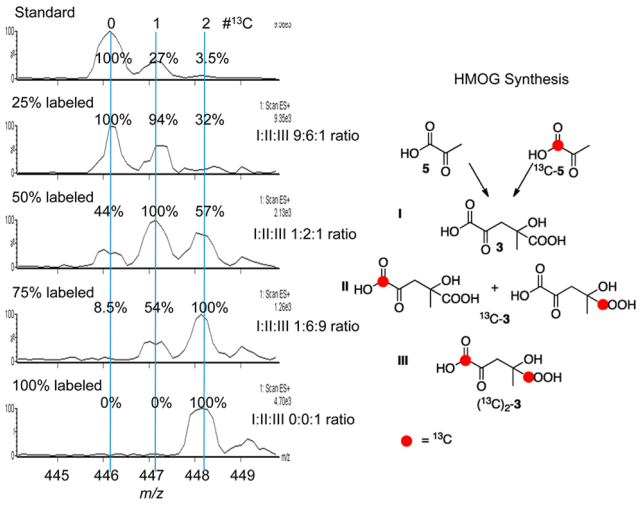


Figure 3. Labeled HMOG incorporation. HMOG was synthesized using different ratios of ^{13}C labeled and unlabeled pyruvate (right). These synthetic HMOG derivatives were fed to cultures containing *tasS* + *tasC*, but lacking aldolase. Analysis by HPLC-MS in continuous mode (left) shows expected isotope incorporation. Isotope ratio % expected is calculated from the expected values for different ratios of unlabeled, singly labeled, and doubly labeled HMOG, normalized to 100% for the expected largest peak.

incorporation. Moreover, the stoichiometry of the label was consistent with incorporation cleanly into the amino acid portion of **1** (one, and only one, HMOG unit was incorporated into the compound).

We investigated the catalytic activity of the aldolase, which was recombinantly expressed in *E. coli* as the His-tagged construct. In all conditions attempted, selective HMOG fission could be achieved, but the forward reaction to synthesize HMOG from pyruvate was not observed. This was identical to the previous reverse reaction observed with plant aldolases in the 1960s and reinforces these earlier findings associating such aldolases with HMOG synthesis in nature.[3,4] It also confirms that the protein acts as aldolase *in vitro*. In this instance, genetic data by heterologous expression in fungi enables us to provide evidence that HMG is produced via the aldolase route since a protein shown to act as an aldolase *in vitro* is required for HMG incorporation *in vivo*.

In summary, we report the biosynthetic pathway to the cytokine receptor inhibitors Sch210971 and Sch210972, identified by genome sequencing and heterologous pathway expression. We demonstrate the novel aldolase route to a nonproteinogenic amino acid found in plants and filamentous fungi, expanding the known routes to nonproteinogenic amino acids in NRPS metabolism. This also adds to the known amino acid precursors in fungal PKS-NRPS metabolism, which currently include proteinogenic amino acids serine, threonine, tryptophan, phenylalanine, and tyrosine.

■ ASSOCIATED CONTENT

Supporting Information

Plasmid construction; fungal transformation procedure; chemical analysis of mutants; and HRESIMS and NMR spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ews1@utah.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was supported by NSF 0957791 and a Utah State University Research Catalyst grant.

■ REFERENCES

(1) Walsh, C. T.; O'Brien, R. V.; Khosla, C. *Angew. Chem., Int. Ed.* **2013**, *52*, 7098.
(2) Grobbelaar, N.; Pollard, J. K.; Steward, F. C. *Nature* **1955**, *175*, 703.
(3) Shannon, L. M.; Marcus, A. *J. Biol. Chem.* **1962**, *237*, 3342.
(4) Marcus, A.; Shannon, L. M. *J. Biol. Chem.* **1962**, *237*, 3348.
(5) Neumann, K.; Kehraus, S.; Gutschow, M.; Konig, G. M. *Nat. Prod. Commun.* **2009**, *4*, 347.
(6) Yang, S.-W.; Mierzwa, R.; Terracciano, J.; Patel, M.; Gullo, V.; Wagner, N.; Baroudy, B.; Puar, M.; Chan, T.-M.; McPhail, A. T.; Chu, M. *J. Nat. Prod.* **2006**, *69*, 1025.
(7) Chooi, Y. H.; Tang, Y. *J. Org. Chem.* **2012**, *77*, 9933.
(8) Kakule, T. B.; Sardar, D.; Lin, Z.; Schmidt, E. W. *ACS Chem. Biol.* **2013**, *8*, 1549.
(9) Kennedy, J.; Auclair, K.; Kendrew, S. G.; Park, C.; Vederas, J. C.; Richard Hutchinson, C. *Science* **1999**, *284*, 1368.
(10) Kato, N.; Nogawa, T.; Hirota, H.; Jang, J.-H.; Takahashi, S.; Ahn, J. S.; Osada, H. *Biochem. Biophys. Res. Commun.* **2015**, DOI: 10.1016/j.bbrc.2015.03.011.
(11) Kakule, T. B.; Jadulco, R. C.; Koch, M.; Janso, J. E.; Barrows, L. R.; Schmidt, E. W. *ACS Synth. Biol.* **2014**, DOI: 10.1021/sb500296p.
(12) Kakule, T. B.; Lin, Z.; Schmidt, E. W. *J. Am. Chem. Soc.* **2014**, *136*, 17882.
(13) Jadulco, R. C.; Koch, M.; Kakule, T. B.; Schmidt, E. W.; Orendt, A.; He, H.; Janso, J. E.; Carter, G. T.; Larson, E. C.; Pond, C.; Matainaho, T. K.; Barrows, L. R. *J. Nat. Prod.* **2014**, *77*, 2537.
(14) Baldwin, J. E.; Dyer, R. L.; Ng, S. C.; Pratt, A. J.; Russell, M. A. *Tetrahedron Lett.* **1987**, *28*, 3745.
(15) Senuma, M.; Nakamichi, K.; Nabe, K.; Nishimoto, S.; Tosa, T. *Appl. Biochem. Biotechnol.* **1989**, *22*, 141.
(16) Hélaine, V.; Rossi, J.; Gefflaut, T.; Alaux, S.; Bolte, J. *Adv. Synth. Catal.* **2001**, *343*, 692.

DOI: 10.1021/acs.orglett.5b00715
Org. Lett. XXXX, XXX, XXX−XXX