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Biosynthesis of the Carbonylmethylene Structure Found in a Class of Pseudotripeptides, Ketomemicins

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Abstract

We recently discovered novel pseudotripeptides, ketomemicins, which possess a *C*-terminal pseudodipeptide connected with a carbonyl methylene instead of an amide bond, through heterologous expression of gene clusters identified in actinobacteria. The carbonyl methylene structure is a stable isostere of the amide bond and its biological significance has been shown in several natural and synthetic products. Despite the biological importance of these compounds, little is known about how the carbonyl methylene structure is biosynthesized. In this article, we fully characterized the biosynthetic machinery of the pseudodipeptide. An aldolase, dehydratase, PLP-dependent glycine-*C*-acetyltransferase, and dehydrogenase were involved in the formation of the pseudodipeptide with malonyl-CoA and phenylpyruvate as starter substrates.

Peptides are indispensable for all living organisms and play many important roles in biological processes. Because peptides are labile in biological systems, peptidomimetic compounds that overcome this problem have attracted attention for their potential as enzyme inhibitors or receptor ligands. In drug design, a widely used strategy is to replace the peptide backbone with nonhydrolyzable isosteres such as esters, alkenes, and carbonylmethylenes.[1] Nature employs a similar strategy although such molecules are not widely distributed. The largest group of pseudopeptide natural products is depsipeptides, where the isosteric replacement is an ester bond. Besides depsipeptides, two types of carbonylmethylene-containing pseudopeptides, arphamenines (**1**, **2**) and ketomemicins (**3**, **4**), are known. Arphamenines A and B are pseudodipeptides isolated from *Chromobacterium violaceum* BMG361-CF4 and show potent activity as aminopeptidase B inhibitors.[2] Ketomemicins are pseudotripeptides that we recently discovered through heterologous expression of gene clusters identified in actinobacteria from three genera, *Micromonospora* sp. ATCC 39149, *Streptomyces mobaraensis* NBRC 13819, and *Salinispora tropica* CNB-440 (Figure 1).[3] Ketomemicins contain *C*-terminal pseudodipeptides closely related to arphamenines. Although arphamenines and ketomemicins are the only known examples of naturally occurring pseudopeptides with a carbonylmethylene moiety, synthetic peptide isosteres with a carbonylmethylene structure have been developed as protease inhibitors in a variety of synthetic studies such as rupintrivir (AG7088).[4] Despite the biological importance of these molecules, little is known about how the carbonylmethylene structure is biosynthesized. Taking advantage of the genetic information on ketomemicin biosynthesis, in this study, we performed the *in vitro* functional characterization of the biosynthetic pathway of the pseudopeptide moiety of ketomemicins B3 (**3**) and B4 (**4**).

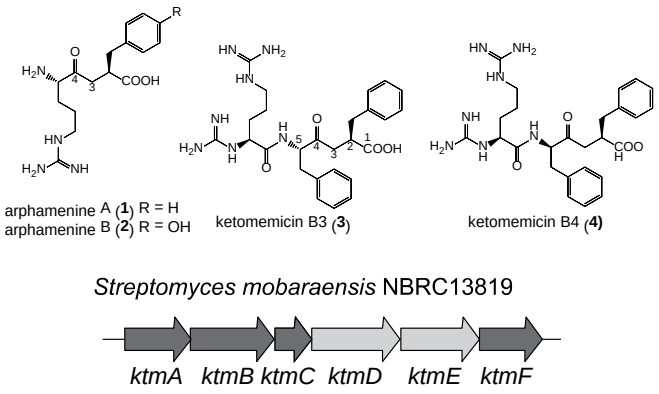

Figure 1. (A) Structures of arphamenines and ketomemicins, and (B) the ketomemicin biosynthetic gene cluster of *S. mobaraensis*.

There are six genes in each of the three clusters and we have assigned each gene a systematic name. The names, their corresponding locus tags, and proposed functions are summarized in Table 1. We reported that KtmD, a homolog of peptide ligase PGM1 involved in pheganomycin biosynthesis[5], is the (pseudo)dipeptide ligase responsible for the amide bond formation between the amidino-amino acid and pseudodipeptide in the final step of ketomemicin biosynthesis.[3] The *N*-terminal substrates of KtmD are generated by KtmE catalyzing an amidinotransfer reaction.[6] These results suggested that the remaining four genes in the *ktm* cluster—*ktmA*, *ktmB*, *ktmC*, and *ktmF*—are involved in the biosynthesis of the pseudodipeptide.

Table 1. The genes in the clusters, proposed functions, and corresponding locus tags.

		ORFs locus tag		
	Proposed function	*S. mobaraensis*	*Micromonospora* sp.	*S. tropica*
ktmA	aldolase	H340_08846	MCAG_05282	Strop_1719
ktmB	amino acid *C*-acyltransferase	H340_08841	MCAG_05283	Strop_1720
ktmC	dehydratase	H340_08836	MCAG_05284	Strop_1721
ktmD	dipeptide ligase	H340_08831	MCAG_05280	Strop_1717
ktmE	amidinotransferase	H340_08826	MCAG_05285	Strop_1716
ktmF	dehydrogenase	H340_08821	MCAG_05281	Strop_1718

The amino acid sequence of KtmA showed significant homology to CitE family enzymes. The original CitE is the β-subunit of the bacterial ATP-independent citrate lyase and consists of α, β, and γ subunits.[7] Using an acyl carrier protein (ACP, γ subunit) as a scaffold, CitE catalyzes a retro-aldol reaction from citryl-ACP to oxaloacetate and acetyl-ACP (Figure S1). Other CitE-like enzymes include malyl-CoA lyases[8], malyl-CoA thioesterases[9], and malate synthases[10], which utilize acyl-CoA thioesters as substrates. The translated gene product of *ktmB* showed homology to various PLP-dependent amino acid *C*α-acyltransferases (Figure S2).[11] This family of enzymes catalyzes C–C bond formation between the Cα of an amino acid and the carbonyl carbon of an acyl-CoA thioester, followed by decarboxylation to generate an α-oxoamine structure. KtmC and KtmF are homologs of dehydratases and NAD(P)H-dependent reductase, respectively.

On the basis of homology analysis, we propose a possible biosynthetic pathway to generate the pseudodipeptide structure (Figure 2). This pathway includes 1) an aldol reaction between acetyl-CoA and phenylpyruvate catalyzed by KtmA, 2) a dehydration (KtmC) and an enoyl reduction (KtmF) to reduce the -OH group generated by the aldolase reaction, and 3) a C–C bond formation between phenylalanine and acyl-CoA to produce an α-oxoamine structure by

KtmB. However, three possible pathways can be envisioned depending on the timing of the KtmB reaction (Pathways A–C).

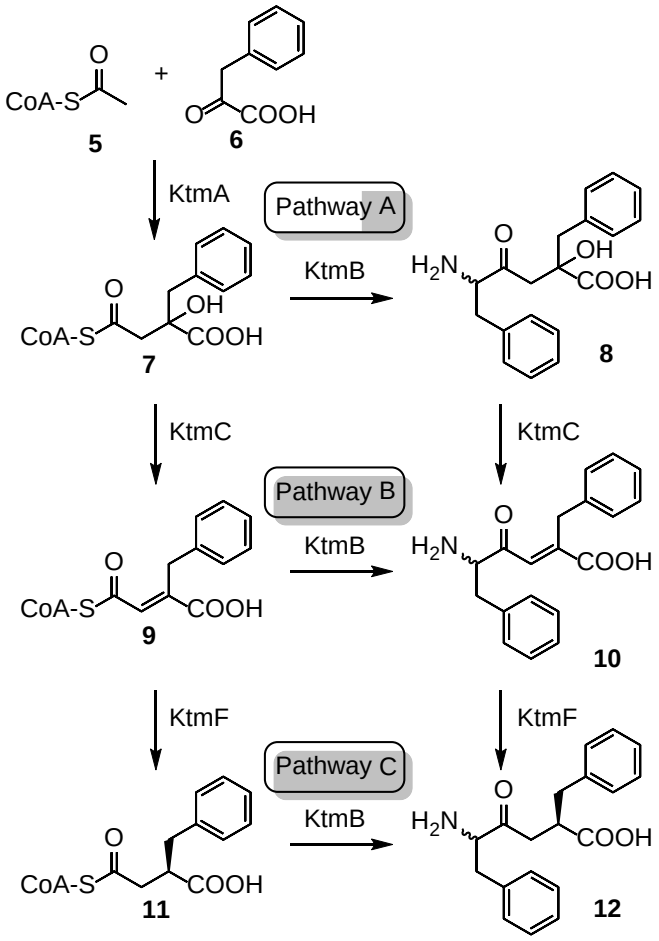

Figure 2. Proposed biosynthetic pathways for the pseudodipeptide in ketomemicins B3 and B4.

To confirm the proposed pathways, the four genes in the *ktm* cluster of *S. mobaraensis* were individually cloned into pET28b (+) expression vectors. Each of them was heterologously expressed in *Escherichia coli* BL21 (DE3) and purified as an *N*-terminal His-tagged protein by affinity chromatography using Ni-NTA resin to near homogeneity. SDS-PAGE of the purified proteins clearly showed production of the recombinant proteins (Figure S3).

To examine the aldolase reaction, the purified KtmA was incubated with acetyl-CoA (**5**) and phenylpyruvate (**6**) in the presence of $MgCl_2$ because other CitE-like family enzymes requires a divalent metal. However, no products were formed (Figure 3, trace A). Then, malonyl-CoA (**13**) was used as a substrate instead of acetyl-CoA because the malonyl-moiety is known to serve as a surrogate of acetate, exemplified by ketosynthases in fatty acid synthases that catalyze a decarboxylation-induced Claisen condensation reaction, and reactions of aldolases driven by decarboxylation are also known.[12] KtmA was incubated with phenylpyruvate (**6**) and malonyl-CoA (**13**) and the reaction mixture was subjected to HPLC analysis. A new product peak was observed in the reaction mixture (Figure 3, traces B and C). The molecular mass of this new product was consistent with that of the proposed benzylmalyl-CoA (**7**) product (calcd. for $C_{32}H_{45}N_7O_{20}P_3S^-$ 972.1658, found 972.1670). These results indicated that KtmA produces benzylmalyl-CoA (**7**) from malonyl-CoA (**13**) and phenylpyruvate (**6**). Screening of divalent metal ions (Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Ca^{2+}) revealed that KtmA exclusively requires the Mg^{2+} ion for its activity (Figure S4). We also determined the kinetic parameters of the KtmA reaction. As shown in Figures S5 and S6, the KtmA reaction followed Michaelis-Menten kinetics and the kinetic parameters (Table 2) were determined by fitting the data points to the Hanes-Woolf plot equation. However, it is worthwhile noting that the K_M value for the malonyl-CoA of KtmA is high compared to those of other malonyl-CoA specific enzymes from secondary metabolite

biosynthesis. We thus cannot completely rule out the possibility that KtmA generates benzylmalyl-ACP from malonyl-ACP and phenylpyruvate (**6**) in a similar manner with citrate lyase reaction.

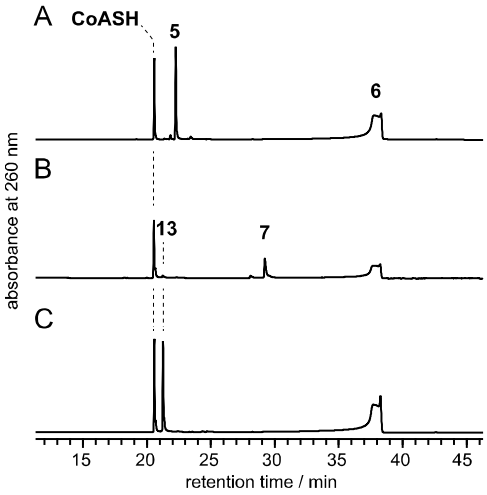

Figure 3. HPLC analysis of the recombinant KtmA reaction. (A) Reaction between acetyl-CoA (**5**) and phenylpyruvate (**6**). (B) Reaction between malonyl-CoA (**13**) and phenylpyruvate. (C) Control reaction without the enzyme.

Table 2. Kinetic parameters for KtmA.

	*k*cat (s^{-1})	K_M (mM)	*K*cat/ K_M ($mM^{-1}s^{-1}$)
malonyl-CoA	0.58 ± 0.06	0.70 ± 0.07	0.83 ± 0.08
phenylpyruvate	0.67 ± 0.05	0.27 ± 0.03	2.5 ± 0.2

As illustrated in Figure 2, the second C–C bond formation by KtmB may occur before or after the dehydratase (KtmC) reaction. Therefore, the reaction mixture of the aldolase reaction was first incubated with KtmC and analyzed by LC-MS. A new peak at *m/z* = 954 in negative ion mode was observed (Figure 4, traces A and B). The results of HR-ESI MS analysis of the

product were in good agreement with the proposed benzylfumaryl-CoA (**9**) product (calcd. for $C_{32}H_{43}N_7O_{19}P_3S^-$ 954. 1553, found 954.1534). However, no new peak was observed in the HPLC trace when the KtmA reaction mixture was incubated with KtmB in the presence of phenylalanine and PLP (Figure 4, traces C and D). These results clearly suggested that the dehydratase reaction by KtmC precedes the C–C bond formation catalyzed by KtmB.

We next examined the third reaction (KtmB or KtmF). When KtmF was added into the KtmA and KtmC reaction mixture with NADPH, we detected a new reaction product by LC-MS (Figure 4, trace E). This product was absent in a control reaction in which KtmF was omitted (Figure 4, trace F). Although the molecular mass of the observed mass signal was consistent with that of the proposed product, the amount of product was too small for further analysis. Therefore, we chemically synthesized benzylsuccinyl-CoA (**11**). The synthetic scheme is shown in Scheme S1. The KtmF product was confirmed to coelute with the synthetic benzylsuccinyl-CoA (**11**) by LC-MS, indicating the KtmF product was benzylsuccinyl-CoA (Figure 4, trace G). We then incubated synthetic benzylsuccinyl-CoA (**11**) with KtmB in the presence of L-phenylalanine and PLP (Figure 4, trace H). Unexpectedly, no new peak was observed in any reaction conditions tested. These results suggested that pathway C is not the biosynthetic route.

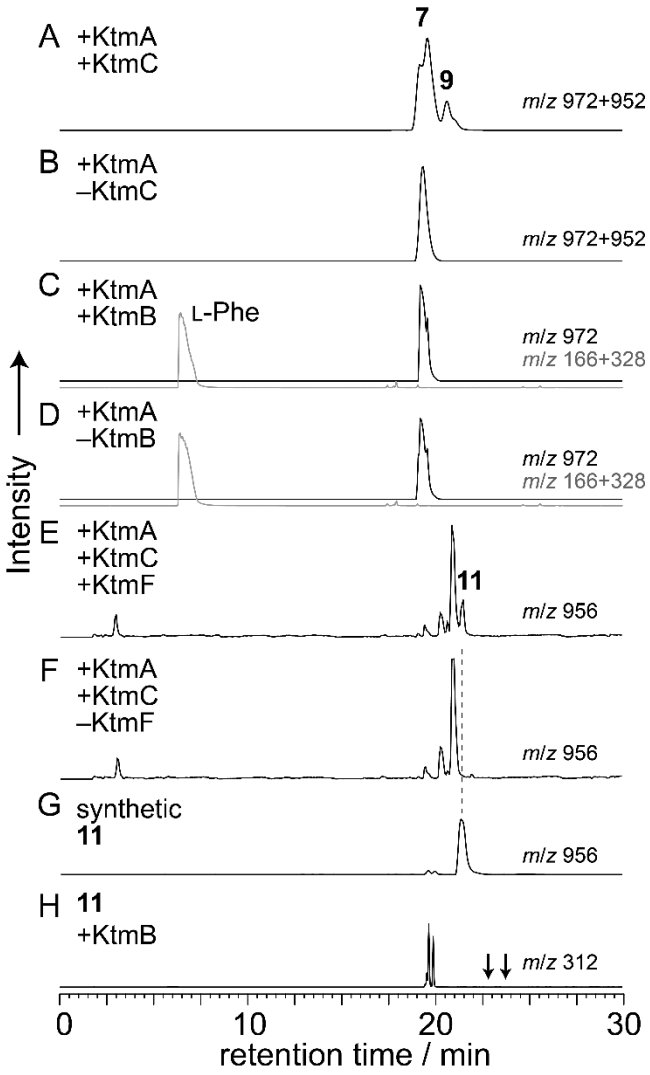

Figure 4. LC-MS analysis of enzyme reactions. (A) Reaction of KtmA + KtmC and (B) control reaction without KtmC. The LC-MS profile was monitored at *m*/*z* 972 (**7**, $[M-H]^-$) and 954 (**9**, $[M-H]^-$). (C) Reaction of KtmA + KtmB and (D) control reaction without KtmB. The LC-MS profile was monitored with ESI-negative mode (black trace, *m*/*z* 972 for **7**, $[M-H]^-$) and with ESI-positive mode (gray trace, *m*/*z* 166 for phenylalanine and 328 for **8**, $[M+H]^+$). (E) Reaction of KtmA + KtmC + KtmF, (F) control reaction without KtmF, and (G) synthetic standard **11**. The LC-MS profile was monitored at *m*/*z* 956 (**11**, $[M-H]^-$). (H) Reaction of KtmB with synthetic **11**.

The MS profile was monitored at *m*/*z* 312 (**12**, $[M+H]^+$). Arrows indicates the retention times of predicted pseudodipeptide products (Figure 5, trace E).

To examine if pathway B is operative, we added KtmB into the KtmA and KtmC reaction mixture together with L-phenylalanine and PLP. In this case, the formation of a new peaks (**10** and peaks with an asterisk) was observed (Figure 5, traces A and B). Compound **10** was collected and characterized by HR-ESI-MS. The molecular mass of the observed mass signal agreed with that of the proposed product (calcd. for $C_{19}H_{20}NO_3^+$ 310.1438, found 310.1436). We next carried out the KtmF reaction using purified **10**. As shown in trace C, incubation of **10** with KtmF in the presence of NADPH resulted in the disappearance of the substrate (**10**) concomitant with the appearance of two new peaks that coeluted with diastereomers of synthetic pseudo-phenylalanyl-phenylalanine (**12**)[3]. NADH also functioned as a hydride source. Taken together, our results unequivocally established the formation of the pseudodipeptide in ketomemicin biosynthesis (Figure 6). This pathway is consistent with early feeding experiments using ^{13}C-labeled acetate in the biosynthesis of arphamenines, where the C-3 and C-4 carbons are derived from C-2 and C-1 of acetate, respectively.[13]

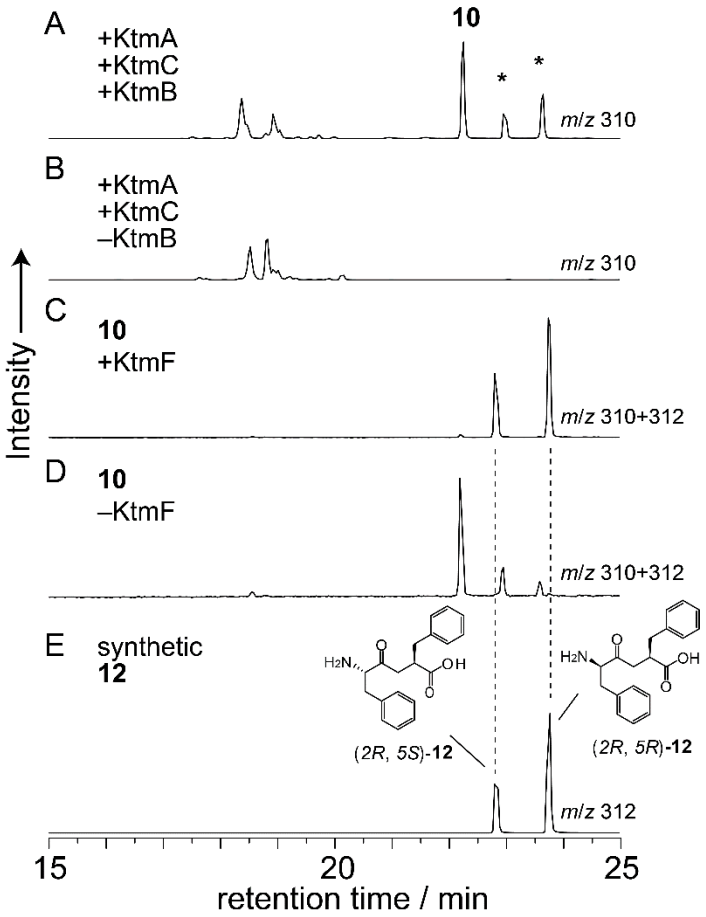

Figure 5. LC-MS analysis of enzyme reactions. (A) Reaction of KtmA + KtmC + KtmB and (B) control reaction without KtmB. The LC-MS profile was monitored at *m*/*z* 310 (**10**, $[M+H]^+$). Compounds with an asterisk are likely stereo- and positional isomers of **10** and we observed interconversion among three compounds upon incubation in the reaction buffer. (C) Reaction of KtmF with purified **10**, (D) control reaction without KtmF. The LC-MS profile was monitored at *m*/*z* 312 (**12**, $[M+H]^+$) and 310 (**10**, $[M+H]^+$). (E) Synthetic standard **12**. The LC-MS profile was monitored at *m*/*z* 312 (**12**, $[M+H]^+$).

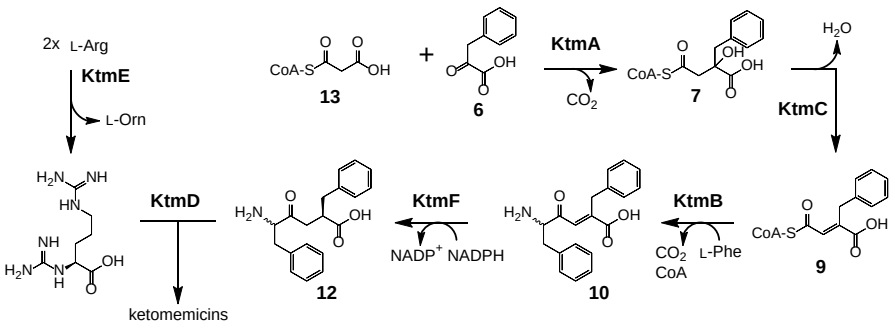

Figure 6. Biosynthesis of ketomemicins

In conclusion, we performed an *in vitro* characterization of KtmA, KtmC, KtmB, and KtmF, and fully established the biosynthetic machinery for the pseudodipeptide structure in ketomemicins. Our results demonstrate the involvement of two different types of C–C bond formation driven by decarboxylation. KtmA catalyzes a rare aldol reaction concomitant with decarboxylation of malonyl-CoA and generates benzylmalyl-CoA. KtmB, a PLP-dependent amino acid-*C*-acyltransferase homolog, catalyzes a Claisen-type condensation between phenylalanine and benzylfumaryl-CoA, which determines the *N*-terminal residue of the pseudodipeptide. We also revealed the reaction order of the four enzymes (KtmA → KtmC → KtmB → KtmF). Significantly, this is the first report to clarify the biosynthesis of a carbonylmethylene-containing pseudodipeptide. Our findings set the stage to explore and generate other carbonylmethylene-containing pseudodipeptide natural products.

The authors declare no competing financial interest.

Supporting Information available: This material is available free of charge via the Internet.

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References

[1] a) A. Choudhary, R. T. Raines, *Chembiochem* **2011**, *12*, 1801; b) N. A. Meanwell, *J. Med. Chem.* **2011**, *54*, 2529.

[2] a) S. Ohuchi, H. Suda, H. Naganawa, T. Takita, T. Aoyagi, H. Umezawa, H. Nakamura, Y. Iitaka, *J. Antibiot*. **1983**, *36*, 1576; b) H. Umezawa, T. Aoyagi, S. Ohuchi, A. Okuyama, H. Suda, T. Takita, M. Hamada, T. Takeuchi, *J. Antibiot*. **1983**, *36*, 1572; c) H. Umezawa, T. Nakamura, S. Fukatsu, T. Aoyagi, K. Tatsuta, *J. Antibiot*. **1983**, *36*, 1787.

[3] Y. Ogasawara, J. Kawata, M. Noike, Y. Satoh, K. Furihata, T. Dairi, *ACS Chem. Biol*. **2016**, *11*, 1686.

[4] a) X. N. Zhang, Z. G. Song, T. Jiang, B. S. Shi, Y. W. Hu, Z. H. Yuan, *World J. Gastroenterol* **2010**, *16*, 201; b) L. S. Zalman, M. A. Brothers, P. S. Dragovich, R. Zhou, T. J. Prins, S. T. Worland, A. K. Patick, *Antimicrob Agents Chemother* **2000**, *44*, 1236; c) D. A. Matthews, P. S. Dragovich, S. E. Webber, S. A. Fuhrman, A. K. Patick, L. S. Zalman, T. F. Hendrickson, R. A. Love, T. J. Prins, J. T. Marakovits, R. Zhou, J. Tikhe, C. E. Ford, J. W. Meador, R. A. Ferre, E. L. Brown, S. L. Binford, M. A. Brothers, D. M. DeLisle, S. T. Worland, *Proc. Natl. Acad. Sci. U S A* **1999**, *96*, 11000.

[5] M. Noike, T. Matsui, K. Ooya, I. Sasaki, S. Ohtaki, Y. Hamano, C. Maruyama, J. Ishikawa, Y. Satoh, H. Ito, H. Morita, T. Dairi, *Nat. Chem. Biol*. **2015**, *11*, 71.

[6] Y. Ogasawara, M. Fujimori, J. Kawata, T. Dairi, *Bioorg. Med. Chem. Lett*. **2016**, *26*, 3662.

[7] a) S. Bekal, J. Van Beeumen, B. Samyn, D. Garmyn, S. Henini, C. Divies, H. Prevost, *J. Bacteriol*. **1998**, *180*, 647; b) M. Bott, P. Dimroth, *Mol Microbiol* **1994**, *14*, 347; c) M. Bott, *Arch. Microbiol*. **1997**, *167*, 78.

[8] a) S. Herter, A. Busch, G. Fuchs, *J. Bacteriol*. **2002**, *184*, 5999; b) M. Meister, S. Saum, B. E. Alber, G. Fuchs, *J. Bacteriol*. **2005**, *187*, 1415.

[9] T. J. Erb, L. Frerichs-Revermann, G. Fuchs, B. E. Alber, *J. Bacteriol*. **2010**, *192*, 1249.

[10] J. A. Serrano, M. J. Bonete, *Biochim. Biophys. Acta*. **2001**, *1520*, 154.

[11] a) R. S. Ajioka, J. D. Phillips, J. P. Kushner, *Biochim. Biophys. Acta* **2006**, *1763*, 723; b) H. Ikushiro, H. Hayashi, *Biochim. Biophys. Acta* **2011**, *1814*, 1474; c) B. A. Yard, L. G. Carter, K. A. Johnson, I. M. Overton, M. Dorward, H. Liu, S. A. McMahon, M. Oke, D. Puech, G. J. Barton, J. H. Naismith, D. J. Campopiano, *J. Mol. Biol*. **2007**, *370*, 870; d) A. Schmidt, J. Sivaraman, Y. Li, R. Larocque, J. A. Barbosa, C. Smith, A. Matte, J. D. Schrag, M. Cygler, *Biochemistry* **2001**, *40*, 5151.

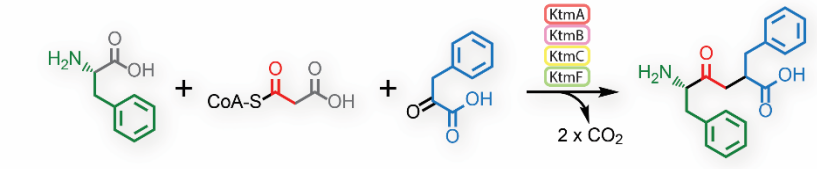
[12] S. A. Borisova, B. T. Circello, J. K. Zhang, W. A. van der Donk, W. W. Metcalf, *Chem. Biol*. **2010**, *17*, 28.

[13] S. Ohuchi, A. Okuyama, H. Naganawa, T. Aoyagi, H. Umezawa, *J. Antibiot*. **1984**, *37*, 518.

TOC Summary

The biosynthetic pathway of the carbonylmethylene-containing pseudopeptide in ketomemicins was fully elucidated *in vitro*. The pathway involves two unique C–C bond formations. KtmA catalyzes an aldol reaction concomitant with decarboxylation starting from malonyl-CoA and phenylpyruvate. A Claisen-type condensation catalyzed by a PLP-dependent amino acid-*C*-acyltransferase homolog, KtmB, is also involved.

TOC Figure



Keywords

Biosynthesis

Enzyme catalysis

Natural product

Pseudopeptide