Characterization of a methyltransferase for iterative *N*-methylation at the leucinostatin termini in *Purpureocillium lilacinum*

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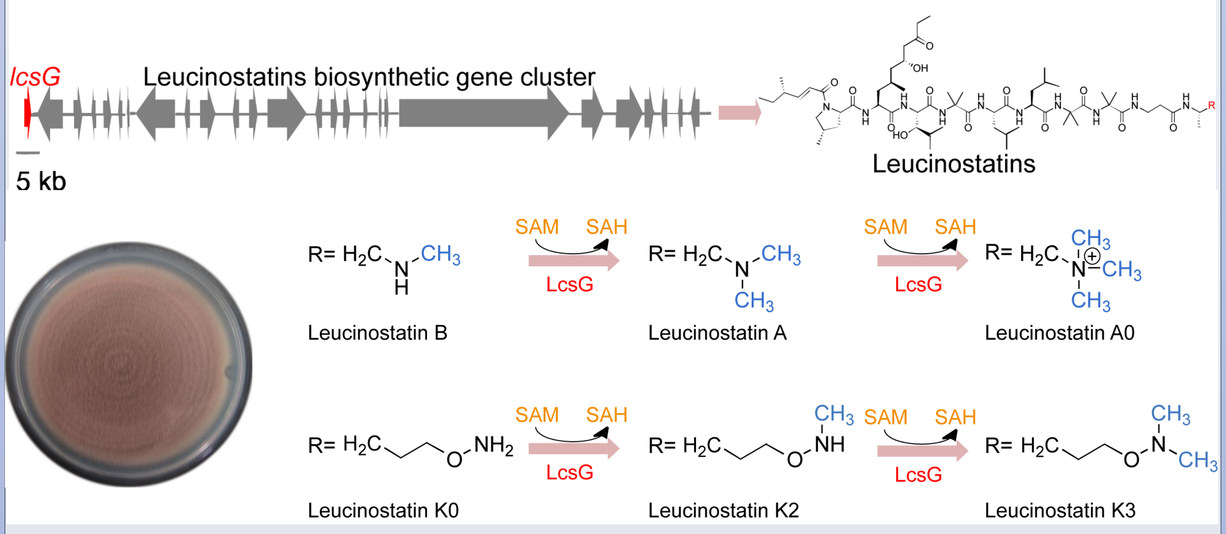
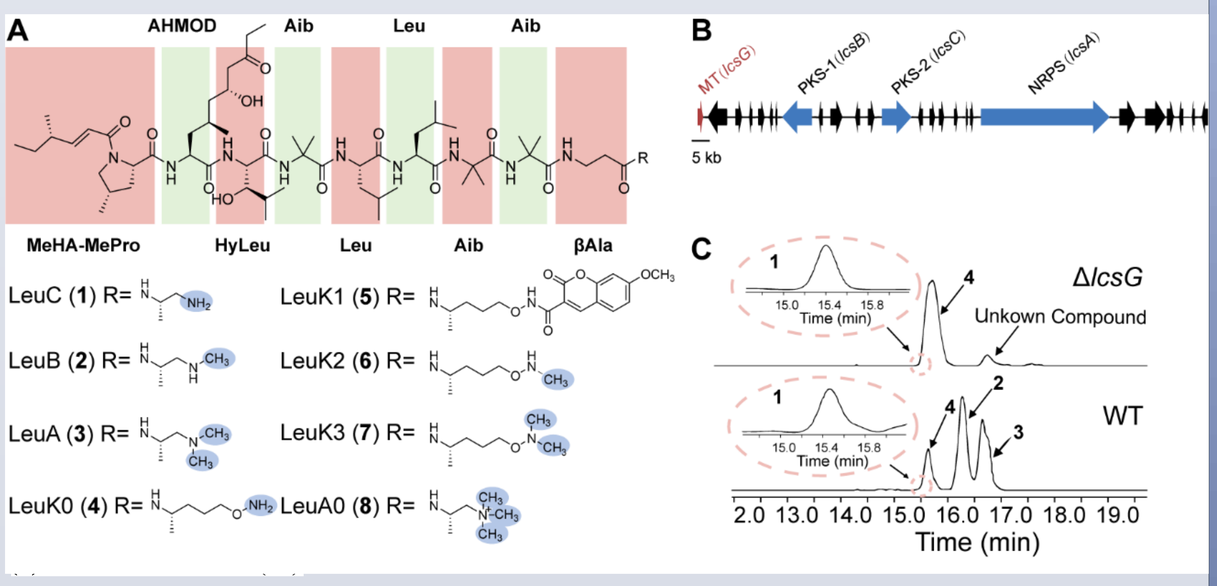
ABSTRACT

N-methyltransferase (NMT)-catalyzed methylation at the termini of nonribosomal peptides (NRPs) has rarely been reported. Here, we discovered a fungal NMT, LcsG, for the iterative terminal *N*-methylation of a family of NRPs, leucinostatins. Gene deletion results suggested that LcsG is essential for leucinostatin methylation. In vitro assay and HRESI-MS-MS analysis revealed the methylation sites were the NH_2, $NHCH_3$ and $N(CH_3)_2$ in the C-terminus of various leucinostatins. LcsG catalysis yielded new lipopeptides, some of which demonstrated effective antibiotic properties against the human pathogen *Cryptococcus neoformans* and the plant pathogen *Phytophthora infestans*. Multiple sequence alignments and site-directed mutagenesis of LcsG indicated the presence of a highly conserved SAM-binding pocket, along with two possible active site residues (D368 and D395). Molecular docking and molecular dynamics simulations showed that the targeted N can be docked between these two residues. Thus, this study suggests a method for increasing the variety of natural bioactivity of NPRs and a possible catalytic mechanism underlying the *N*-methylation of NRPs.



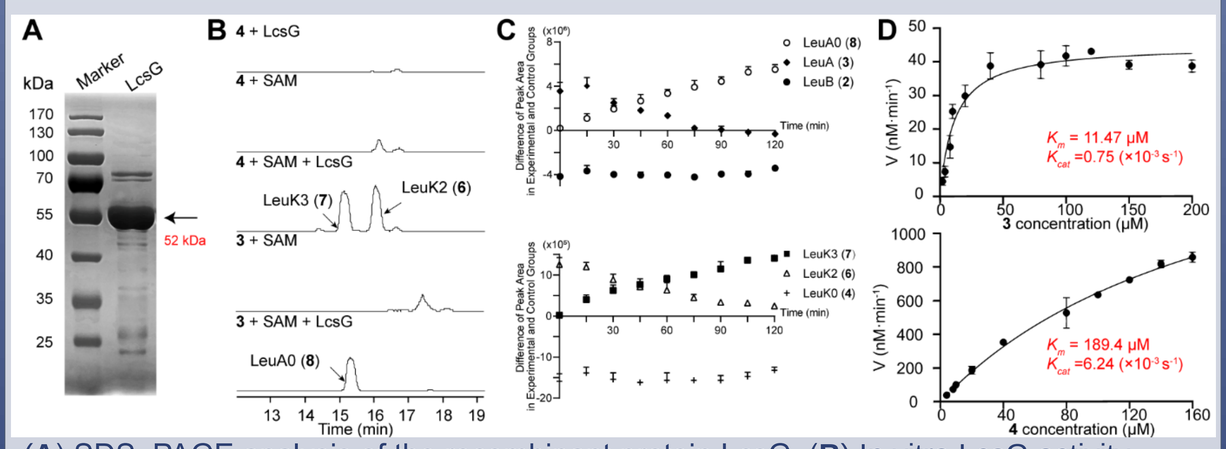
RESULTS

1. Identification of LcsG from *Purpureocillium lilacinum*



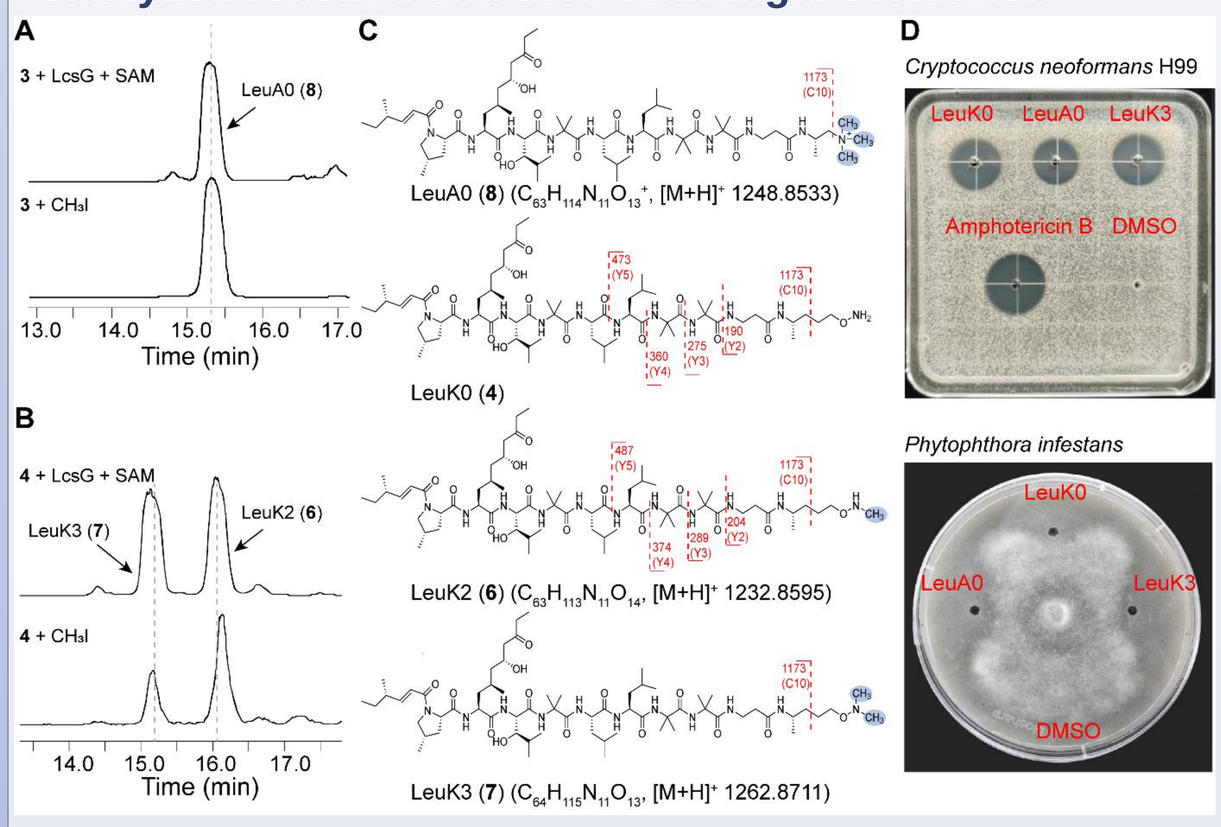
(**A**) Structures of leucinostatins (**1**-**8**). (**B**) Genetic organization of the leucinostatin BGC in *P. lilacinum* PLBJ-1. (**C**) LC–MS analysis of the lcsG knockout (Δ*lcsG*) mutant and the wild-type (WT) strain.

2. LcsG functions as a SAM-dependent methyltransferase



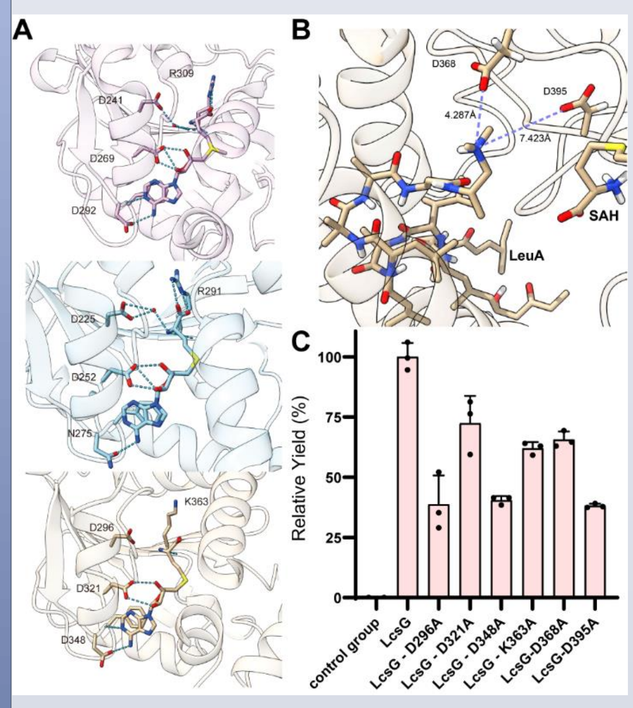
(**A**) SDS–PAGE analysis of the recombinant protein LcsG. (**B**) In vitro LcsG activity analysis using LeuK0 (**4**) and LeuA (**3**) as the substrates and the $[M+H]^+$ ions of three new product peaks at positions **6**-**8**. (**C**) Time dependency of the variations in each component. (**D**) Kinetic analysis of LcsG using LeuA (**3**, top) and LeuK0 (**4**, bottom) as substrates.

3. Characterizations of the products of the LcsG-catalyzed reaction and their antifungal evaluation



(**A**) LC–MS analysis of the *N*-methylation of LeuA (**3**) to the same trimethylammonium compound (**8**, LeuA0) by LcsG and CH_3I. (**B**) LC–MS analysis of two methylation reactions using LeuK0 (4) as the substrate and LcsG and CH_3I. (**C**) Structures and the HERSI-MS-MS data of LeuA0 (**8**), LeuK0 (**4**), LeuK2 (**6**), and LeuK3 (**7**). (**D**) Growth inhibition of eukaryotic microorganisms by 25 μg/well LeuK0 (**4**), LeuA0 (**8**), and LeuK3 (**7**) according to the agar diffusion assay.

4. Catalytic mechanism of the *N*-methyltransferase LcsG



(**A**) SAH binding sites of OxaC (PDB code: 5w7p, marked in pink), LepI (PDB code: 6ix7, marked in blue), and LcsG (marked in golden). (**B**) Modified LeuA binding sites of LcsG (Maestro, Schrödinger, LLC). (**C**) Comparison of wild-type LcsG and mutated LcsG-mediated methylation. The yield of the product was quantified by comparison with the peak area of the product catalyzed by LcsG (100%). All the data are presented as the means of n = 3 biologically independent samples, and the error bars show the standard deviations.

CONCLUSION

An OMT-like enzyme from *P. lilacinum*, LcsG, was identified as a discrete SAM-dependent NMT that can iteratively catalyze the formation of primary amines, secondary amines, and tertiary amines in the unique terminal unit of leucinostatins. Furthermore, one new secondary metabolite (LeuK0) and two enzymatic products (LeuK2 and LeuK3) were identified as new leucinostatins. In addition, the methylated compounds were observed to display greater antifungal activity than that of their parent molecules. To our knowledge, LcsG is a rare NMT that can methylate the terminal residues of NRPs. We expect that the results of this study will provide deeper insights into the mechanisms underlying the *N*-methylation of peptides and increase the possibility of engineering new methylated molecules for exploring more potent antibiotics.

Acknowledgments

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