JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Synthesis of the Rosette-Inducing Factor RIF-1 and Analogs

Christine Beemelmanns,[†,§] Arielle Woznica,[‡] Rosanna A. Alegado,[‡,∥] Alexandra M. Cantley,[†] Nicole King,[*,‡] and Jon Clardy[*,†]

[†] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115, United States

[‡] Howard Hughes Medical Institute and Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: Studies on the origin of animal multicellularity have increasingly focused on one of the closest living relatives of animals, the choanoflagellate *Salpingoeca rosetta*. Single cells of *S. rosetta* can develop into multicellular rosette-shaped colonies through a process of incomplete cytokinesis. Unexpectedly, the initiation of rosette development requires bacterially produced small molecules. Previously, our laboratories reported the planar structure and femtomolar rosette-inducing activity of one rosette-inducing small molecule, dubbed rosette-inducing factor 1 (RIF-1), produced by the Gram-negative Bacteroidetes bacterium *Algoriphagus machipongonensis*. RIF-1 belongs to the small and poorly explored class of sulfonolipids. Here, we report a modular total synthesis of RIF-1 stereoisomers and structural analogs. Rosette-induction assays using synthetic RIF-1 stereoisomers and naturally occurring analogs defined the absolute stereochemistry of RIF-1 and revealed a remarkably restrictive set of structural requirements for inducing rosette development.

Multicellularity, the transition from a unicellular to a multicellular organism, evolved at least 25 times within eukaryotes, but it evolved only once in the animal lineage.[1] Choanoflagellates, the closest living relatives of animals, have emerged as important model organisms for reconstructing the transition to multicellularity.[2] Choanoflagellate cells have a spherical to prolate spheroid cell body and an apical collar of microvilli surrounding a single flagellum (Figure 1)[3] that resembles the feeding cells (choanocytes) of sponges.

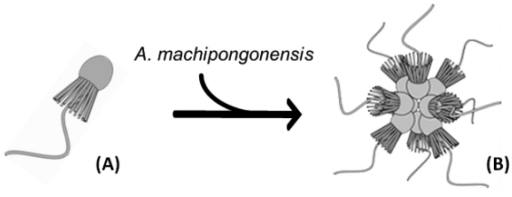


Figure 1. Morphogenesis of the choanoflagellate *S. rosetta* upon exposure to the prey bacterium *A. machipongonensis*: (A) unicellular slow swimmer and (B) multicellular colonial rosette form (drawing: courtesy of Mark Dayel).

Undulation of the apical flagellum generates water currents that sweep bacteria against the microvillar collar, where they are trapped and ultimately phagocytosed. One species of choanoflagellate, *Salpingoeca rosetta*, exhibits both free-living and multicellular colonial forms called rosettes; and this transition provides the basis of our study (Figure 1).[2]

The rosette-shaped colonies formed by *S. rosetta* resemble early stage morula embryos of diverse animals and develop through a process of incomplete cytokinesis from a single founding cell.[4] The induction of rosette development requires a bacterially produced signal from its prey *Algoriphagus machipongonensis*.[5] In a previous publication we identified the planar structure of the first rosette-inducing factor (RIF-1, **1**), and we demonstrated its extraordinary femtomolar potency.[6] In this report, we describe a modular total synthesis that defines the three-dimensional structure of RIF-1, the isolation of some naturally occurring analogs, and a rosette-inducing assay to establish initial structure−activity relations. In addition, we note that synthetic RIF-1 by itself does not completely recapitulate the activity of RIF-1 isolated from bacterial extract.

RIF-1 belongs to the small and poorly explored class of sulfonolipids.[7] Sulfonolipids have been reported as constituents of the cell envelopes of Bacteroidetes bacteria and are thought to contribute to the gliding motility frequently found in this group.[8] Sulfonolipids (**2**−**4**) closely resemble sphingolipids, such as (dihydro)ceramides (Figure 2, **5**), that are important membrane components in eukaryotes and act as both structural components and signaling molecules for cell death, survival, differentiation, and migration.[9] Sphingolipids and sulfonolipids have been reported rarely in bacteria and so far have only been isolated from the Bacteroidetes phylum and *Sphingomonas* genus, where their biological functions are poorly understood.[10] Both are amides of a fatty acid and an amine base called either sphingosine (for sphingolipids) or capnine (for sulfonolipids). Whereas sphingosine originates in the condensation of serine with a fatty acid followed by reduction and dehydrogenation, labeling studies with deuterated amino acids suggest that the capnine base is biosynthesized via the condensation of a fatty acyl-CoA with cysteic acid.[11]

To elucidate the stereochemistry of RIF-1 (Figure 2, **1**), we designed a flexible synthetic approach so that multiple derivatives of RIF-1 could be produced without changing the

Received: May 9, 2014
Published: July 1, 2014

   dx.doi.org/10.1021/ja5046692 | J. Am. Chem. Soc. 2014, 136, 10210−10213

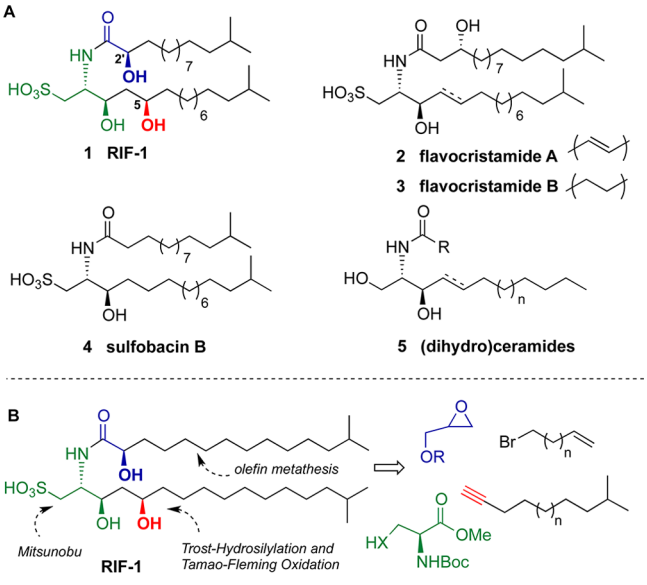
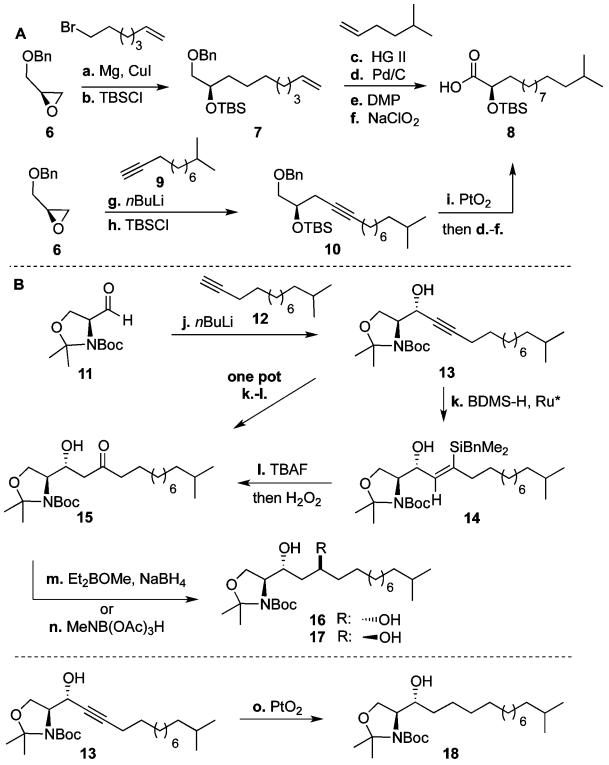


Figure 2. (A) RIF-1 and known sulfonolipids; (B) retrosynthesis of RIF-1.

main synthetic route. The absolute stereochemistry of RIF-1 was unknown, but we assumed it to be homologous to reported sulfonolipids (**2**–**4**).[7,12] Therefore, we first focused on the two completely unknown stereocenters at C-2′ and C-5, which generates four possible diastereoisomeric targets.

The synthesis of the α-hydroxy fatty acid commenced with the addition of cuprate reagent to benzyl-protected *R*(−) or *S*(+)-glycidol (Scheme 1).[13] After TBS protection, chain elongation was pursued by metathesis reaction with a second generation Hoveyda-Grubbs catalyst. A metathesis reaction at this point allowed access to other fatty acid precursors with different chain length and substitution pattern. The newly generated double bond and the Bn-protecting group were removed with Pd/C under hydrogen atmosphere in one step. The primary alcohol was then treated with Dess-Martin reagent and directly oxidized under standard reaction conditions with $NaClO_2$ in the presence of 2-methyl-2-butene yielding the desired α-hydroxy fatty acid **8**. The alternative fatty acid precursor **10** could be obtained by addition of alkyne **9** to glycidol ether **6**, subsequent TBS protection of the secondary alcohol, and reduction of the triple bond with PtO_2. Alkynes like **9** were synthesized according to literature procedures.[14] To assemble the sphingosine/capnine moiety, alkyne **12** was treated with *n*BuLi and reacted with Garner's aldehyde **11** in the presence of HMPA to yield compound **13** in acceptable 75% (*syn*:*anti* 20:80) yield (Scheme 1).[15] Over the course of the synthesis, it became apparent that a late stage Mitsunobu reaction for the introduction of the sulfonic acid group was a more attractive synthetic approach than using a cysteine-derived Garner's aldehyde as described by Takikawa et al.[12d] The next step involved a hydrosilylation reaction of **13** with benzyldimethylsilane (BDMS-H) as reported by Trost.[16] The reaction proceeded with excellent regiocontrol (>95:5) and afforded the desired silylated compound **14** in 90% yield. Subsequent Tamao-Fleming oxidation using TBAF and H_2O_2 (2 h) yielded ketone **15**. Gratifyingly, a one-pot procedure as reported by Trost starting from compound **13** could be accomplished affording ketone **15** in slightly higher overall yield (82%). Finally, β-hydroxy ketone was stereoselectively reduced using either Et_2BOMe as chelating reagent to give almost

Scheme 1. Representative Synthesis of (A) α-Hydroxy Acid and (B) Precursor of Capnine Base[a]



[a]Conditions: (a) Mg, CuI, THF, −20 °C, 84%; (b) TBSCl, TEA, DMAP, DMF, quant.; (c) 5-methyl-1-hexene, 5 mol % Hoveyda-Grubbs II catalyst, CH_2Cl_2, 40 °C, then; (d) Pd/C, H_2, EtOAc:EtOH 1:1, 2 d, 79% over 2 steps; (e) DMP, 30 mol % $NaHCO_3$, CH_2Cl_2, 0 °C → RT; then (f) $NaClO_2$, 2-methyl-butene, THF:BuOH:H_2O (3:1:1), RT, 3 h, 65% over 2 steps; (g) *n*BuLi, HMPA, THF, −78 °C, 82%; (h) TBSCl, TEA, DMAP, DMF, quant.; (i) PtO_2, H_2, EtOAc, 1 d, quant.; (j) *n*BuLi, HMPA, THF, −78 °C, 75% (*syn*:*anti* 20:80); (k) $[Cp^*Ru(NCCH_3)_3]^+PF_6^-$, BDMS-H, acetone, 0 °C → RT, 1 h, 90%; (l) TBAF, THF, 15 min, 0 °C; then H_2O_2, MeOH, K_2CO_3, 12 h, RT, 87%; (m) Et_2BOMe, $NaBH_4$, THF:MeOH 4:1, 77% (*syn*:*anti* > 90:10); (n) $Me_4NB(OAc)_3H$, MeOH:AcOH, −40 °C, 91% (*syn*:*anti* 20:80); (o) PtO_2, H_2, EtOAc, 1 d, quant.

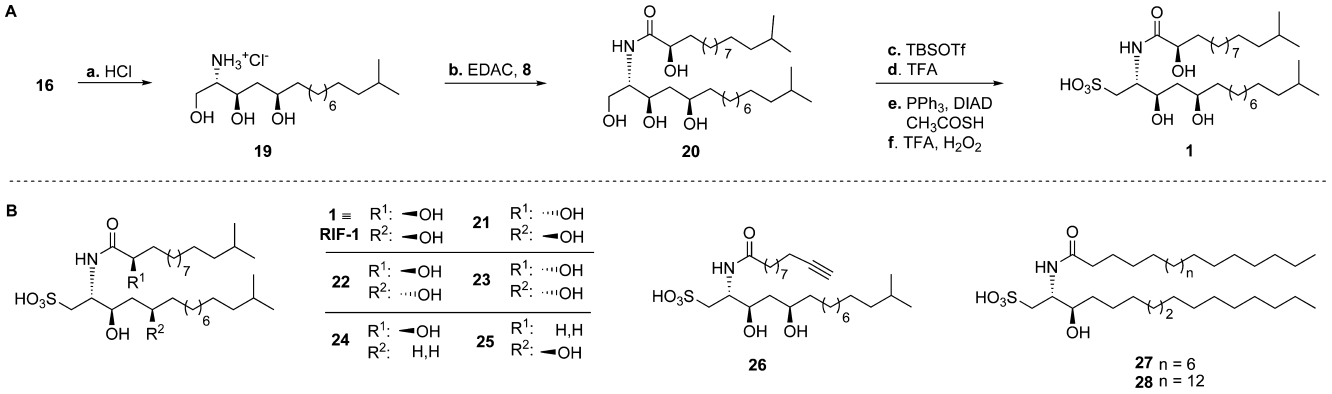
exclusively *syn*-diol **16** in 77% (dr, *syn*:*anti* > 90:10),[17] or $Me_4NB(OAc)_3H$ to furnish *anti*-diol **17** with lower but satisfactory diastereoselectivity.[18] In addition, the alkyne moiety of **13** was hydrogenated using PtO_2 in nearly quantitative yield.

Since the stereochemistries of RIF-1's hydroxy groups at C-2′ and C-5 were unknown, we first continued our synthetic approach with α-hydroxy fatty acid **8** and *syn*-diol **16**. The cyclic isopropylaminal and Boc protecting groups were removed in 6 N HCl at 60 °C yielding free sphingoid base **19**, which was suitable for condensation with a fatty acid (Scheme 2).

The sphingolipid core structure **20** was assembled by treatment of **19** and fatty acid **8** with peptide coupling reagent EDAC (Scheme 2). Subsequent protection with TBSOTf and selective deprotection with TFA of the primary alcohol yielded the key precursor for RIF-1. Finally, a Mitsunobu reaction of **20** with thioacetic acid, one-pot deprotection and oxidation of the primary thiol with H_2O_2 afforded sulfonolipid **1** in an overall yield of 8% (9 steps) starting from Garner's aldehyde **11**. The spectroscopic data of **1** were in full agreement with the reported

dx.doi.org/10.1021/ja5046692 | J. Am. Chem. Soc. 2014, 136, 10210−10213

Scheme 2. (A) Completion of the Total Synthesis and (B) Synthesis of Structurally Related Sulfonolipids[a]



[a]Conditions: (a) 6 N HCl, MeOH, 60 °C, 6 h; then (b) EDAC, compound **8**, CH_2Cl_2, 2 h, 58% over 2 steps; (c) TBSOTf, 2,6-lutidine, DMAP, CH_2Cl_2, 89%; (d) 10% TFA in H_2O, THF, 0 °C → RT, 6 h, 58% + 30% sm; (e) PPh_3, DIAD, CH_2Cl_2, 1 h, 0 °C, then CH_3COSH, 81%; (f) TFA, H_2O_2, 4 h, RT, 65%.

data on natural RIF-1.[6] For sulfonolipids **21**–**28** an analogous synthetic route was performed.[19]

In a complementary approach we also investigated the diversity of sulfonolipids produced by *A. machipongonensis*.[5,6] The already reported sulfonolipids flavochristamide A and B (**2**, **3**) were only detected in negligible amounts by LC-MS. However, sulfobacin B (**4**) turned out to be one of the major sulfonolipid products under our standard growth condition.[19]

By detailed analysis of the lipid extracts we were able to isolate and characterize four unknown sulfonolipids **24**, **29**–**31**, which we named accordingly (sulfobacins C–F, Figure 3A). Synthetic compound **24**, missing the C-5 hydroxy group, had identical spectroscopic data as isolated sulfonolipid sulfobacin D, suggesting the depicted absolute stereochemistry. These synthetic and isolated materials allowed a preliminary structure–activity analysis for rosette induction. All synthesized (**1**, **21**–**28**) and isolated sulfonolipids (**1**, **4**, **24**, **29**–**31**) as well as sphingolipid intermediates of type **20** were tested over a broad concentration range (μM to fM) in a robust rosette colony-induction assay with the *S. rosetta* RCA cell line.[19] We also tested the corresponding capnine bases (**32**–**34**), which were obtained by hydrolysis with methanolic HCl. However, RIF-1 diastereomers (**21**–**23**), RIF-1 analogs (**24**–**31**), and capnine bases (**32**–**34**) did not induce rosette formation in *S. rosetta*. Small amounts of isomers of sulfonolipids **24** and **31** were also tested, but they too showed no rosette-inducing activity.[19] Only synthetic and natural RIF-1 (**1**) stimulated the development of solitary slow swimmers into rosette colonies. This unexpectedly restricted set of structural requirements indicates a highly specific substrate–receptor interaction. Synthetic RIF-1 does not completely replicate the biological activity of RIF-1 isolated from *A. machipongonensis* as shown by quantitative comparison (Figure 3B). We are currently exploring the reasons for this discrepancy by investigating additional bacterially produced molecules with rosette-inducing activity, molecules that synergize with RIF-1, and methods of delivering these highly hydrophobic signals.

In summary, we have defined the three-dimensional structure of RIF-1 through a modular total synthesis, characterized four new naturally occurring sulfonolipids, established the tight structural requirements for RIF-1's biological activity, and discovered that signals beyond RIF-1 may be needed for full activity.

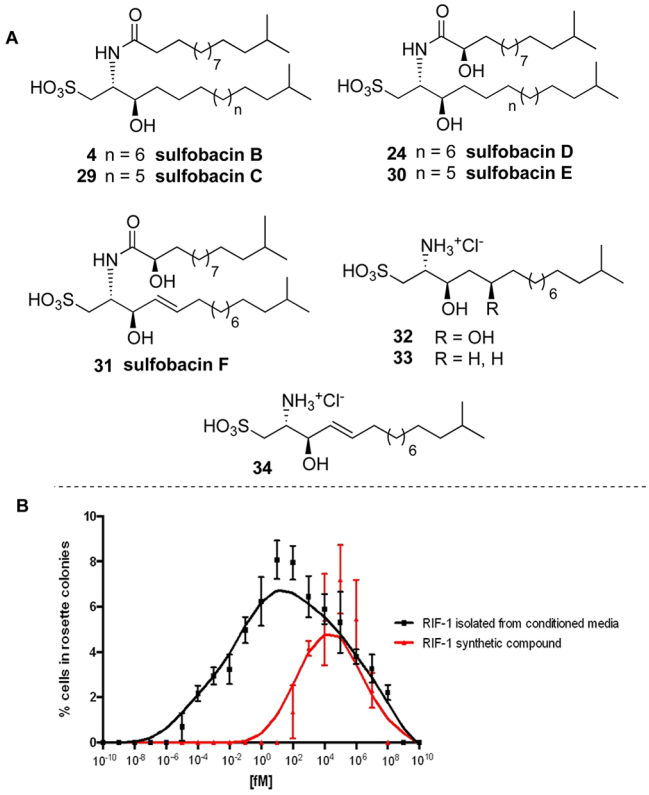


Figure 3. (A) Isolated sulfonolipids from *A. machipongonensis*, and corresponding capnine bases; and (B) dose–response curve of *S. rosetta* (fM concentration range) after treatment with natural isolate RIF-1 (black) and synthetic RIF-1 (red); error bars indicate standard deviation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Syntheses, isolation procedures, compound characterization, and assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

jon_clardy@hms.harvard.edu; nking@berkeley.edu

dx.doi.org/10.1021/ja5046692 | *J. Am. Chem. Soc.* 2014, 136, 10210–10213

Present Addresses

[§]Leibniz Institute for Natural Product Research and Infection Biology e.V., Hans-Knöll-Institute (HKI), Beutenbergstrasse 11a, 07745 Jena, Germany.

[‖]Center for Microbial Oceanography Research and Education, 1950 East-west Rd., Honolulu, HI 96822.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NIH GM099533 (N.K.) and GM086258 (J.C.). C.B. was supported by a Leopoldina Research Fellowship (LPDS 2011-2) from the German Academy of Sciences Leopoldina. A.W. is supported by the National Science Foundation Graduate Research Fellowship Program under grant no. DGE 1106400. N.K. is an Investigator in the Howard Hughes Medical Institute and a Senior Scholar in the Integrated Microbial Biodiversity Program of the Canadian Institute for Advanced Research. The authors thank Dr. P. Stallforth for insightful discussions on synthetic strategy, and Mark Dayel (http://www.dayel.com) for choanoflagellate illustrations.

■ REFERENCES

(1) (a) Grosberg, R. K.; Strathmann, R. R. *Annu. Rev. Ecol. Syst.* **2007**, *38*, 621. (b) King, N. *Dev. Cell* **2004**, *7*, 313.

(2) For recent reviews, see: (a) Richter, D. J.; King, N. *Annu. Rev. Genet.* **2013**, *47*, 527. (b) Rokas, A. *Annu. Rev. Genet.* **2008**, *42*, 235. (c) Ruiz-Trillo, I.; Burger, G.; Peter, W. H.; Holland, P. W. H.; King, N.; Lang, B. F.; Roger, A. J.; Gray, M. W. *Trends Genet.* **2007**, *23*, 113.

(3) (a) del Campo, J.; Massana, R. *Protist* **2011**, *162*, 435. (b) Nielsen, C. *Evol. Dev.* **2008**, *10*, 241. (c) Wainright, P. O.; Hinkle, G.; Sogin, M. L.; Stickel, S. K. *Science* **1993**, *260*, 340. (d) Haeckel, E.; Quart, J. *Microscop. Sci.* **1874**, *14*, 142. (e) Haeckel, E. *Ann. Mag. Nat. Hist.* **1873**, *4*, 241.

(4) (a) Fairclough, S.; Chen, Z.; Kramer, E.; Zhen, Q.; Young, S.; Robertson, H.; Begovic, E.; Richter, D. J.; Russ, C.; Westbrook, M. J.; Manning, G.; Lang, B. F.; Haas, B.; Nusbaum, C.; King, N. *Genome Biol.* **2013**, *14*, R15. (b) Dayel, M. J.; Alegado, R. A.; Fairclough, S. R.; Levin, T. C.; Nichols, S. A.; McDonald, K.; King. *N. Dev. Biol.* **2011**, *357*, 73. (c) Fairclough, S. R.; Dayel, M. J.; King, N. *Curr. Biol.* **2010**, *20*, R875.

(5) (a) Alegado, R. A.; Grabenstatter, J. D.; Zuzow, R.; Morris, A.; Huang, S. Y.; Summons, R. E.; King, N. *Int. J. Syst. Evol. Microbiol.* **2012**, *63*, 163. (b) Alegado, R. A.; Ferriera, S.; Nusbaum, C.; Young, S. K.; Zeng, Q.; Imamovic, A.; Fairclough, S. R.; King, N. *J. Bacteriol.* **2011**, *193*, 1485.

(6) Alegado, R. A.; Brown, L. W.; Cao, S.; Dermenjian, R. K.; Zuzow, R.; Fairclough, S. R.; Clardy, J.; King, N. *eLife* **2012**, *1*, e00013.

(7) For examples of sulfonolipid isolation, see: (a) Baronio, M.; Lattanzio, V. M. T.; Vaisman, N.; Oren, A.; Corcelli, A. *J. Lipid Res.* **2010**, *51*, 1878. (b) Kamiyama, T.; Umino, T.; Itezono, Y.; Nakamura, Y.; Satoh, T.; Yokose, K. *J. Antibiot. (Tokyo)* **1995**, *48*, 929. (c) Kamiyama, T.; Umino, T.; Satoh, T.; Sawairi, S.; Shirane, M.; Ohshima, S.; Yokose, K. *J. Antibiot.* **1995**, *48*, 924. (d) Godchaux, W.; Leadbetter, E. R. *J. Bacteriol.* **1983**, *153*, 1238. (d) Godchaux, W., III; Leadbetter, E. R. *J. Bacteriol.* **1980**, *144*, 592.

(8) (a) Agarwal, S.; Hunnicutt, D. W.; McBride, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12139. (b) Godchaux, W.; Leadbetter, E. *J. Biol. Chem.* **1984**, *259*, 2982.

(9) For a recent review on bacterial lipids, see: Parsons, J. B.; Rock, C. O. *Prog. Lipid Res.* **2013**, *52*, 249. For selected reviews on sphingolipids, see: (a) Pruett, S. T.; Bushnev, A.; Hagedirn, K.; Adiga, M.; Haynes, C. A.; Sullards, M. C.; Liotta, C. D.; Merrill, A. H., Jr. *J. Lipid Res.* **2008**, *49*, 1621. (b) Hannun, Y. A.; Obeid, L. M. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 139. (c) Futerman, A. H.; Hannun, Y. A. *EMBO Rep.* **2004**, *5*, 777. (d) Merrill, A. H. *J. Biol. Chem.* **2002**, *277*, 25843.

(10) For a recent example of the role of bacterial sphingolipids, see: (a) Wieland-Brown, L. C.; Penaranda, C.; Kashyap, P. C.; Williams, B. B.; Clardy, J.; Kronenberg, M.; Sonnenburg, J. L.; Comstock, L. E.; Bluestone, J. A.; Fischbach, M. A. *PLoS Biol.* **2013**, *11*, e1001610. (b) An, D.; Na, C.; Bielawski, J.; Hannun, Y. A.; Kasper, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 4666.

(11) (a) Raman, M. C.; Johnson, K. A.; Clarke, D. J.; Naismith, J. H.; Campopiano, D. J. *Biopolymers* **2010**, *93*, 811. (b) White, R. H. *J. Bacteriol.* **1984**, *159*, 42. Analogous results were obtained by feeding ^{2}H- and ^{13}C-labeled amino acids to a culture of *A. machipongonensis* and will be reported in due course.

(12) For synthesis of known sulfonolipids, see: (a) Sharma, A.; Gamre, S.; Chattopadhyay, S. *Tetrahedron Lett.* **2007**, *48*, 3705. (b) Gupta, P.; Naidu, S. V.; Kumar, P. *Tetrahedron Lett.* **2004**, *45*, 9641. (c) Labeeuw, O.; Phansavath, P.; Genêt, J. *Tetrahedron Lett.* **2003**, *44*, 6383. (d) Takikawa, H.; Nozawa, D.; Kayo, A.; Muto, S.; Mori, K. *J. Chem. Soc., Perkin Trans.* **1999**, 2467. (e) Irako, N.; Shioiri, T. *Tetrahedron Lett.* **1998**, *39*, 5797. (f) Irako, N.; Shioiri, T. *Tetrahedron Lett.* **1998**, *39*, 5793. (g) Ohashi, K.; Kosai, S.; Arizuka, M.; Watanabe, T.; Yamagiwa, Y.; Kamikawa, T.; Kates, M. *Tetrahedron* **1989**, *45*, 2557.

(13) Bonini, C.; Chiummiento, L.; Lopardo, M. T.; Pullez, M.; Colobert, F.; Solladié, G. *Tetrahedron Lett.* **2003**, *44*, 2695.

(14) Iwayama, Y.; Ando, H.; Ishida, H.; Kiso, M. *Chem.—Eur. J.* **2009**, *15*, 4637.

(15) Fujisawa, T.; Nagai, M.; Koike, Y.; Shimizu, M. *J. Org. Lett.* **1994**, *59*, 5865.

(16) Trost, B. M.; Ball, Z. T.; Jöge, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 3415.

(17) (a) Chen, K.; Hardtmann, G. E.; Prasad, K.; Repic, O.; Shapiro, M. J. *Tetrahedron Lett.* **1987**, *28*, 155. (b) Narasaka, K.; Pai, F.-C. *Tetrahedron* **1984**, *12*, 2238.

(18) Evans, D. A.; Chapman, K. T.; Carreira, E. M. *J. Am. Chem. Soc.* **1988**, *110*, 3560.

(19) For further details, see Supporting Information.