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

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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 euakaryotes, 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.

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.2 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

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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).
main synthetic route. The absolute stereochemistry of RIF-1 was unknown, but we assumed it to be homologous to reported sulfonolipids (2–4). 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). 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 NaClO2 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 PtO2. Alkynes like 9 were synthesized according to literature procedures. To assemble the sphingosine/capnine moiety, alkyne 12 was treated with nBuLi and reacted with Garner’s aldehyde 11 in the presence of HMPA to yield compound 13 in acceptable 78% (syn:anti 20:80) yield (Scheme 1). 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-based reaction. The newly generated double bond and the Bn-protecting group were removed with Pd/C under hydrogen atmosphere in one step.

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 isopropyraminal 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 cyclic isopropyraminal 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).

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

"Conditions: (a) Mg, CuI, THF, −20 °C, 84%; (b) TBSCI, TEA, DMAP, DMF, quant.; (c) 5-methyl-1-hexene, 5 mol % Hoveyda-Grubbs II catalyst, CH2Cl2, 40 °C, then; (d) Pd/C, H2, EtOAc:EtOH 1:1, 2 d, 79% over 2 steps; (e) DMP, 30 mol % NaHCO3, CH2Cl2, 0 °C → RT; then (f) NaClO2, 2-methyl-butenone, THF:BuOH:H2O (3:1:1), RT, 3 h, 65% over 2 steps; (g) nBuLi, HMPA, THF, −78 °C, 82%; (h) TBSCI, TEA, DMAP, DMF, quant.; (i) PtO2, H2, EtOAc, 1 d, quant.; (j) nBuLi, HMPA, THF, −78 °C, 75% (syn:anti 20:80); (k) [OsRu(NCCH3)6]3+PF6−, BDMS-H, acetone, 0 °C → RT, 1 h, 90%; (l) TBAF, THF, 15 min, 0 °C; then H2O2, MeOH, K2CO3, 12 h, RT, 87%; (m) Et3BOMe, NaBH4, THF:MeOH 4:1, 77% (syn:anti > 90:10); (n) Me2NB(OAc)2H, MeOH:AcOH, −40 °C, 91% (syn:anti 20:80); (o) PtO2, H2, EtOAc, 1 d, quant.

exclusively syn-diol 16 in 77% (dr, syn:anti > 90:10),17 or Me2NB(OAc)2H to furnish anti-diol 17 with lower but satisfactory diastereoselectivity. In addition, the alkyne moiety of 13 was hydrogenated using PtO2 in nearly quantitative yield.

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 H2O2 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.
For sulfonolipids 21–28 an analogous synthetic route was performed. In a complementary approach we also investigated the diversity of sulfonolipids produced by A. machipongonensis. 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.

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. 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. 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.

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.

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(11) (a) Raman, M. C.; Johnson, K. A.; Clarke, D. J.; Naismith, J. H.; Campoppiano, D. J. Biopolymers 2010, 93, 811. (b) White, R. H. J. Bacteriol. 1984, 159, 42. Analogous results were obtained by feeding 1H- and 13C-labeled amino acids to a culture of A. machipongonensis and will be reported in due course.