

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

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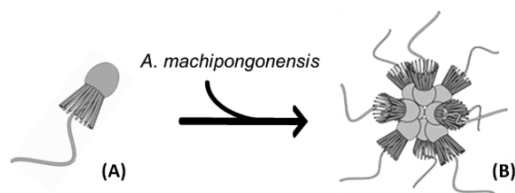
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### Supporting Information

**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.<sup>1</sup> Choanoflagellates, the closest living relatives of animals, have emerged as important model organisms for reconstructing the transition to multicellularity.<sup>2</sup> Choanoflagellate cells have a spherical to prolate spheroid cell body and an apical collar of microvilli surrounding a single flagellum (Figure 1)<sup>3</sup> 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).<sup>2</sup>

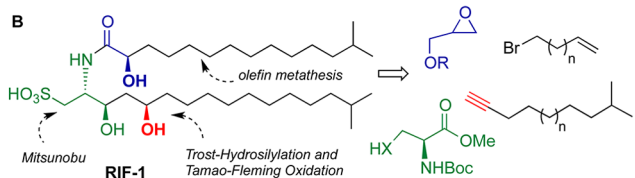
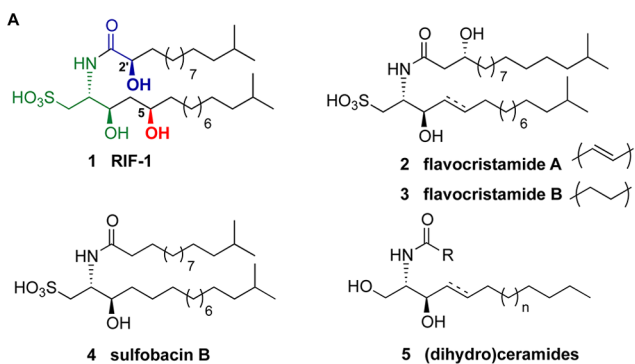
The rosette-shaped colonies formed by *S. rosetta* resemble early stage morula embryos of diverse animals and develop through a process of incomplete cytokinesis from a single founding cell.<sup>4</sup> The induction of rosette development requires a bacterially produced signal from its prey *Algoriphagus machipongonensis*.<sup>5</sup> 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.<sup>6</sup> 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.<sup>7</sup> 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.<sup>8</sup> 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.<sup>9</sup> 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.<sup>10</sup> 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.<sup>11</sup>

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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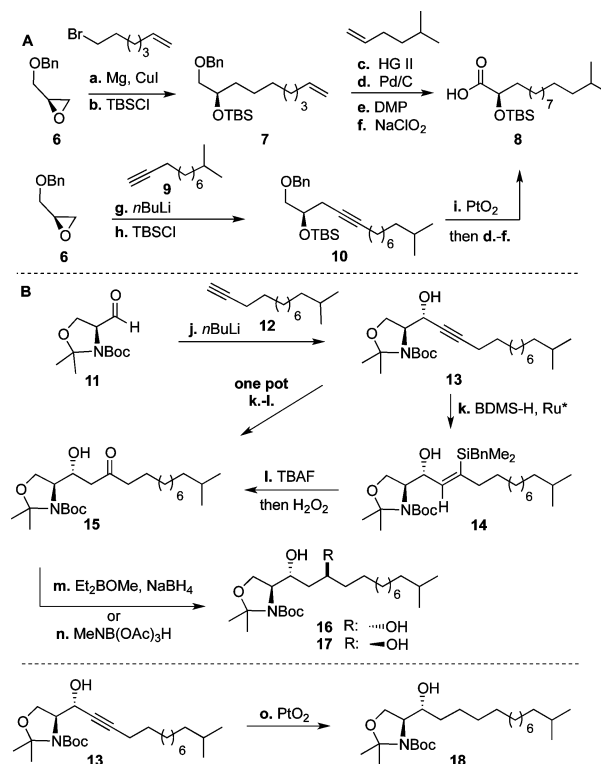


**Figure 2.** (A) RIF-1 and known sulfonolipids; (B) retrosynthesis of RIF-1.

main synthetic route. The absolute stereochemistry of RIF-1 was unknown, but we assumed it to be homologous to reported sulfonolipids (2–4).<sup>7,12</sup> 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  $\alpha$ -hydroxy fatty acid commenced with the addition of cuprate reagent to benzyl-protected *R*(–) or *S*(+)-glycidol (Scheme 1).<sup>13</sup> 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<sub>2</sub> in the presence of 2-methyl-2-butene yielding the desired  $\alpha$ -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<sub>2</sub>. Alkynes like 9 were synthesized according to literature procedures.<sup>14</sup> 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).<sup>15</sup> 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.<sup>12d</sup> The next step involved a hydrosilylation reaction of 13 with benzyldimethylsilane (BDMS-H) as reported by Trost.<sup>16</sup> 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<sub>2</sub>O<sub>2</sub> (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,  $\beta$ -hydroxy ketone was stereoselectively reduced using either Et<sub>2</sub>BOMe as chelating reagent to give almost

**Scheme 1.** Representative Synthesis of (A)  $\alpha$ -Hydroxy Acid and (B) Precursor of Capnine Base<sup>a</sup>

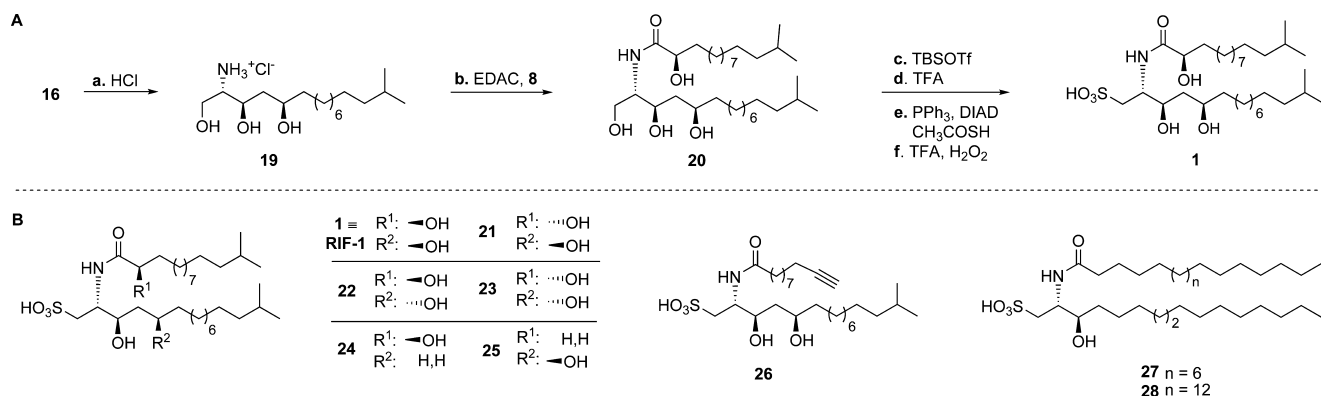


<sup>a</sup>Conditions: (a) Mg, CuI, THF, –20 °C, 84%; (b) TBSCl, TEA, DMAP, DMF, quant.; (c) 5-methyl-1-hexene, 5 mol % Hoveyda-Grubbs II catalyst, CH<sub>2</sub>Cl<sub>2</sub>, 40 °C, then; (d) Pd/C, H<sub>2</sub>, EtOAc:EtOH 1:1, 2 d, 79% over 2 steps; (e) DMP, 30 mol % NaHCO<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C → RT; then (f) NaClO<sub>2</sub>, 2-methyl-butene, THF:BuOH:H<sub>2</sub>O (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<sub>2</sub>, H<sub>2</sub>, EtOAc, 1 d, quant.; (j) *n*BuLi, HMPA, THF, –78 °C, 75% (*syn:anti* 20:80); (k) [Cp\*<sup>+</sup>Ru(NCCH<sub>3</sub>)<sub>3</sub>]<sup>+</sup>PF<sub>6</sub><sup>–</sup>, BDMS-H, acetone, 0 °C → RT, 1 h, 90%; (l) TBAF, THF, 15 min, 0 °C; then H<sub>2</sub>O<sub>2</sub>, MeOH, K<sub>2</sub>CO<sub>3</sub>, 12 h, RT, 87%; (m) Et<sub>2</sub>BOMe, NaBH<sub>4</sub>, THF:MeOH 4:1, 77% (*syn:anti* > 90:10); (n) Me<sub>4</sub>NB(OAc)<sub>3</sub>H, MeOH:AcOH, –40 °C, 91% (*syn:anti* 20:80); (o) PtO<sub>2</sub>, H<sub>2</sub>, EtOAc, 1 d, quant.

exclusively *syn*-diol 16 in 77% (*dr*, *syn:anti* > 90:10),<sup>17</sup> or Me<sub>4</sub>NB(OAc)<sub>3</sub>H to furnish *anti*-diol 17 with lower but satisfactory diastereoselectivity.<sup>18</sup> In addition, the alkyne moiety of 13 was hydrogenated using PtO<sub>2</sub> 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  $\alpha$ -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<sub>2</sub>O<sub>2</sub> 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

Scheme 2. (A) Completion of the Total Synthesis and (B) Synthesis of Structurally Related Sulfonolipids<sup>a</sup>

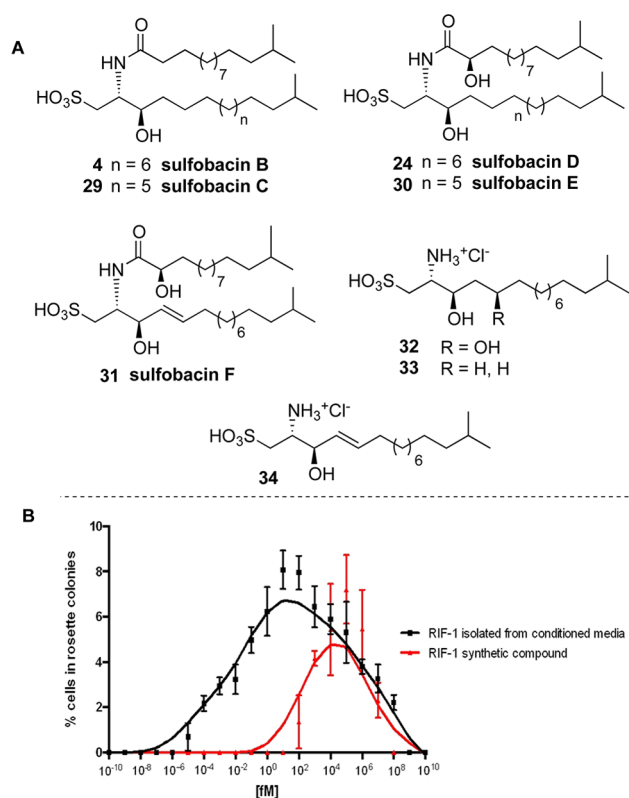
<sup>a</sup>Conditions: (a) 6 N HCl, MeOH, 60 °C, 6 h; then (b) EDAC, compound **8**, CH<sub>2</sub>Cl<sub>2</sub>, 2 h, 58% over 2 steps; (c) TBSOTf, 2,6-lutidine, DMAP, CH<sub>2</sub>Cl<sub>2</sub>, 89%; (d) 10% TFA in H<sub>2</sub>O, THF, 0 °C → RT, 6 h, 58% + 30% sm; (e) PPh<sub>3</sub>, DIAD, CH<sub>2</sub>Cl<sub>2</sub>, 1 h, 0 °C, then CH<sub>3</sub>COSH, 81%; (f) TFA, H<sub>2</sub>O<sub>2</sub>, 4 h, RT, 65%.

data on natural RIF-1.<sup>6</sup> For sulfonolipids **21–28** an analogous synthetic route was performed.<sup>19</sup>

In a complementary approach we also investigated the diversity of sulfonolipids produced by *A. machipongonensis*.<sup>5,6</sup> 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.<sup>19</sup>

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.<sup>19</sup> 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.<sup>19</sup> 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>.

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## Notes

The authors declare no competing financial interest.

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