Engineering Fluorometabolite Production: Fluorinase Expression in *Salinispora tropica* Yields Fluorosalinosporamide[†]

Alessandra S. Eustáquio,[‡] David O'Hagan,[*,§] and Bradley S. Moore[*,‡,⊥]

Scripps Institution of Oceanography and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California at San Diego, La Jolla, California 92093-0204, and Centre for Biomolecular Sciences, School of Chemistry, University of St Andrews, KY16 9ST, U.K.

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Organofluorine compounds play an important role in medicinal chemistry, where they are responsible for up to 15% of the pharmaceutical products on the market. While natural products are valuable sources of new chemical entities, natural fluorinated molecules are extremely rare and the pharmaceutical industry has not benefited from a microbial source of this class of compounds. *Streptomyces cattleya* is an unusual bacterium in that it elaborates fluoroacetate and the amino acid 4-fluorothreonine. The discovery in 2002 of the fluorination enzyme FlA responsible for C−F bond formation in *S. cattleya*, and its subsequent characterization, opened up for the first time the prospect of genetically engineering fluorometabolite production from fluoride ion in host organisms. As a proof of principle, we report here the induced production of fluorosalinosporamide by replacing the chlorinase gene *salL* from *Salinispora tropica* with the fluorinase gene *flA*.

Fluorinated natural products are extremely rare, with only a handful of such metabolites identified. Fluoroacetate is the most common, found in a variety of plants; however despite an increased sophistication in high-throughput screening technology and analytical methods aimed at natural product identification, it has been over 20 years since the last fluorinated microbial products were identified. These were fluoroacetate and 4-fluorothreonine from the soil bacterium *Streptomyces cattleya*.[1] This is in stark contrast to metabolites containing the other halogens.[2,3] While numerous biochemical methods have evolved for the activation and incorporation of inorganic chloride, bromide, and iodide into organo-halo compounds, the unique physical properties of fluoride prevent oxidative activation to F^+. Fluoride ion is also a poor nucleophile in water. The process of enzymatic fluorination in *S. cattleya* has been characterized, and it has been shown that fluoride ion acts as a nucleophile and reacts with *S*-adenosyl-L-methionine (SAM) to generate the C−F bond in 5′-fluorodeoxyadenosine (5′-FDA). 5′-FDA is subsequently metabolized into the mammalian toxin fluoroacetate and the antibiotic 4-fluorothreonine (Figure 1).[4,5] We now report the first example whereby the fluorinase gene has been incorporated into a host organism by genetic engineering, such that the heterologous host has a new capacity to generate a fluorinated natural product when grown in the presence of inorganic fluoride.

Organofluorine compounds represent an important class of drugs in which the halogen can impart favorable pharmacokinetic properties.[6,7] While fluorinated molecules are routinely synthesized in the laboratory, the incorporation of fluorine into structurally complex natural products can prove problematic, as most fluorination reagents are nonselective, often noxious, and difficult to handle. To circumvent these problems, precursor-directed biosynthesis and mutasynthesis have contributed to the generation of a host of fluorinated natural product derivatives in which simple, synthetic organofluorine precursors are metabolized into more complex biosynthetic products.[8] The discovery of fluorinase (5′-FDA synthase) and the characterization of its initiating role in the biosynthesis of fluorinated metabolites in *S. cattleya*[4,5] have provided an opportunity to reprogram biosynthesis in a heterologous host for the assembly of an engineered organofluorine metabolite. We thus selected the marine bacterium *Salinispora tropica* as a suitable test organism since it uniquely biosynthesizes the potent anticancer agent salinosporamide A from a related halogenation pathway involving chlorine assimilation via 5′-ClDA in a reaction catalyzed by the chlorinase SalL (Figure 1).[9,10]

Results and Discussion

We previously prepared fluorosalinosporamide by a standard mutasynthesis approach in which the chlorinase gene *salL* was inactivated and the ensuing mutant was chemically complemented with synthetic 5′-FDA, 5-fluororibose,[11] or 4-fluorocrotonic acid.[12] In order to engineer fluorosalinosporamide biosynthesis from inorganic fluoride, we generated a *S. tropica* mutant in which the *salL* chlorinase gene was chromosomally replaced with the *S. cattleya flA* fluorinase gene by λ-Red-mediated recombination[13] so that *flA* was placed under the control of the native *salL* promoter and ribosome binding site (Figure 2A). The correct gene replacement was confirmed by PCR (Figure 2B) and sequencing.

Cultivation of the *salL*$^-$ *flA*$^+$ mutant in seawater-based medium initially showed no detectable salinosporamide A nor fluorosalinosporamide production (Figure 3) despite *flA* transcription being established in the mutant by reverse transcription PCR (RT-PCR) (Figure 2C). Previously it was reported that fluorinase can also function as a chlorinase *in vitro* when coupled to a L-amino acid oxidase or to an adenylic acid deaminase in order to drive the equilibrium of the reaction toward chlorinated products.[14] Although the rate of the reaction is at least 2 orders of magnitude lower with chloride as a substrate compared to fluoride, we did expect at least some salinosporamide A production in the mutant. Upon semipurification of a large-scale fermentation extract followed by LC/APCI-MS analysis, we ultimately detected salinosporamide A. Yet, the amount produced (35 μg/L) was 3 orders of magnitude lower compared to the wild-type *S. tropica* (50 mg/L), reflecting the differential specialization of SalL and FlA as chlorinase and fluorinase, respectively.

The inability to biosynthesize fluorosalinosporamide, on the other hand, was likely due to the low concentration of fluoride in seawater (1.3 ppm). Cultivation of the *S. tropica salL*$^-$ *flA*$^+$ mutant in seawater-based medium supplemented with 2 mM potassium fluoride as reported for *S. cattleya*[15] caused growth inhibition,

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[*] To whom correspondence should be addressed. (B.S.M.) Tel: +1 858 822 6650. Fax: +1 858 558 3702. E-mail: bsmoore@ucsd.edu. (D.O'H.) Tel: +44 1334 467176. Fax: +44 1334 463808. E-mail: do1@st-andrews.ac.uk.

[‡] Scripps Institution of Oceanography.

[§] University of St Andrews.

[⊥] Skaggs School of Pharmacy and Pharmaceutical Sciences.

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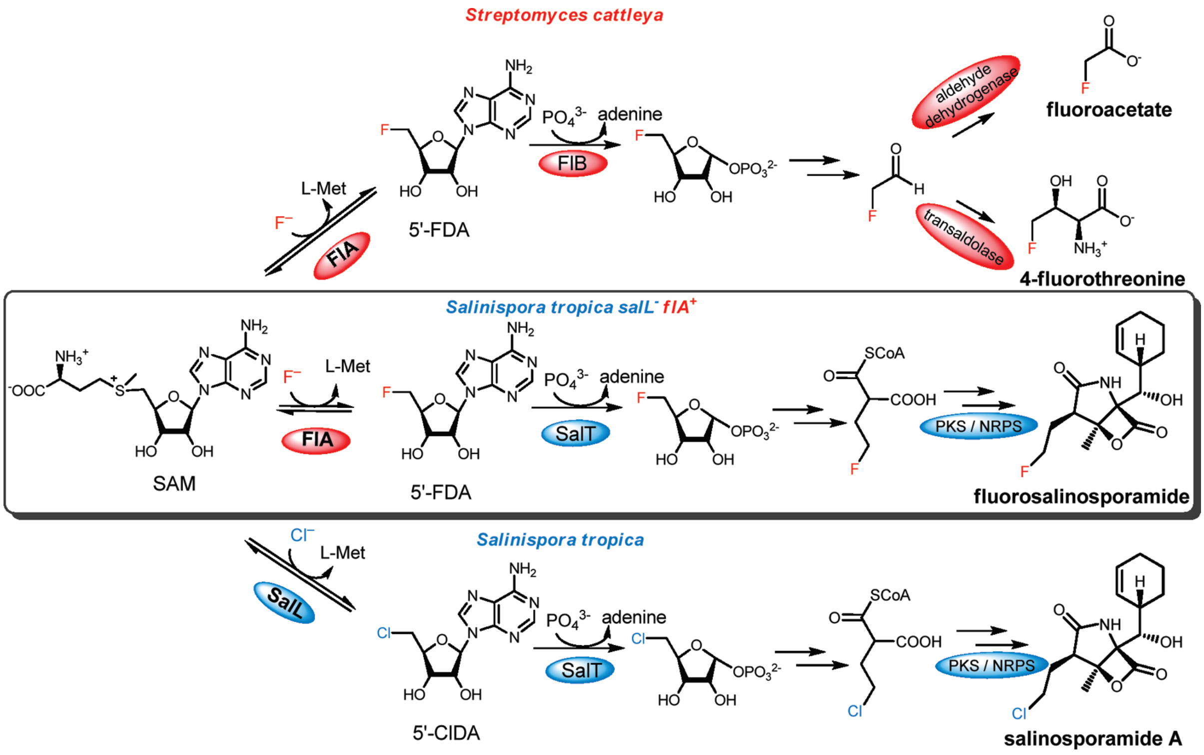

Figure 1. Biosynthesis of fluorometabolites by *Streptomyces cattleya*[21,22] (top) and of salinosporamide A by *Salinispora tropica*[10] (bottom) and fluorosalinosporamide engineering (middle).

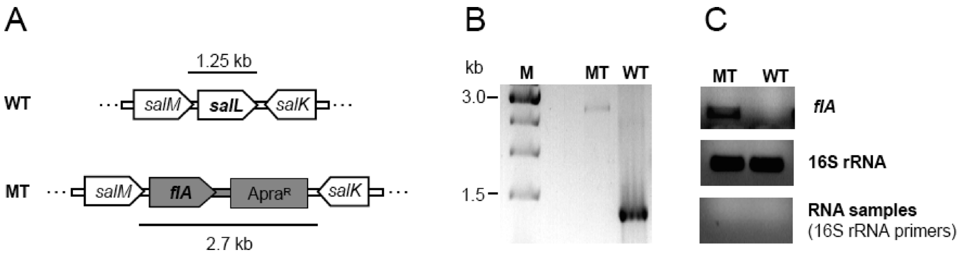

Figure 2. Fluorinase expression in *S. tropica*. (A) The chlorinase gene *salL* was chromosomally replaced with fluorinase *flA* and the apramycin resistance/*oriT* cassette from pIJ773 (Apra^R) using PCR targeting.[10,13] *flA* is thus under control of the natural *salL* promoter and ribosome binding site. The targeted locus of the wild-type (WT) and mutant (MT) chromosomes is shown for comparison. (B) The authenticity of mutants was confirmed by PCR. (C) *flA* transcription was analyzed by semiquantitative RT-PCR. 16S rRNA was used as control of cDNA quality and integrity. The negative control with RNA instead of cDNA samples confirms the absence of genomic DNA contamination.

preventing the detection of fluorosalinosporamide in a timely manner. To circumvent the fluoride toxicity issue, potassium fluoride was not added until the culture reached an early to mid exponential phase. Under these conditions, fluorosalinosporamide was clearly detectable in the culture broth by LC/(ESI)MS (Figure 3) and ^{19}F NMR (Figures 4 and 5).

Fluoride uptake and fluorometabolite production by *S. cattleya* has been shown to be pH-dependent, with maximum rates at pH 6.0 and a sharp decline at higher and lower pH values.[15] Likewise, maximum yields of fluorosalinosporamide production were detected at pH 6.0 (4 mg/L), while lower pH values (5.5 was tested) inhibited growth, preventing fluorometabolite production, and higher pH values (7−8) led to, on average, a 5-fold and 12-fold decrease in yields, respectively (Table 1).

The fluorosalinosporamide production yields obtained at optimum pH were comparable to those attained through mutasynthesis,[11] which however was still 1 order of magnitude lower than the amount of salinosporamide A produced by the parent strain. In order to verify if there were any significant competing pathways diverting fluorinated intermediates into other side products, ^{19}F NMR analyses of the culture extracts were carried out. Figure 4 shows both ^{19}F{^{1}H} and ^{19}F spectra, revealing that at least five fluorinated metabolites were produced. The major fluorinated component in the *S. tropica salL$^-$ flA$^+$* extract is fluorosalinosporamide (at −220.7 ppm), which was confirmed by NMR comparison to a reference sample generated from previous mutasynthesis studies.[11] In particular, the 2D ^{1}H/^{19}F-HMBC experiment comparing the extract with a reference sample of fluorosalinosporamide showed identical chemical shifts and correlations between ^{19}F and ^{1}H signals in the two samples (Figure 5).

Although the structures of the other fluorinated compounds have not yet been elucidated, the minor metabolites corresponding to ^{19}F NMR signals at −217.5 and −219.4 ppm have very similar ^{1}H/^{19}F NMR coupling patterns to those observed with fluorosalinosporamide (Figure 4). Hence they are most likely close structural analogues. Natural structural modifications reported for salinosporamide A include the C-2 epimer salinosporamide F, the C-3 ethyl derivative salinosporamide I, the C-5 deoxy analogue salinosporamide J, and the C-5 isopropyl derivative antiprotealide.[16−18] The presence of salinosporamide degradation products in culture extracts has also been described, which include the hydrolyzed β-lactone and decarboxylated products.[19,20]

Because ^{19}F NMR analysis showed that fluorosalinosporamide is indeed the major fluorinated metabolite produced by *S. tropica*

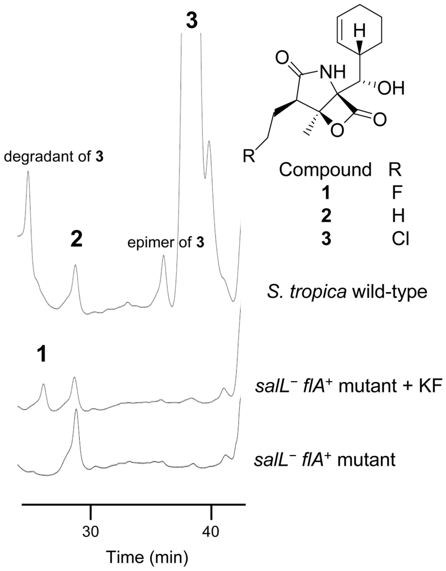

Figure 3. HPLC-MS analysis of culture extracts of *S. tropica* wild-type and the *flA*$^+$ *salL*$^-$ mutant grown in A1 seawater-based medium and of the mutant grown in A1 seawater-based medium pH 6.0 to which 2 mM potassium fluoride was added at day 2 (+ KF). HPLC was monitored at 210 nm.

salL$^-$ *flA*$^+$, an explanation for the lower yield of fluorosalinosporamide produced by the mutant compared to salinosporamide A accumulated by the wild-type is not consistent with parallel pathways diverting fluorinated intermediates. Rather fluoride toxicity and the differential preference for chlorinated versus fluorinated substrates by downstream pathway enzymes in the salinosporamide biosynthetic pathway likely account for the observed disparate yields, especially considering that the mutasynthesis experiment gave similar results.

In conclusion, we have presented an example of the biosynthesis of fluorinated natural product analogues from fluoride ion by introducing the fluorinase gene from *S. cattleya* into a suitable heterologous host. Broader applicability of fluorometabolite engineering in different organisms may come from the concomitant expression of other pathway enzymes involved in the production of polyketide and/or nonribosomal peptide building blocks such as fluoroacetyl-CoA, fluorothreonine, or fluoroethylmalonyl-CoA.[10,12,21,22]

Experimental Section

General Experimental Procedures. *Salinispora tropica* CNB-440[23] was cultured in A1 seawater-based medium as described.[24] The pH of the medium was set up using 50 mM MES buffer (pH 5.5 to 7.0) or phosphate buffer (pH 8.0). The REDIRECT technology kit for PCR targeting[13] was obtained from Plant Bioscience Limited (Norwich, UK). pCC1FOS-based (Epicenter) fosmid BHXS1782 was obtained from the Joint Genome Institute. Apramycin (200 μg/mL for *S. tropica*; 50 μg/mL for *E. coli*), chloramphenicol (12−25 μg/mL), carbenicillin (100 μg/mL), and nalidixic acid (100 μg/mL) were used for selection of recombinant strains. DNA manipulation and PCR were performed according to standard procedures.[25,26] Details of gene replacement in *S. tropica* including a conjugation protocol can be found in refs 9 and 10.

Replacement of *salL* by *flA* in *S. tropica*. The cassette for replacement of *salL* with *flA* was generated by a two-step PCR strategy in which *flA* was fused to the apramycin resistance cassette from pIJ773.[13] In the first step, *flA* and the apramycin resistance cassette were amplified in two parallel reactions termed here "PCR 1" and "PCR 2", respectively. Primers used in PCR 1 were P1_flAki (GTC GGT TTC CGA CCG ATA AAA CGG AGG TCA CTC ACC ATG GCT GCC AAC AGC ACA CG) and P2_flAki (TT CGA ACT GCA GGT CGA CGG ATC CCC GGA AT GGT ACG TCG TCG CGC GTT C) and in PCR 2, P3_flAki (ATT CCG GGG ATC CGT CGA CC) and P4_flAki (GAC AGG AGC TAC CCA GCA GCA TGC CGC CCC CTG GGT CAG TGT AGG CTG GAG CTG CTT C), in which priming sites are underlined. PCR 1 was subject to 30 cycles of 94 °C (30 s), 58 °C (45 s), and 72 °C (90 s) using Pfu Turbo DNA polymerase (Stratagene) and FlA_HT plasmid[27] as template. PCR 2 was carried out using the High Fidelity PCR system (Roche) as reported.[13] PCR products were digested with DpnI to eliminate template plasmid, purified using the QIAQuick PCR purification kit (Qiagen), and mixed to approximately equimolar concentrations for the second round, which was carried out using the High Fidelity PCR system and primers P1_flAki and P4_flAki. Cycle conditions consisted of 1 cycle of 94 °C (2 min), followed by a cycle of 94 °C (45 s) and 72 °C (5 min), 10 cycles of 94 °C (45 s), 60 °C (45 s), and 72 °C (2.5 min), 15 cycles of 94 °C (45 s), 65 °C (45 s), and 72 °C (2.5 min), and a final extension

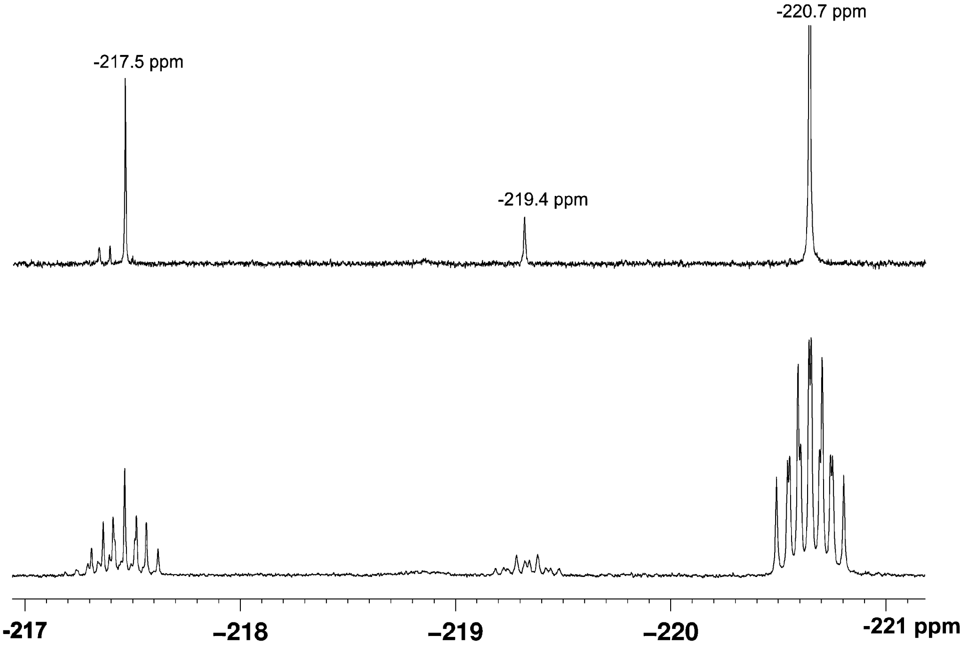

Figure 4. ^{19}F NMR analysis of *S. tropica salL*$^-$ *flA*$^+$ culture extracts. The upper trace shows the decoupled ^{19}F{^{1}H} NMR spectrum of the organic fraction of the extract, whereas the lower trace is the proton-coupled ^{19}F NMR spectrum. In all, five fluorometabolites were detected, with the major product as fluorosalinosporamide (−220.7 ppm).

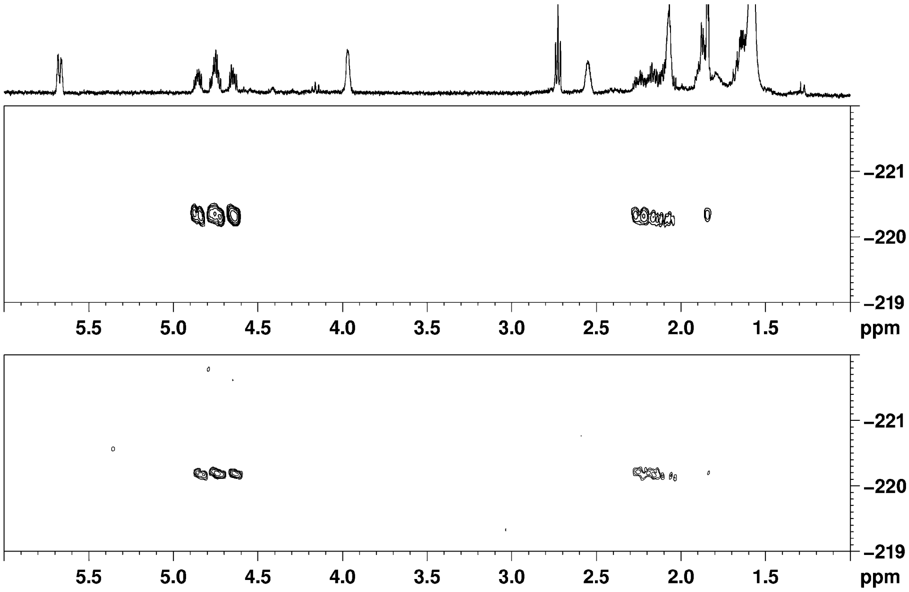

Figure 5. ^{1}H/^{19}F-HMBC (500 MHz, $CDCl_3$) correlation spectra. The upper trace is the spectrum of a reference sample of fluorosalinosporamide. The lower spectrum is a focused section illustrating fluorosalinosporamide in the *S. tropica salL*$^-$ *flA*$^+$ extract grown in limiting fluoride. The fluorine atom is coupling to three sets of protons with chemical shifts consistent with the FCH_2CH_2CH-R fragment.

Table 1. Effect of pH on Fluorosalinosporamide Yields

pH of culture broth	fluorosalinosporamide yield (%)
5.5	– (poor growth)
6.0	100 ± 8[a]
7.0	18 ± 5
8.0	8.5 ± 2

[a] 100% is equivalent to 4 mg/L.

step at 72 °C (10 min). The desired fragment was then gel purified using the QIAQuick gel extraction kit (Qiagen) and used for replacement of *salL* in fosmid BHXS1782 by PCR targeting as previously described.[9,13] Gene replacement in the fosmid was confirmed by PCR and sequencing using primers PflAseq_f (GAG AAG CCT GCG GTT CGT G) and PflAseq_r (GA ACT GCA GGT CGA CGG ATC). *S. tropica* mutants were confirmed by PCR.

RT-PCR Analysis. RNA was isolated from cells collected from a second-generation *S. tropica* A1 liquid culture grown for 67 h (late exponential phase) using the RiboPure-Bacteria kit (Ambion). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as previously described.[10] Primers used in the PCR step to interrogate *flA* transcription were PflArt_f (GAG ATC GTC CGC TTC AAC C) and PflArt_r (TGA GGT AGA TGG CGA TGT TG). Absence of genomic DNA contamination in the RNA samples and cDNA quality were assessed using 16S rRNA primers.[10]

Analysis of Fluorosalinosporamide Production. Best yields of fluorosalinosporamide production were obtained by culturing the *S. tropica salL*$^-$ *flA*$^+$ mutant in A1 pH 6.0 for two days (early exponential phase) before adding 2 mM of potassium fluoride. XAD 7 resin (Sigma) was added at the time of fluoride supplementation and extracted with acetone after two weeks. The dried extract was then subject to EtOAc–H_2O partition and the organic phase analyzed by HPLC with a Phenomenex C18 column (150 × 4.6 mm; 5 μm particle size) at a flow rate of 0.7 mL min^{-1}, using MeCN (B) in H_2O (A) as mobile phase (0 to 30% B in 5 min, then 30% B for 20 min, 30 to 35% B in 5 min, 35% B for 8 min, 35 to 100% B in 5 min, 100% B for 5 min) and monitoring at 210 nm. Fluorosalinosporamide identity was confirmed with an authentic standard by LC/(+)ESIMS ($[M + H]^+$: *m/z* 298).[11]

Salinosporamide A Detection in the *S. tropica salL*$^-$ *flA*$^+$ Mutant. A dried crude extract of the *S. tropica salL*$^-$ *flA*$^+$ mutant grown in A1 seawater-based medium (no fluoride added) was fractioned by silica gel flash chromatography using increasing concentrations of acetone in CH_2Cl_2 as mobile phase. Fractions containing salinosporamides (eluted at 90:10 CH_2Cl_2–acetone) were combined, dried *in vacuo*, and analyzed by LC/(APCI)-MS using a Michrom Magic C-18 column (150 × 1.0 mm, 5 μm particle size, 200 Å pore size) and a gradient of 7.5 to 97.5% MeCN in H_2O (50 μL/min flow rate) as mobile phase (salinosporamide A retention time of 18.5 min, *m/z* 314 and 316, 3:1, $[M + H]^+$).

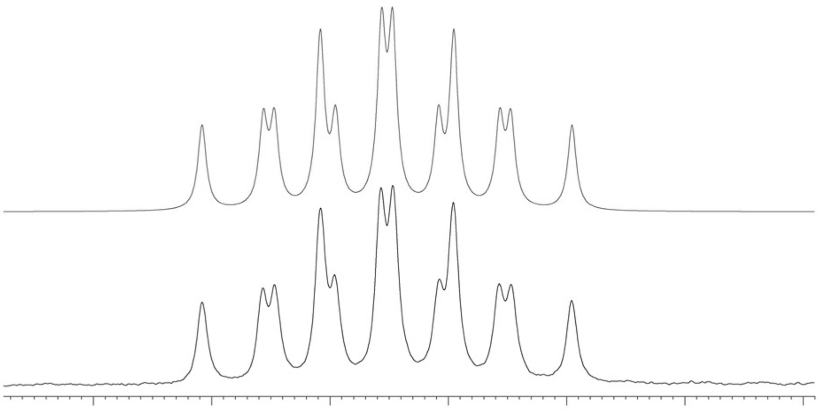

Figure 6. Simulation of the ^{19}F NMR spectrum of fluorosalinosporamide (upper trace) compared to the experimental spectrum (lower trace). $^2J(^1H,^{19}F) = 47.0$ Hz (2 equivalent H), $^3J(^1H,^{19}F) = 28.8$ and 24.3 Hz (2 nonequivalent diastereotopic H).

^{19}F NMR Analysis. The dried acetone extracts of *S. tropica salL$^-$ flA$^+$* were resuspended directly in $CDCl_3$, and ^{19}F NMR (and HMBC) spectra were run on a Bruker AV-500 MHz instrument (^{19}F at 470.33 MHz). ^{19}F NMR spectra were recorded with and without proton decoupling. ^{19}F NMR chemical shifts were calculated relative to $CFCl_3$. Figure 6 shows that the experimental ^{19}F NMR fluorosalinosporamide spectrum matches well with a DAISY simulation[28] of the spectrum (−220.7 ppm; $^2J_{H, F} = 47.0$ Hz, 2 equivalent H, and $^3J_{H, F} = 28.8$ and 24.3 Hz, 2 nonequivalent diastereotopic H).

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