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Supporting Information

69451 Weinheim, Germany

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Production of fluorosalinosporamide by mutasynthesis

Alessandra S. Eustáquio and Bradley S. Moore

Supplementary Methods

Chemicals. 5'-FDA (**7**) and 5-FDR were kindly provided by M. Onega and D. O'Hagan (University of St. Andrews) and synthesized as described[1;2]. Salinosporamide A (**2**) was a gift from W. Fenical (UCSD). Fluorosalinosporamide (**1**) and salinosporamide B (**3**) were obtained and/or purified in this study, respectively. All other chemicals were of analytical grade.

Bacterial strains and culture conditions. The *salL*$^-$ mutant of *Salinispora tropica* CNB-440 was constructed by PCR targeting as described[3] and routinely cultured in 50 mL A1 medium (10 g of starch, 4 g of yeast extract, and 2 g of peptone per liter seawater) in 250 mL flasks containing a stainless steel spring. Cultivation was carried out at 28 ºC and 200 rpm for three to four days. For analysis of secondary metabolites, 2 mL of a 3-4 day old pre-culture were inoculated into 50 mL of A1 medium containing 1% KBr, 0.4% Fe_2SO_4 and 0.1% $CaCO_3$. Cultivation was carried out at 28 ºC and 200 rpm for one day before adding 0.5 – 1 g of XAD7 resin and then continued for 5–6 days. 5'-FDA or 5-FDR were added at the same time as the XAD7 resin at a concentration of 10 mg L^{-1}.

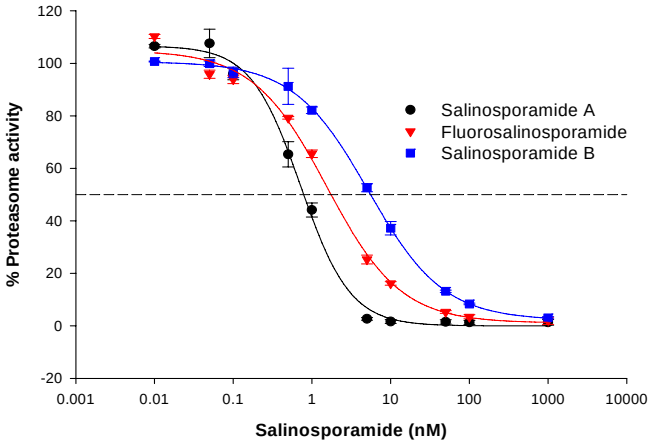
Analysis of secondary metabolites. For analytical work, mutants and the wild-type strains of *S. tropica* were cultured as described above. The XAD7 resin was collected and extracted with 25 mL acetone. The crude extract was dried, redissolved in 2 mL MeCN, filtered, then analyzed by HPLC and/or LC/(+)ESI-MS with a phenomenex C18 column (150 × 4.6 mm; 5 µm particle size) at flow rate of 0.7 ml min^{-1}, using following MeCN (B) in water gradient: 0% B for 1 min, 0-35% B over 7 min, isocratic 35% B over 11min, 35-100% B over 8 min, with detection at 210 nm.

Isolation and characterization of fluorosalinosporamide. Crude extract from a 3 L culture of the *S. tropica salL*$^-$ mutant supplemented with 30 mg of 5-FDR was fractionated by silica gel (20 g) vacuum column chromatography eluting with increasing amounts of acetone in dichloromethane, i.e. eleven 50-ml fractions starting with 100% CH_2Cl_2 and increasing the acetone amount stepwise by 10%. Fraction number four eluting with 30% acetone was purified by RP-HPLC [Prep Hydro RP C18, 250 mm × 21.20 mm, 10 µm particle size, flow rate 13 ml min^{-1}, detection at 210 nm, isocratic 35% MeCN over 30 min] to afford fluorosalinosporamide A (**1**) (t_R = 21 min, 4.5 mg). Inspection by LC/MS showed that the compound was however partially degraded during isolation (hydrolysis of the β–lactone characterized by a $[M+H_3O^+]^+$ peak) and was therefore further purified using a pre-packed silica column (1g, Alltech) and the following stepwise gradient of acetone in CH_2Cl_2: i) 10 ml 100% CH_2Cl_2, ii) 2 × 5 ml 95% CH_2Cl_2, 5% acetone, iii) 10 ml 90% CH_2Cl_2, 10% acetone, iv) 10 ml 75% CH_2Cl_2, 25% acetone, v) 10 ml 100% acetone. Fractions ii (second 5-ml fraction) and iii were combined to afford

fluorosalinosporamide A (**1**) (1 mg): white solid; ESI-MS *m/z* 298 $[M+H]^+$; HR ESI-TOF-MS *m/z* 298.1445 [expected for $C_{15}H_{20}NO_4F^+$, 298.1449]; NMR data recorded on a Varian Inova 500-MHz spectrometer, see Supplementary Table S1 and Figures S2-S3.

Isolation of salinosporamide B. Salinosporamide B (**3**) was isolated during purification of fluorosalinosporamide as described above and its identity confirmed by LC/ESI(+)MS in comparison to authentic standard[4].

Proteasome inhibition assays. Proteasome inhibition assays were carried out using yeast 20S proteasome and the fluorogenic substrate Suc-LLVY-AMC for chymotrypsin-like activity, both from Biomol International, LP. Assay conditions were adapted from ref.[5;6]. Serial dilutions of each inhibitor were added in duplicates to 0.5 nM proteasome in assay buffer (25 mM Tris-HCl pH 7.5, 0.5 mM EDTA, and 0.03% SDS) and incubated at 37 ºC for 15 min. The 96-well plate was placed on ice, and substrate was added to a final concentration of 40 µM. Plates were incubated in the dark at 37 ºC for 30 min, and then placed on ice. Proteasome activities were measured by reading the fluorescence of the cleaved substrate at 355 nm (excitation) and 460 nm (emission) at 37 ºC using Spectra Max M2 (Molecular Devices). IC_{50} values (compound concentration at which 50% maximal relative activity is inhibited) were calculated using SigmaPlot software and a standard four parameter sigmoidal fit curve, i.e. "Logistic, 4 Parameter" (Supplementary Figure S1). Two independent experiments were carried out, and IC_{50} values were obtained using the mean of all collected data sets ± standard deviation. Reversibility assays were carried out using 1 µM inhibitor and 1 nM proteasome. Substrate was either added as described above (standard assay) or after loading the mixture to a protein filter (Microcon 100 kDa MW cut off, Millipore) previously equilibrated with assay buffer, centrifuging for 3 min at 11,000 ×*g*, washing twice with assay buffer to eliminate excess inhibitor, and reconstituting the proteasome in assay buffer.



Supplementary Figure S1. *In vitro* 20S proteasome inhibition by salinosporamides. The obtained IC_{50} values (i.e. compound concentration at which 50% maximal relative activity is inhibited, dashed line) are displayed in Table 2.

Cytotoxicity assay. The colorimetric assay used to assess growth inhibition is based on the reduction of the tetrazolium salt MTS − in the presence of phenazine methosulfate (PMS) − by living cells to a formazan product which can be followed by measuring the optical density (OD) at 490 nm[7]. Human colon carcinoma cell line HCT-116 was plated on 96-well plates at a concentration of 2.5×10^4 cells ml^{-1} McCoy's 5A medium and incubated overnight at 37 ºC and 5% CO_2. Serial dilutions of test compounds were added in triplicates, and the plates further incubated at 37 ºC and 5% CO_2 for 72 hours, before MTS/PMS indicator solution was added. After incubation at 37 ºC and 5% CO_2 for 3 hours, the OD at 490 nm was measured on an Emax precision microplate reader (Molecular Devices). IC_{50} values (the compound concentration that allows 50% cell survival compared to the control) were calculated using the Softmax Pro 2.4 software and a standard sigmoidal four-parameter dose-response fit curve. Two independent experiments were carried out. IC_{50} values were obtained using the mean of the collected data sets ± standard deviation.

References

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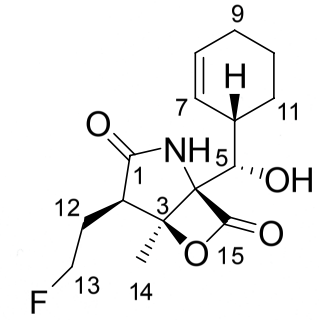
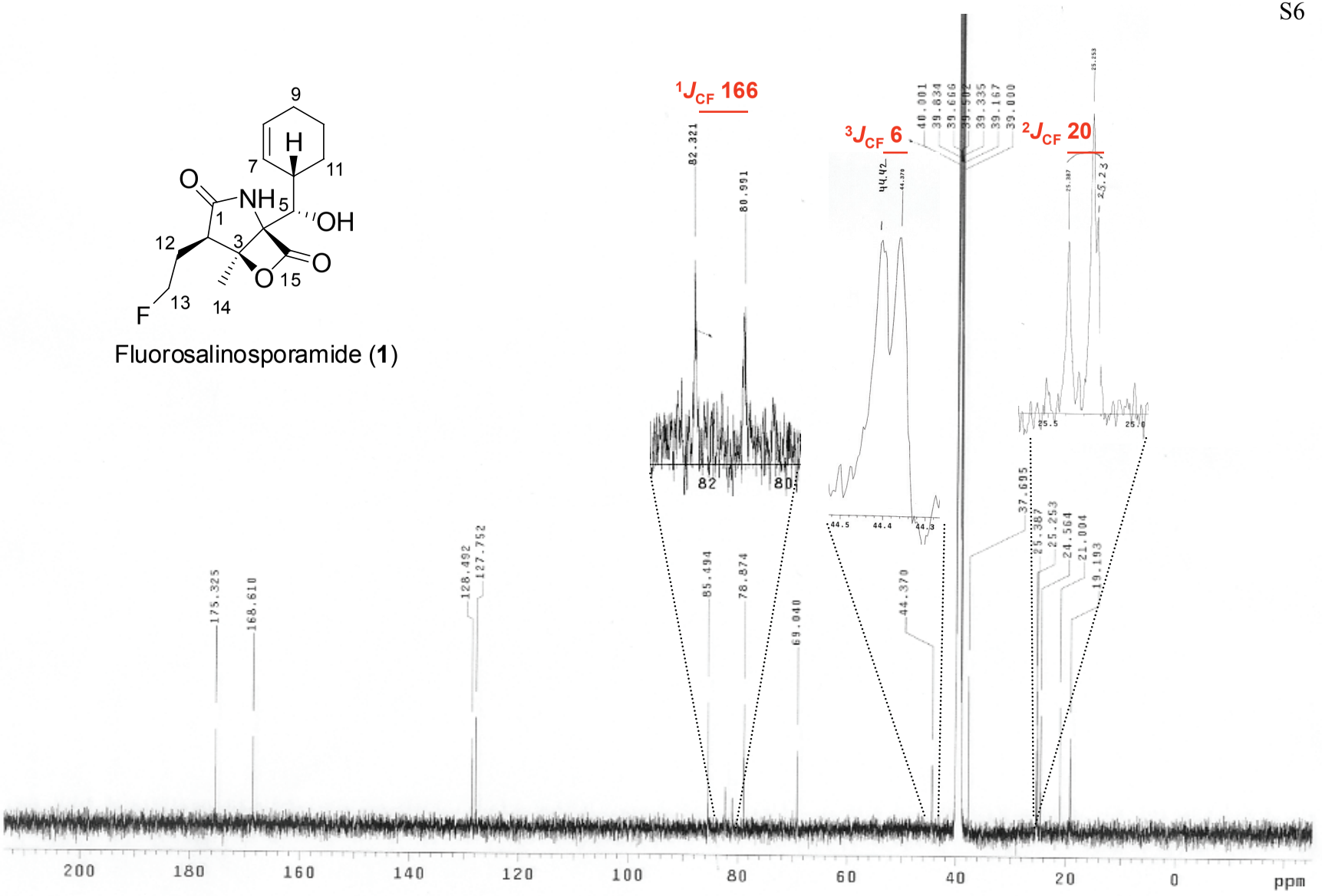
Supplementary Table S1. NMR spectral data for fluorosalinosporamide (**1**) in DMSO-d_6.

C/H #	δ_H (*J* Hz)	δ_C
1	---	175.3
2	2.59, br t (7)	44.4 d ($^3J_{CF}$ 6)
3	---	85.5
4	---	78.9
5	3.67, br t (8)	69.0
6	2.28, m	37.7
7	5.80, br d (10.5)	128.5
8	5.71, dq (2.5; 9.5)	127.8
9	1.91, m	24.6
10a	1.70, m	21.0
10b	1.40, m	
11a	1.82, m	25.2
11b	1.22, m	
12	1.94, dm ($^3J_{HF}$ 23)	25.3, d ($^2J_{CF}$ 20)
13	4.67, dm ($^2J_{HF}$ 47)	81.7, d ($^1J_{CF}$ 166)
14	1.73, s	19.2
15	---	168.6
NH	9.07, s	---
OH	5.53, d (7.5)	---



Fluorosalinosporamide (**1**)

Supplementary Figure S2. ^{1}H NMR spectrum (500 MHz) of fluorosalinosporamide (**1**) in DMSO-*d6*.



Supplementary Figure S3. ^{13}C NMR spectrum (125 MHz) of fluorosalinosporamide (**1**) in DMSO-*d6*.