Halogenation

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Mutasynthesis of Fluorosalinosporamide, a Potent and Reversible Inhibitor of the Proteasome**

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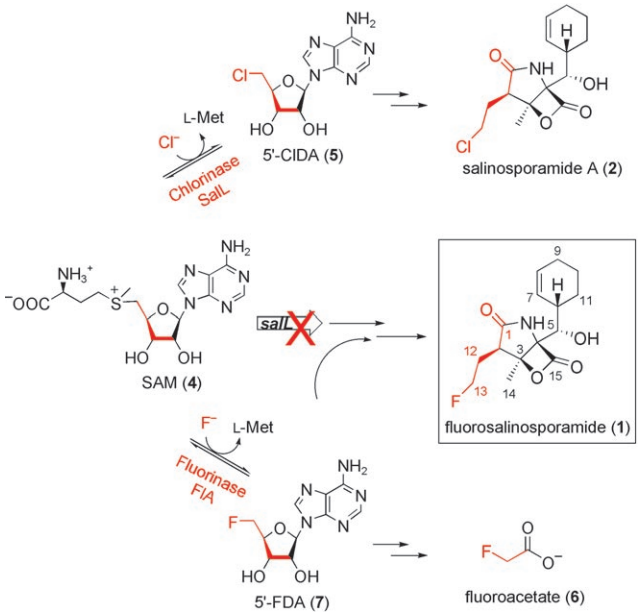
In memory of Richard E. Moore

Fluorinated compounds make up approximately 20% of all drugs in the market, which reflects the favorable effect of fluorine substitution on pharmacological and pharmacokinetic properties of lead compounds.[1] However, natural organofluorides are the least abundant of all halogenated natural products and are exceedingly rare.[2] Fluorine substituents are rather generally introduced by chemical synthesis.[1] Herein, we report the production of fluorosalinosporamide (**1**, Scheme 1) by a rational combination of genetic engineering and precursor-directed biosynthesis.

Salinosporamide A (**2**, Scheme 1), a chlorinated natural product from the marine bacterium *Salinispora tropica*, is a potent proteasome inhibitor currently in development for the treatment of multiple myeloma and other cancers.[3–5] Besides the major analogue **2**, *S. tropica* also accumulates the deschloro compound salinosporamide B (**3**), which is however at least two orders of magnitude less active against tumor cell lines.[6] This large difference in bioactivity is reflective of the respective mechanisms of the two compounds. With **2**, after the drug becomes covalently tethered to the 20S proteasome by attack of the catalytic threonine residue on the electrophilic β-lactone unit, the resulting 3-hydroxy group displaces the 13-chloro group to yield an irreversibly bound adduct.[7] The absence of a leaving group at the C13 position in deschloro compound **3** renders a less stable complex that is prone to hydrolysis. Hence, the nature of the C13 substituent greatly influences the reactivity of the salinosporamide molecule and its residence time on the proteasome.[7]

Production of salinosporamide analogues containing halogen atoms other than chlorine has been attempted by replacing seawater in the production medium with sodium halides.[8] While this strategy was successful for the generation of bromosalinosporamide, the same result could not be achieved with NaI or NaF, which was toxic to the bacterium.[8] Iodosalinosporamide was ultimately produced semisynthetically from **2**,[9] whereas the generation of fluorosalinosporamide has not yet been reported. Structure–activity relationship studies[9] revealed that the bromo and iodo analogues were just as active as **2** due to the similar reactivity of these halogens. The bioactivity of **1**, however, is anticipated to differ from that of the other halogenated salinosporamides since the fluoro group in **1** is likely to be maintained. Hence, we sought a method to prepare **1** for biological evaluation.



Scheme 1. Biosynthesis of salinosporamide A (**2**) by *Salinispora tropica* and of fluoroacetate (**6**) by *Streptomyces cattleya*. Fluorosalinosporamide (**1**) was generated from 5′-FDA in a *salL*⁻ mutant of *S. tropica*. SAM: *S*-adenosyl-L-methionine; 5′-ClDA: 5′-chloro-5′-deoxyadenosine; 5′-FDA: 5′-fluoro-5′-deoxyadenosine.

We recently reported that the salinosporamides are products of a hybrid polyketide synthase–nonribosomal peptide synthetase (PKS-NRPS) and that the four-carbon units comprising C1/C2/C12/C13 in **2** and **3** have different biosynthetic origins involving SAM (**4**) and butyrate, respectively.[10–12] In the biosynthesis of **2**, the chlorinase SalL[12] halogenates SAM to generate 5′-ClDA (**5**; Scheme 1), in a rarely observed nucleophilic substitution strategy analogous to that of the fluorinase in the fluoroacetate (**6**) producer *Streptomyces cattleya*.[13] SalL also accepts bromide and iodide as substrates, which provides the molecular basis for the in

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Supporting information for this article (detailed experimental methods and NMR spectra) is available on the WWW under http://www.angewandte.org or from the author.

vivo production of bromosalinosporamide from NaBr supplementation, but it does not accept fluoride. The substrate discrimination of subsequent pathway enzymes may account for the failure observed with NaI. Moreover, it is clear now that SalL's halide specificity would prevent fluoride substitution by this fermentation method, even in nontoxic concentrations.

However, the discovery of common committed steps in the biosynthesis of **2** and **6** opened the door for a rational mutasynthetic approach. Administration of synthetic 5′-FDA [14] (**7**) to a *salL*-knockout mutant of *S. tropica*[12] devoid of **2** led to the production of a new salinosporamide derivative (**1**), as detected by LC/(+)ESIMS (Figure 1). Interestingly, 5-fluoro-5-deoxyribose[15] (5-FDR) also supported the biotransformation to form **1**, which strongly suggests that its chlorinated analogue is a biosynthetic precursor of **2**.

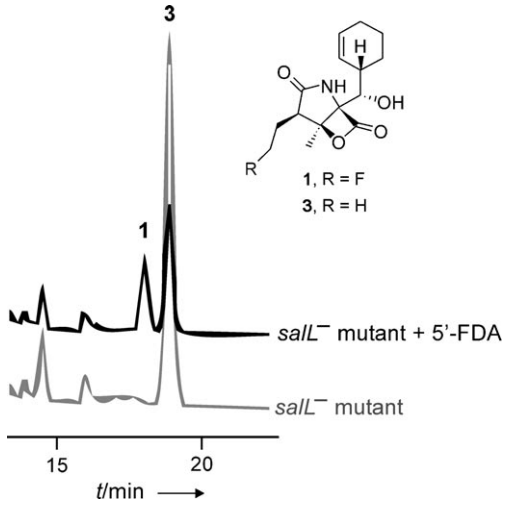

Figure 1. HPLC chromatograms of culture extracts of the *salL*⁻ mutant and of the *salL*⁻ mutant supplemented with 5′-FDA showing production of fluorosalinosporamide (**1**) and salinosporamide B (**3**).

Workup of a 3 L fermentation supplemented with 30 mg of 5-FDR yielded 4.5 mg of **1** with the molecular composition $C_{15}H_{19}NO_4F$, as revealed by high-resolution MS (*m*/*z* calcd for $[M+H]^+$: 298.1449; found: 298.1445). The ^1H and ^{13}C NMR spectra of **1** were nearly identical to those reported for **2**[16] except for anticipated differences in the chemical shifts and coupling constants associated with carbon atoms 2, 12, and 13; these results unequivocally confirmed the presence of fluorine at the C13 position.

We next examined the biological activity of the new salinosporamide analogue **1** in comparison with those of **2** and **3** in inhibition assays against purified yeast 20S proteasome. Moreover, the cytotoxicity of the three compounds was tested ex vivo against the human-colon-carcinoma cell line HCT-116 (Table 1). While the replacement of the chlorine atom in **2** by a fluorine atom slightly reduces purified proteasome inhibition by a factor of 2 and cytotoxicity by a factor of 20, these activities are increased 3- and 30-fold, respectively, relative to those of deschloro **3**.

Table 1: IC_{50} values for purified 20S proteasome inhibition and cytotoxicity of the HCT-116 cell line.[a]

Compound	Proteasome inhibition [nM]	Cytotoxicity [nM]
salinosporamide A (**2**)	0.7 ± 0.05	9.5 ± 1.6
fluorosalinosporamide (**1**)	1.5 ± 0.05	210 ± 21
salinosporamide B (**3**)	5.2 ± 0.34	6100 ± 300

[a] ± standard deviation. The observed IC_{50} values for **2** and **3** are in the same order of magnitude as those previously reported.[3,16]

The high energy necessary to break the C–F bond strongly suggests that, unlike the mechanism with **2**, the halide will not be displaced by the 3-hydroxy group and that binding of **1** will be reversible.[7] Indeed, we provide experimental proof for the reversible binding of **1**, based on the recovery of proteasome activity in a wash-out assay, in comparison with the irreversible binding of **2** (Figure 2). The data support enhanced

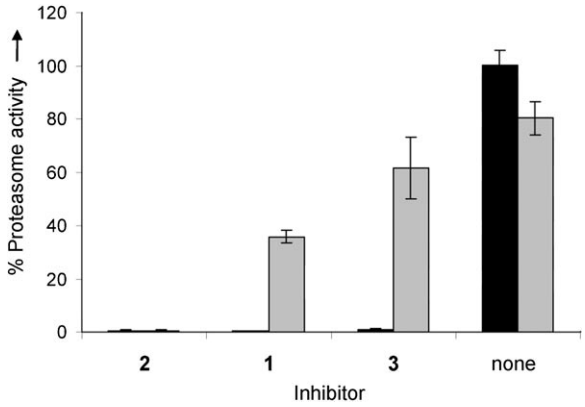

Figure 2. Reversibility of fluorosalinosporamide binding. Standard (black) and wash-out (gray) assays were performed with purified 20S proteasome. See the Experimental Section for details.

binding of **1** versus that of **3**, which is indicative of the beneficial effects of fluorine substitution.[17] A close look into the proteasome active site with bound **2**[7] reveals potential candidates (Thr21, Tyr168, and Ser129) for H-bond formation with the fluorine atom of **1** that may explain its increased activity compared with that of **3**.

Polyketides represent one of the major classes of biologically active natural products. Although various mutasynthesis examples exist for this compound class, they primarily include variations of the starter units.[18,19] The biosynthesis of **1**, on the other hand, targets the introduction of a halogenated extender unit for the first time. As halogenation often increases the biological activity of drugs,[20] as in the case of salinosporamides, the newly discovered SAM-dependent halogenation pathways[12,13] may prove to be valuable tools for the generation of bioactive polyketides.

Experimental Section

1: Crude extract[10] was fractionated by silica gel vacuum chromatography with acetone in dichloromethane and further purified by

reversed-phase HPLC (Prep Hydro RP C18 column, 250 × 21.20 mm, 10 μm, flow rate of 13 mL min^{-1}, detection at 210 nm, isocratic 35% MeCN over 30 min) to afford **1** (t_R = 21 min) as a white solid: ^{1}H NMR (500 MHz, [D$_6$]dimethylsulfoxide): δ = 9.07 (s, NH), 5.80 (br d, J = 10.5 Hz, H7), 5.71 (dq, J = 9.5, 2.5 Hz, H8), 5.53 (d, J = 7.5 Hz, OH), 4.67 (dm, $^2J_{HF}$ = 47 Hz, H13), 3.67 (br t, J = 8 Hz, H5), 2.59 (br t, J = 7 Hz, H2), 2.28 (m, H6), 1.94 (dm, $^3J_{HF}$ = 23 Hz, H12), 1.91 (m, H9), 1.82 (m, H11a), 1.73 (s, H14), 1.70 (m, H10a), 1.40 (m, H10b), 1.22 ppm (m, H11b); ^{13}C NMR (125 MHz, [D$_6$]dimethylsulfoxide): δ = 175.3 (C1), 168.6 (C15), 128.5 (C7), 127.8 (C8), 85.5 (C3), 81.7 (d, $^1J_{CF}$ = 166 Hz, C13), 78.9 (C4), 69.0 (C5), 44.4 (d, $^3J_{CF}$ = 6 Hz, C2), 37.7 (C6), 25.3 (d, $^2J_{CF}$ = 20 Hz, C12), 25.2 (C11), 24.6 (C9), 21.0 (C10), 19.2 ppm (C14).

Proteasome inhibition assays were carried out by using yeast 20S proteasome and the fluorogenic substrate *N*-succinyl-Leu-Leu-Val-Tyr-(7-amino-4-methylcoumarin) for chymotrypsin-like activity (Biomol International, LP).[21,22] For reversibility assays, 1 nM proteasome was incubated with 1 μM inhibitor for 15 min at 37 °C; substrate was then either added immediately (standard assay) or after loading the mixture onto a protein filter (Microcon, 100 kDa molecular-weight cut off), centrifuging for 3 min at 11000 *g*, washing twice with assay buffer to eliminate excess inhibitor, and reconstituting the proteasome in assay buffer (wash out assay).

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