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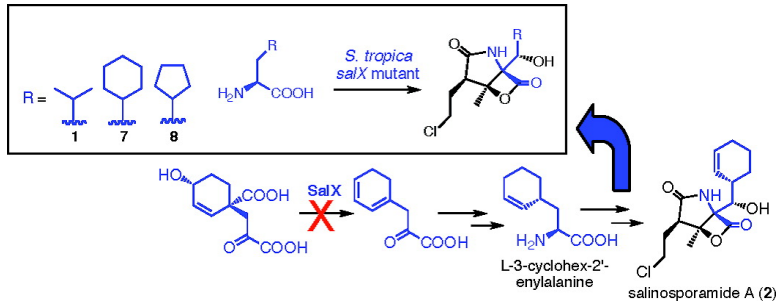

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Engineered Biosynthesis of Antiprotealide and Other Unnatural Salinosporamide Proteasome Inhibitors

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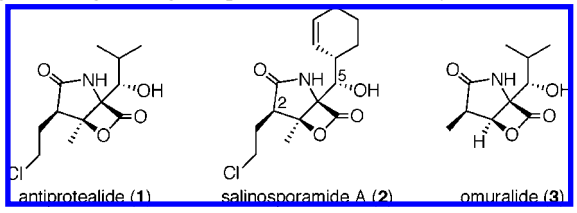
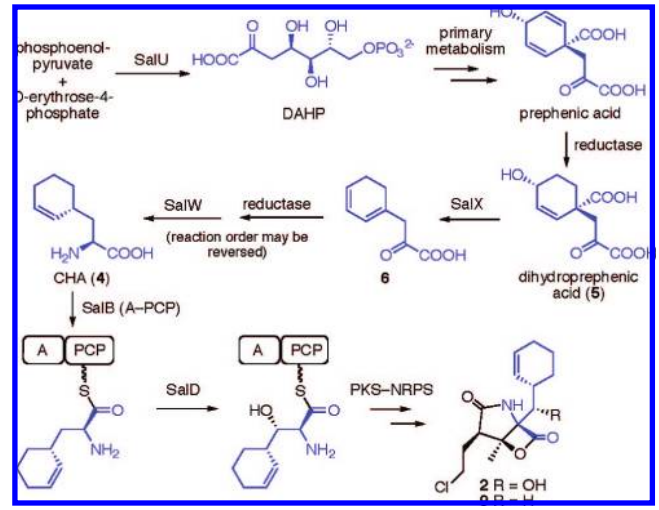
Antiprotealide[1] (**1**) is a low nanomolar synthetic proteasome inhibitor inspired by two β-lactone natural products in which the cyclohexenyl ring of salinosporamide A[2] (**2**) was replaced with the isopropyl functional group of omuralide[3] (**3**). The mechanistically important C2 chloroethyl side chain on the lactam ring of **2**, which is key to its irreversible binding to the 20S proteasome,[4] is retained in **1**, while the C5 isopropyl substitution alters its binding affinity and proteasomal β-subunit selectivity. Owing to these favorable properties, **1** has emerged as a new biochemical tool in cellular biology. Previous studies on the biosynthesis of salinosporamide A[5] and omuralide (indirectly through its precursor lactacystin[6]) suggest that **1** may be alternatively accessible through biotechnology in a suitable host organism from three precursors, namely chlorobutyrate, acetate, and leucine. While the latter two are common intermediates of primary metabolism, chlorobutyrate is unique to the salinosporamide-producing marine actinomycete *Salinispora tropica*.[7] This observation and the recent availability of its genome sequence[8] made *S. tropica* CNB-440 an attractive host for the bioengineering of **1**. Herein, we report a simplified approach to generate not only **1**, but other unnatural salinosporamide derivatives with C5 modifications, by employing a combination of genetic engineering and precursor-directed biosynthesis.[9]



Isotopic experiments established the biosynthetic origin of the building blocks to **2** as acetate, a chlorobutyrate unit, and the novel nonproteinogenic amino acid L-3-cyclohex-2′-enylalanine (CHA, **4**), which directly correlate to the distinctive side chains on its trisubstituted γ-lactam-β-lactone nucleus.[5] Bioinformatic analysis of the 41-kb salinosporamide biosynthetic gene cluster[8] (*sal*) revealed a subset of genes putatively involved in the biosynthesis of CHA via a shunt in the phenylalanine pathway presumptively at the stage of prephenic acid (Scheme 1).[5] As with other secondary metabolic pathways involving a shikimate-derived shunt product,[10] the *sal* locus encodes a pathway specific DAHP synthase (SalU) to initiate shikimate biosynthesis. At the stage of prephenic acid, the CHA pathway presumably deviates via reduction to give dihydroprephenate (**5**) followed by decarboxylative dehydration to the diene **6**. Further reduction and transamination would provide CHA (Scheme 1). The *sal* locus harbors genes consistent with these transformations, including the prephenate dehydratase homologue *salX* and the aliphatic L-amino acid aminotransferase *salW*. Assimilation of the CHA unit into **2** is facilitated by nonribosomal peptide synthetase biochemistry in which

Scheme 1. Proposed Biosynthesis of the L-3-cyclohex-2′-enylalanine (CHA, **4**) Building Block of Salinosporamide A (**2**) in *S. tropica*



amino acid selection and activation is conducted by the adenylation−peptidyl carrier protein (A−PCP) didomain SalB protein. Subsequent β-hydroxylation of the tethered substrate is putatively catalyzed by the cytochrome P450 SalD, which belongs to a family of amino acyl-PCP β-hydroxylases.[11] The β-hydroxy CHA unit is then primed for condensation with the diketide product of the bimodular polyketide synthase SalA protein for final assembly to **2** (Scheme 1).

While the amino acid specificity code of the SalB A domain is unique and likely correlates with the novelty of CHA, it most closely aligns with known A domains specific for L-Phe and L-Tyr.[12] Thus in order to bioengineer **1** in *S. tropica*, we first had to eliminate the background biosynthesis of CHA. To do so, we targeted *salX* for genetic disruption via PCR-based mutagenesis[7] due to its relatedness to other secondary metabolic prephenate dehydratase homologues.[13] Fermentation of the *S. tropica salX*$^-$ disruption mutant revealed its central role in salinosporamide biosynthesis, as LC/(+)ESI-MS analysis of the organic extract clearly showed the complete abolishment of **2** and all other known salinosporamide derivatives. Further inspection of the *salX* mutant revealed a new salinosporamide derivative, which was absent in the wild-type organism. The new analogue was purified and fully characterized by NMR and high-resolution MS. To our surprise and delight, the identity of the new analogue was unequivocally confirmed as our intended target antiprotealide (**1**). Supplementation of the culture with 0.38 mM L-leucine showed a 2-fold increase in the production of **1**, with yields of ~1 mg/L, establishing leucine as the biosynthetic precursor to **1** even though the specificity code of the SalB A domain better correlated with aromatic amino acids. Close analysis of the mutant failed to identify additional structural analogues with alternative C5 substituents derived from the other proteinogenic amino acids, including phenylalanine. As a consequence, the SalB A

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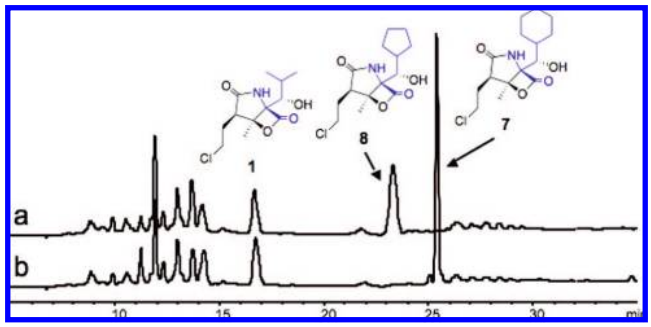

Figure 1. HPLC analysis at 220 nm of organic fractions of S. tropica salX$^-$ supplemented with (a) 0.32 mM DL-3-cyclopentylalanine and (b) 0.29 mM L-3-cyclohexylalanine. All products shown were purified and characterized by NMR and MS analyses.

domain must have a strong preference for aliphatic rather than aromatic amino acid substrates. We thus set out to explore the flexibility of the system to generate additional salinosporamide A C5 derivatives from nonproteinogenic cycloalkyl amino acids.

We first attempted a chemical complementation experiment of the *salX*$^-$ mutant with saturated L-3-cyclohexylalanine since it most closely resembled the presumptive natural precursor CHA (**4**). Analysis of the ensuing fermentation yielded the dihydro analogue of **2**, salinosporamide X1 (**7**), in increased biosynthetic yield at ~5 mg/L with background production of **1** (Figure 1). Biosynthetic **7** was identical in all regards to semisynthetic **7** (NPI-2056), which was alternatively prepared by hydrogenation of natural **2**.[14]

With the successful mutasynthesis of **7**, we sought next to explore the biosynthesis of an unnatural salinosporamide derivative not accessible semisynthetically. Administration of DL-3-cyclopentylalanine to the *salX* knockout mutant yielded a new aliphatic analogue (Figure 1) with the anticipated mass of *m*/*z* 301 as shown by LC/MS analysis. The new analogue, salinosporamide X2 (**8**), was purified and characterized, which unequivocally confirmed the presence of the cyclopentane ring in **8**. The biosynthetic yield of **8** at ~1 mg/L was 5× lower than **7** yet comparable to **1**, suggesting that the natural substrate specificity of the *sal* biosynthetic enzymes is paramount in the production of these unnatural variants.

The biosynthesis of **1**, **7**, and **8** provided analogues consistently hydroxylated at C5, suggesting that since we did not encounter corresponding unoxidized products, the associated hydroxylase must have broad substrate tolerance. To confirm our initial assumption that SalD is indeed the oxygenase responsible for this hydroxylation chemistry, we similarly disrupted the 1278 bp *salD* gene in *S. tropica*. Once again the disruption mutant lost its ability to produce **2** with concomitant production of a new analogue (**9**). LC/MS analysis showed a molecular ion $[M+H]^+$ at *m*/*z* 298 for **9**, consistent with the loss of an oxygen atom in comparison to **2**. Analogue **9** was purified and characterized, which confirmed its structure as 5-deoxy **2** (salinosporamide J) that was independently produced by large-scale fermentation of wild-type *S. tropica* NPS000465.[15] The mutant produces **9** at >10× over the parental strain, which enabled us to readily generate **9** in a small-scale fermentation. The data further proves the function of SalD as the β-hydroxylase responsible for the oxidation of the CHA residue (Scheme 1).

The availability of the salinosporamide analogues provided us the opportunity to further explore the importance of the C5 substituent toward proteasome inhibition. We evaluated **1**−**2**, **7**−**9** both in vitro against the yeast 20S proteasome chymotrypsin-like activity (β5-subunit) and ex vivo against the human colon carcinoma cell line HCT-116 (Table 1). All changes exacted to the C5 cyclohexenyl or hydroxy units in **2** resulted in reduced potency confirming the importance of these groups in proteasome binding. Antiprotealide (**1**) with the smallest

Table 1. IC_{50} Values for Inhibition of Chymotrypsin-like Activity of Purified Yeast 20S Proteasome β5-Subunit and Cytotoxicity of the Cancer Cell Line HCT-116

inhibitor	proteasome (nM)	cytotoxicity (μM)
antiprotealide (**1**)	38.2 ± 4.9	0.856
salinosporamide A (**2**)	1.2 ± 0.1	0.005
salinosporamide X1 (**7**)	16.0 ± 1.9	0.649
salinosporamide X2 (**8**)	14.8 ± 0.7	0.282
salinosporamide J (**9**)	20.8 ± 0.8	2.184

C5 side chain was the weakest inhibitor tested in this study, which is supported by structural data showing that larger C5 substituents such as the cyclohexenyl unit of **2** afford additional hydrophobic interactions with the S1 binding pocket of the β5-subunit.[4] This increased residence time in the binding pocket allows for covalent addition by the catalytic *N*-terminal threonine to occur. Salinosporamide X2 (**8**) was the most potent analogue tested here, suggesting that an olefinic derivative as in **2** may have enhanced activity.

In summary, we discovered a new shunt in the phenylalanine pathway to the CHA residue in salinosporamide A (**2**) that through mutagenesis and precursor-directed biosynthesis allowed us to readily access new C5 structural derivatives in the salinosporamide family of potent proteasome inhibitors. By this method, we were able to produce biosynthetically a previously synthetic analogue (**1**), a semisynthetic analogue (**7**), as well as a novel analogue (**8**). Together with our recent ability to engineer C2 derivatives in the "chlorobutyrate" fragment by a similar strategy,[16] the door is wide open to further engineer new *sal*-based proteasome inhibitors with enhanced potency and altered specificity.

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Supporting Information Available: Materials and methods, NMR and proteasome bioactivity data for **1**, **7**−**9**, and HPLC chromatogram for the *salD* mutant. This material is available free of charge via the Internet at http://pubs.acs.org.

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