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Effects of Halogens on the Production of Salinosporamides by the Obligate Marine Actinomycete *Salinispora tropica*

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Abstract We examined the effects of halogens on the production of salinosporamide A (NPI-0052) by the obligate marine actinomycete *Salinispora tropica* NPS465, specifically the production of analogs containing halogens other than chlorine. Adding NaF, NaBr and NaI directly to the production medium prepared in seawater containing ~3% NaCl did not induce the production of the corresponding analogs. Replacing seawater with 2~3% NaI in the production medium enhanced the production of NPI-0052 by 2.1 fold. Replacing seawater with 2~3% NaBr in the production medium suppressed the production of NPI-0052 but induced the production of a brominated analog at very low yield. Using a stepwise enrichment of bromide in the seed cultures in order to reduce the chloride ion carried over to the production medium, the production of the brominated analog was enhanced by 4 fold. We also demonstrated that the growth of this obligate marine actinomycete is dependent upon sodium concentration, not chloride concentration.

Keywords NPI-0052, salinosporamide A, *Salinispora tropica*, marine actinomycete, halogens

Introduction

Salinosporamide A (NPI-0052, Fig. 1), a product of the obligate marine actinomycete, *Salinispora tropica* [1~3], is a highly potent inhibitor of the 20S proteasome [3~5]. It is currently undergoing Phase I clinical studies for the treatment of various cancers. NPI-0052 consists of a γ-lactam-β-lactone core structure similar to omuralide [6] and JS360 [7]. However, there are several significant structural differences that distinguish NPI-0052 from these structurally related proteasome inhibitors. One such difference is that while omuralide and JS360 do not possess any halogen atom, NPI-0052 contains a chlorine atom at the ethyl side chain. The chemical diversity of halogenated metabolites is impressive [8], and is particularly important in the commercial production of many antibiotics such as chlorinated metabolites vancomycin [9] and chlortetracycline [10], and brominated compound brodimoprim [11]. Different halogens possess different degrees of electronegativity [12], and incorporation of different halogens into NPI-0052 may change its potency and/or spectrum of activity. For example, it has been determined that the presence of bromine in the commercial antibiotic brodimoprim facilitated its penetration into the Gram-positive bacterial cells more readily than the non-brominated analog [13]. Furthermore, we have demonstrated that salinosporamides containing halogens have a spectrum of biological activity that is distinct from their non-halogenated congeners [14, 15]. During our studies on the biosynthesis of NPI-0052, we examined the effect of halogens on the production of NPI-0052, specifically the production of analogs containing halogens other than chlorine.

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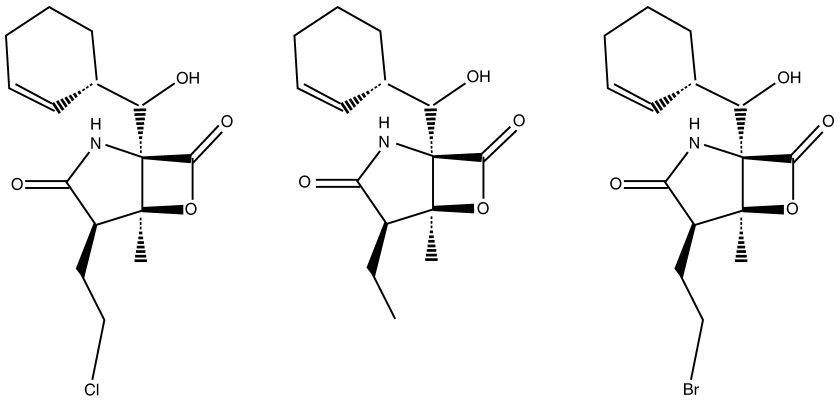

NPI-0052 NPI-0047 NPI-2053

Fig. 1 Structures of salinosporamides NPI-0052, NPI-0047 and NPI-2053.

Materials and Methods

Microorganism

The producing strain, *Salinispora tropica* NPS465, was isolated from a sediment sample collected from Cross Harbor, Abaco, Bahamas, by Jensen, Dwight and Fenical [16]. Strain NPS465 was deposited with the American Type Culture Collection and assigned the accession number PTA-5275.

Media and Culture Conditions

To prepare an inoculum for shake flask culture, a frozen stock culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of glucose 0.4%, Bacto tryptone 3%, Bacto casitone 0.5% in seawater. The seed culture was incubated at 28°C and 250 rpm on a rotary shaker. After 3 days, 5-ml aliquots were transferred to 500-ml Erlenmeyer flasks containing 100 ml production medium, prepared using soluble starch 1%, yeast extract 0.4%, Bacto peptone 0.2%, ferric sulfate 0.004%, potassium bromide 0.01% and calcium carbonate 0.1% in seawater. After 1 day incubation of the production culture at 28°C and 250 rpm, 2 g Amberlite XAD-7 resin was added to the production culture. The production culture was returned to the shaker and further incubated at 28°C and 250 rpm for an additional 4 to 7 days before analysis for the production of salinosporamides. For the NaBr enrichment study, seawater in the seed cultures was replaced by 3% NaBr in deioinized water. The seed cultures were incubated at 28°C and 250 rpm until good growth yields were observed before inoculating into the next seed medium or production medium. Seawater in the production medium was also replaced by 3% NaBr in the enrichment study.

Growth Analysis

The growth of the culture was determined by centrifuging 10 ml of culture in a 15-ml centrifuge tube at 3000 rpm for 15 minutes in a Beckman centrifuge (Allegra model 6). The growth was expressed as % packed cell volume defined as volume of packed cell/volume of culture ×100%.

Extraction and Analytical Methods

The production of salinosporamides in the fermentation was monitored by HPLC using an ACE C-18 reversed-phase column (4.6×150 mm). A step gradient solvent system was employed which consisted of water (0.01% TFA) as solvent A and acetonitrile (0.01% TFA) as solvent B. The elution was started at 100% solvent A for 1 minute, 100% solvent A to 100% solvent B linear gradient in 16 minutes, held at 100% solvent B for 9 minutes at a flow rate of 1 ml/minute with the detector wavelength set at 210 nm. The fermentation extracts for HPLC analysis were prepared by extracting 3.5 ml of culture broth with an equal volume of ethyl acetate for 1 hour. A 1 ml aliquot was evaporated to dryness under a stream of nitrogen and redissolved in 320 μl of DMSO. Five μl of the extract was used for HPLC analysis. For LC-MS analysis of the crude extract, a Micromass Q-TOF2 mass spectrometer was used with a Waters Symmetry column (2.1×150 mm, 3.5 μm) using the same mobile phase above except replacing TFA with 0.05% formic acid.

Table 1 Effects of halide ions on the production of salinosporamides in seawater-based seed and production media

Seawater	Halogen added	NPI-0052* (mg/liter)	NPI-0047* (mg/liter)	%PCV**
100% (~3% NaCl)	0	57	7	4
	1% NaF	0	0	0
	2% NaF	0	0	0
	1% NaBr	38	6	4
	2% NaBr	10	2	3.5
	1% NaI	61	7	4
	2% NaI	21	6	4

* Titers of NPI-0047 and NPI-0052 were determined on day 4 and day 7. Maximum titers were detected on day 7.
** %PCV (percentage packed cell volume) was determined on day 7.

Results

Effects of Halide Ions on the Production of Salinosporamides in Seawater-based Seed and Production Media

The salinosporamide-producing strain, NPS465, is an obligate marine actinomycete that requires seawater or 3% NaCl in the culture medium for optimal growth and production of NPI-0052 [1, 3]. The NaCl requirement complicated the design of the halogen feeding study. Table 1 summarizes the production of salinosporamides in the seawater medium upon adding 1% or 2% of NaF, NaBr or NaI to the production medium. Seawater contains about 3% NaCl [1]. Adding 1% or 2% NaF, NaBr or NaI directly into the seawater medium did not induce the formation of the corresponding halogenated analogs. Addition of NaF, both at 1% and 2%, to the seawater production medium inhibited the growth of the organism completely. One percent and 2% NaBr inhibited the production of NPI-0052 by 33% and 83%, respectively, without affecting the growth of the organism. The major metabolite produced under these conditions was NPI-0052. The other salinosporamide metabolite produced was salinosporamide B (NPI-0047, Fig. 1), the deschloro-analog of NPI-0052 [17]. NaI did not affect NPI-0052 production at 1% concentration but inhibited the production of NPI-0052 by 63% at the higher NaI concentration without affecting the growth of the organism.

Production of Salinosporamides in Production Media Containing Different Halide Ions

In order to enhance the production of analogs, seawater in the production medium was replaced by 2% or 3% of NaF, NaBr or NaI to increase the ratio of other halogens to chloride. NaCl was also tested to examine the effectiveness of NaCl to replace seawater for the growth of the organism and the production of salinosporamides. Table 2 summarizes the production of salinosporamides in these media. Again, there was no growth in the NaF media. In the NaCl media, the final growth yield was the same as the seawater medium, at 4.5 to 5% PCV. The production of NPI-0052 in the NaCl media was about 72% of the seawater medium, even though growth was not affected, indicating that additional supplements other than NaCl are required to replace seawater in order to achieve the optimal NPI-0052 production. The production of NPI-0047 was similar in NaCl medium and seawater medium.

There was no visible growth in the media using NaBr or NaI to replace seawater during first 7 days of incubation. Upon further incubation, growth was observed and the production of NPI-0052 and NPI-0047 was detected in both media. After 14 days of incubation, the final growth yield (4.5 to 5%) in these media was the same as the seawater medium. A comparison of the NaI and seawater media showed the production of NPI-0052 and NPI-0047 to be significantly higher (2.1 and 6.1 fold, respectively) in the NaI medium. However, no iodo-analog was detected in the fermentation by HPLC and LC-MS analyses.

The production of NPI-0052 was reduced by 69-78% in the NaBr media; however, the production of NPI-0047 was increased by 2.6~4.9 fold. In addition to the production of NPI-0047 and NPI-0052, a new salinosporamide was detected in the production media containing NaBr. LC-MS analysis of the extract indicated that the new analog contains a bromine atom but no chlorine atom. The production of the new bromo-analog, designated NPI-2053 (bromosalinosporamide, Fig. 1), was very low (4.9 mg/liter) and was about 30% of NPI-0052 in the 3% NaBr medium.

Table 2 Effects of halide ions on the production of salinosporamides in sodium halide-based production medium

Seawater or halide ion in production medium	NPI-0052 (mg/liter)	NPI-0047 (mg/liter)	NPI-2053 (mg/liter)	%PCV
Seawater (~3% NaCl)*	54	7	—	4.5
2% NaF**	0	0	—	0
3% NaF**	0	0	—	0
2% NaCl*	39	5	—	5
3% NaCl*	36	9	—	4.5
2% NaBr**	17	34	4.7	4.5
3% NaBr**	12	18	4.9	4.5
2% NaI**	111	43	—	5
3% NaI**	101	67	—	4.5

* Titers of NPI-0047 and NPI-0052 were determined on day 4, 7 and 10. Maximum titers were observed on day 10. %PCV was determined on day 10.

** Titers of NPI-0047, NPI-0052 and new bromo analog, NPI-2053, were determined on day 10 and day 14. Maximum titers were observed on day 14. %PCV was determined on day 14.

Table 3 Effect of bromide ion on the production of salinosporamides after two cycles of bromide-enrichment in seed cultures

Production cycle (day)	NPI-0052 (mg/liter)	NPI-0047 (mg/liter)	NPI-2053 (mg/liter)	%PCV
4	0.2	2.7	4.7	3.5
5	0.7	24.7	12.3	4.5
6	0.9	38.7	18.3	5
7	1.2	51.9	19.4	5
9	1.1	80.3	17.5	5

Effect of Bromide Ion on the Production of Salinosporamides after Two Cycles of Bromide-enrichment in Seed Cultures

We examined the possibility of improving the production of the new analog, NPI-2053 in the fermentation by implementing additional bromide enrichment steps. The rationale was to reduce the amount of chloride, the precursor for NPI-0052 that can be carried over from the seed medium to the production culture. After growing strain NPS465 in the first seed culture in seawater medium for 3 days to achieve a healthy inoculum, the first seed culture was transferred to the second seed medium containing 3% NaBr in deionized water instead of seawater. The growth rate of strain NPS465 in the second seed culture growing in NaBr medium was very slow. It took 9 days before adequate cell mass (2% PCV) was observed. The second seed culture was inoculated into the third seed medium with the same composition of the second seed medium (3% NaBr). The growth rate in the third seed culture (4 days to achieve good growth of 4% PCV) was better than the second seed culture. After adapting the growth of the organism in successive two seed stages using NaBr medium, the third seed culture was inoculated into the production medium containing 3% NaBr in deionized water. Since the third seed culture was grown in the medium that contained a high concentration of bromide but a very low concentration of chloride, the carried over chloride from the seed culture to the production medium was negligible (estimated at 65 μM).

Table 3 summarizes the production of NPI-0052, NPI-0047 and NPI-2053 in the NaBr production medium after two cycles of enrichment of bromide in the seed medium. There was a 4-fold increase in the production of NPI-2053, the bromo-analog, at a concentration of 19.4 mg/liter in the fermentation of the enrichment scheme as compared to the condition with no enrichment (4.9 mg/liter, Table 2). Most

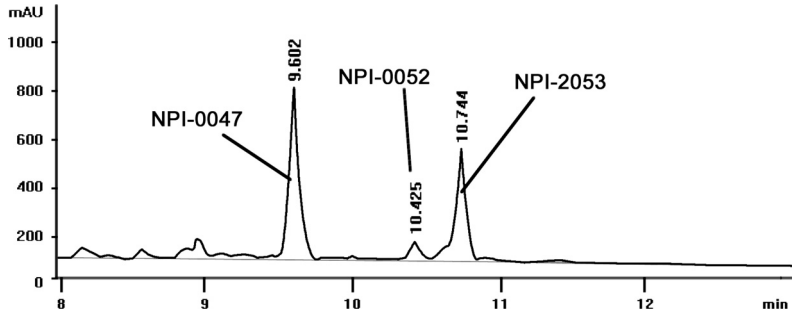

Fig. 2 HPLC chromatogram of extract of enrichment scheme with two stages of seed medium replacing seawater with 3% NaBr and production medium with 3% NaBr in deionized water.

significantly, the production of NPI-0052 was reduced close to the base line level at 1.2 mg/liter in the enrichment condition. The production of NPI-0047 was gradually increased from day 4 to day 9, and was the major salinosporamide metabolite present in the fermentation, at 80.3 mg/liter. Fig. 2 shows the metabolite production profile by strain NPS465 grown in production medium containing 3% NaBr after two cycles of enrichment of bromide in the seed media. The major product was NPI-0047 (retention time 9.6 minutes), followed by the new analog NPI-2053 (retention time 10.7 minutes) and NPI-0052 (retention time 10.4 minutes) as a minor analog in the fermentation.

Physico-chemical Properties of NPI-2053 (Bromosalinosporamide)

The new analog, bromosalinosporamide (NPI-2053) was isolated from the above bromide-enrichment scheme. The isolation and structure elucidation have recently been reported elsewhere [14]. Briefly, the crude extract was purified by Si flash chromatography with a EtOAc/hexanes step gradient. The fraction enriched in NPI-2053 was further purified by RP HPLC using an isocratic solvent system consisting of 35% CH_3CN/65% H_2O. The structure was determined using spectroscopic methods including 1-D and 2-D NMR and mass spectrometry analyses such as HR-APCIMS (Fig. 1); the physico-chemical properties are summarized in Table 4. The molecular formula was calculated from the HR-APCI MS measurement. The molecular ion contained an isotopic ratio of 1 : 1 for $[M+H]^+$ peaks at *m/z* 358 and *m/z* 360, appropriate for a compound containing one bromine atom and no additional halogens. Both ^1H and ^{13}C NMR assignments for the compound compared well with assignments for NPI-0052, except that the chemical shift for C-13 of NPI-2053 was 10 ppm upfield compared to NPI-0052, consistent with replacement of chlorine with bromine.

Table 4 Physico-chemical properties of bromosalinosporamide (NPI-2053)

Appearance	White power
MW	358.23
Molecular formula	$C_{15}H_{21}BrNO_4$
HRAPCIMS (*m/z*)	358.0647 ($M+H^+$)
UV λ_{max} nm (log ε) in CH_3CN	220 (sh) (3.05), 205 (3.25)
IR ν_{max} cm^{-1} (NaCl)	3365, 2918, 1811, 1702

Discussion

From this study, we determined that the growth of the obligate marine actinomycete *Salinispora tropica* NPS465 was not dependent on chloride ion concentration since we observed the same growth yield when replacing the seawater with NaBr or NaI in the production media, even though the growth rates were slower in the replacement media. This supports the claim that obligate marine microorganisms require a certain concentration of sodium for growth and that the chloride ion concentration is not as critical as the sodium ion [1]. It is observed that replacing seawater by NaCl in the production medium led to reduced production of NPI-0052 without affecting the growth of the organism. Therefore, the seawater growth requirement can be fulfilled by NaCl, however, the production of secondary metabolites is more stringent than the growth requirement. Additional nutrients will be needed in addition to NaCl for optimal production of NPI-0052.

NaF inhibited the growth of the organism at 1 to 2% concentration. In contrast, NaI not only replaced seawater in the production medium to support growth of the organism, it also increased the production of NPI-0052 by 2.1-fold. Furthermore NPI-0052 retains its chlorine atom and no iodo-analog was detected. Iodide-enhanced production of secondary metabolites that do not contain

iodide in the molecules has been reported [18, 19]. We do not know the mechanism of improved production of NPI-0052 by iodide. Since there are several biosynthetic reactions involving transient halogenated intermediates while the final products are devoid of halogens [20~23], it is conceivable that the biosynthesis of NPI-0052 may be facilitated by an iodinated intermediate.

Bromine of inorganic origin has been found to be incorporated in place of chlorine in the microbial metabolites chlortetracycline [24], griseofulvin [25], pyrrolinitrin [26], actaplanin [27] and rebeccamycin [28] as a result of supplementing bromide in the fermentation medium. Adding bromide in the medium at about the same concentration of chloride supported the production of the above brominated antibiotics, with titers higher than (about double) the corresponding chlorinated metabolites. The halogenating enzymes that carry out the above bromination/chlorination reaction are haloperoxidases [29, 30]. The energy for the bromination process by haloperoxidases is more favorable than the chlorination process [29]. It is surprising that we did not observe any production of the brominated salinosporamide by *Salinispora tropica* strain NPS465 when 2% NaBr was added to the seawater medium (~3% NaCl). It is even more surprising to observe that upon replacing the seawater by 3% NaBr in the production medium, the production of NPI-0052 (chlorinated metabolite) was 3.7-fold higher than NPI-2053 (brominated analog) even though the molar ratio of bromide to chloride in the production medium was 11 : 1. The majority of the chloride in the production medium was carried over from the seed medium. Using a stepwise NaBr enrichment scheme to reduce the carried over chloride in the production medium, the production of NPI-2053 was increased to 16-fold higher than NPI-0052. The estimated molar ratio of bromide to chloride in the production medium was 4000 : 1. Eliminating one enrichment seed stage reduced the molar ratio of bromide to chloride in the production medium to 200 : 1. This modification lowered the production of NPI-2053 by 66% and increased the production of NPI-0052 by 10-fold as compared to the two-cycle enrichment scheme (data not shown). The increase in ratio of NPI-2053 to NPI-0052 in the fermentation using the two-cycle enrichment scheme facilitated the purification and structure determination of NPI-2053. NPI-2053 is a novel brominated analog of NPI-0052 in which chlorine at the ethyl side chain is replaced by bromine. NPI-2053 has similar biological activities as NPI-0052 [14, 15].

It is clear from the above finding that the halogenating enzyme responsible for the production of NPI-0052 and NPI-2053 from the obligate marine actinomycete *Salinispora tropica* strain NPS465 is different from the terrestrial haloperoxidases. Significant advancement has been made in the understanding of biological halogenation reactions and the enzymes involved during the last few years [8, 30]. New halogenating enzyme systems have been reported [31]. It is of considerable interest to determine what type of halogenating enzyme is involved in the production of NPI-0052 by the obligate marine actinomycete *Salinispora tropica* strain NPS465. This may help explain the differences in production of secondary metabolites by microorganisms from marine and terrestrial environments.

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