Production of Tuberactinamine A by *Streptomyces griseoverticillatus* var. *tuberacticus* NRRL 3482 Fed with (*S*)-2-Aminoethyl-L-cysteine

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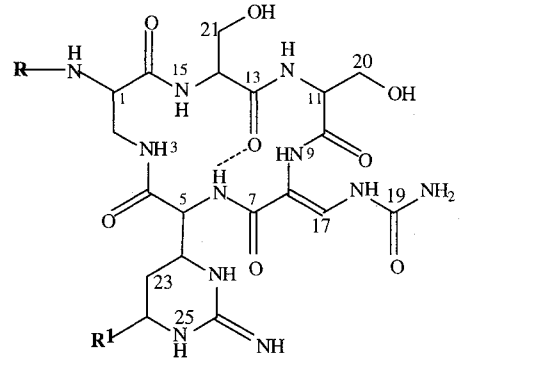
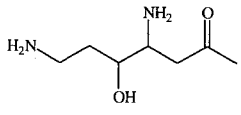
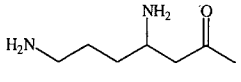
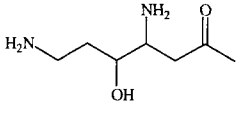
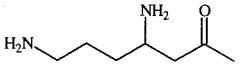
In this paper, we report a new method of tuberactinamine A (**1**) production accomplished by adding an inhibitor of lysine-2,3-aminomutase, (*S*)-2-aminoethyl-L-cysteine (AEC), to the production stage fermentation of *Streptomyces griseoverticillatus* var. *tuberacticus* NRRL 3482. Other workers have shown tuberactinamine A production in a *Nocardia* species, Lederle culture BM547, and in *Streptomyces* sp. 106[1,2]. The strain used in our experiments, NRRL 3482, is known to produce predominantly tuberactinomycin A (**2**), as well as low levels of tuberactinomycins B (viomycin) (**3**), N (**4**), and O (**5**)[3~5]. The structures of these compounds are shown in Figure 1. In general, tuberactinamine A differs from the tuberactinomycins in that it lacks a β-lysine side chain.

Fig. 1. Structures of tuberactinomycins A, B (viomycin), N, O, as well as tuberactinamine A.



	R	R^1
Tuberactinamine A (**1**)	H	OH
Tuberactinomycin A (**2**)		OH
Tuberactinomycin B (viomycin) (**3**)		OH
Tuberactinomycin N (**4**)		H
Tuberactinomycin O (**5**)		H

Based upon this structural difference, we hypothesized that fermenting NRRL 3482 in the presence of (*S*)-2-aminoethyl-L-cysteine (AEC) would result in the production of tuberactinamine A. AEC is known to be a potent inhibitor of lysine-2,3-aminomutase, the enzyme responsible for converting L-lysine to L-β-lysine[6]. In addition, radiolabeling studies done by CARTER *et al.* have shown that the L-β-lysine side chain of viomycin is derived from L-lysine[7]. Therefore, we reasoned that if the γ-hydroxy-β-lysine side chain of tuberactinomycin A was derived from L-β-lysine, then the addition of AEC would inhibit its formation and tuberactinamine A would accumulate as the final product. This hypothesis was demonstrated experimentally.

To test the effect of AEC addition on the production profile of *S. griseoverticillatus* var. *tuberacticus* NRRL 3482, this strain was patched onto YPD agar (1% Difco yeast extract, 1% Difco Bacto peptone, 0.5% dextrose, 1% MOPS, and 1.7% Difco Bacto agar, pH 7.0) from a spore stock stored in 20% glycerol at -20°C. The patch was grown at 28°C for 5 days. After 5 days, a 6 mm diameter plug of the patch was inoculated into 30 ml seed medium (2% soybean meal, 1% corn meal, 2% corn steep liquor, 0.6% $NaNO_3$, and 0.02% KH_2PO_4, pH 7.7) in 300 ml Erlenmeyer flasks and incubated at 29°C, 200 rpm, 48 hours. After 48 hours, the contents of the seed flasks were pooled and 2.5 ml of the pooled cultures were inoculated into 25 ml production medium (3% nutrisoy flour, 3% corn starch, 1.5% NaCl, and 2% dextrose, pH 7.0) in 250 ml Erlenmeyer flasks. AEC was made up to 100 g/liter, filter-sterilized through a 0.2 μm filter, and added either before inoculation of the production stage or at 24 hours into production. The cultures were incubated at 29°C, 200 rpm, for a total of 4 days.

In order to determine whether added AEC had shifted the production profile of the organism from compound **2** to **1**, the samples were harvested and assayed by HPLC. To harvest, the fermentation broth was clarified by centrifugation, and 0.5 ml of the clarified broth was added to 9.5 ml of the mobile phase composed of 50 mM

KH_2PO_4, 0.4 M NaCl (pH to 3.5 with H_3PO_4). Chromatography was carried out using a Thermo Separation Products 3200 pump and detector. Samples were injected at 20 μl onto a Nest Group Polysulfoethyl Aspartamide column (4 mm × 200 mm, 5 μm), eluted at a flow rate of 0.75 ml/minute, and monitored at 240 nm. Following HPLC assay, the chromatograms of AEC-supplemented cultures were compared to the un-supplemented controls. From these results, it was evident that the addition of AEC altered the production profile of the organism. Without added AEC, **2** was the predominant product. When AEC was added at 2 g/liter immediately before inoculation, **2** was only made at low levels and a novel component thought to be compound **1** was observed.

To confirm that the new product was indeed **1**, the compound was isolated using a two-stage purification that consisted of ion exchange chromatography and preparative HPLC. The fermentation broth was clarified by a combination of filtration and centrifugation. The clarified broth was then loaded onto IRC-76 in a mixture of the Na^+ and H^+ form. After washing the column with H_2O and 0.3 N acetic acid, compound **1** was eluted with 0.1 N H_2SO_4. The eluent was subsequently neutralized with IRA-93 resin in the basic form and desalted twice by precipitating in an excess of methanol and filtering. The desalted product was concentrated to approximately 100 ml using a rotary evaporator followed by drying to completion in a vacuum oven. The product was purified further using preparative HPLC on a Waters Millennium System. Samples were monitored at 270 nm using a Waters 996 diode array detector. For recovery of **1**, the dried sample was dissolved in 0.1% TFA and filtered through a 0.2 μm filter. The sample was injected onto an Inertsil C8 column (10 mm × 250 mm, 5 μm) and eluted with 0.1% TFA at a flow rate of 3.5 ml/minutes. The fraction containing **1** was concentrated to approximately 3 ml using a rotary evaporator followed by drying to completion *in vacuo*.

Purified compound **1** was analyzed by Fast Atom Bombardment (FAB) from an MNBA (metanitrobenzoic acid) matrix using Peak Matching technique on a Concept Mass Spectrometer by Kratos. The data obtained ($[M+H]^+$ *m/z* 558 and $[M-H_2O+H]^+$ *m/z* 540) are consistent with the molecular formula of tuberactinamine A, $C_{19}H_{31}N_{11}O_9$, with a calculated MW of 557. In addition, the compound exhibited an absorbance at 267 nm that is characteristic for the vinyl urea group of the cyclic peptides in this class. To confirm the FAB-MS results, ^{13}C NMR spectra were obtained on **1** using a Varian Unity Plus 400 MHz spectrometer with D_2O as a solvent and with dioxan added as an internal reference. Table 1 lists the signals obtained on the putative tuberactinamine A product. The carbon spectrum showed signals that closely matched 18 of the 19 reported for **1** by McGAHREN *et al.* ($\Delta\delta=0.16$)[1]. The signal reported for the urea carbon, C-19, was not observed at the reported value of δ 156.32. However, a signal was observed at δ 154.32. Based upon the chemical shifts reported for other tuberactinomycins at C-19 (tuberactinomycin A = δ 154.35, tuberactinomycin B = δ 154.26, and LL-BM547β = δ 154.26)[1], it is reasoned that the chemical shift of 154.32 represents the urea carbon.

Table 2 summarizes the ratios of tuberactinamine A to tuberactinomycin A produced by NRRL 3482 under the different conditions tested. When AEC was added at 2 g/liter immediately prior to inoculation of the production stage, the tuberactinamine A to tuberactinomycin A ratio was 36±6. Tuberactinomycin A was

Table 1. ^{13}C NMR data obtained on the purified tuberactinamine A product (δ in ppm, dioxan was used as an internal standard).

Carbon	Tuberactinamine A
23	30.07
2	40.36
22	46.79
1	51.73
5, 11, 14	54.93, 55.38, 57
20	59.75
21	63
24	70.78
8	105.62
17	135.98
19	154.32
26	157.35
7	167.97
16	168.02
13	171.56
4	172.8
10	173.24

Table 2. A summary of the tuberactinamine A to tuberactinomycin A ratios obtained when AEC was added at varying levels.

[AEC] added in g/liter	Time of AEC addition	Tuberactinamine A to tuberactinomycin A ratio
0	—	0.07±0.004
2	Immediately before inoculation	36±6
2	24 hours into production	1.8±0.4

only produced at low levels. Adding AEC at 24 hours into the production stage resulted in higher levels of tuberactinomycin A and lower tuberactinamine A to tuberactinomycin A ratios (1.8 ± 0.4). These results would be expected because the absence of inhibitor during the first 24 hours would allow L-lysine to be converted to L-β-lysine, thus providing the precursor needed to form tuberactinomycin A. Adding AEC at 24 hours would block further formation of L-β-lysine, subsequently allowing tuberactinamine A to accumulate. Therefore, we conclude that the addition of AEC to the fermentation of NRRL 3482 results in the production of tuberactinamine A by blocking the synthesis of the precursor necessary to form the final product, tuberactinomycin A.

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