Published in final edited form as:

Org Process Res Dev. 2013 February 15; 17(2): 265–272. doi:10.1021/op3003294.

A practical and scalable manufacturing process for an anti-fungal agent, Nikkomycin Z

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Abstract

A scalable and reliable manufacturing process for Nikkomycin Z HCl on a 170 g scale has been developed and optimized. The process is characterized by a 2.3 g/L fermentation yield, 79% purification yield, and >98% relative purity of the final product. This method is suitable for further scale up and cGMP production. The *Streptomyces tendae* ΔNikQ strain developed during the course of this study is superior to any previously reported strain in terms of higher yield and purity of Nikkomycin Z.

Keywords

Nikkomycin Z

Introduction

Nikkomycin Z (**1**) acts as an anti-fungal agent by blocking the enzyme responsible for the production of chitin, a building block in fungal cell walls.^{1–5} Chitin synthase is absent in humans and animals making this compound an attractive agent for antifungal drug discovery research. It was first isolated from the fermentation broth of *Streptomyces tendae* Tu901 by Dahn and coworkers in 1976; the structure was determined by chemical degradation and mass spectroscopy.⁶

In the past two decades, Nikkomycin Z has been extensively studied and found active against certain endemic mycoses such as blastomycosis, histoplasmosis and coccidioidomycosis.⁷ Coccidioidomycosis, also known as valley fever, is an orphan disease occurring primarily in the southwestern US.^{8,9} The disease was reported also outside the endemic area in places like Australia¹⁰ and Canada¹¹ and, most recently, in Italy.¹² It also occurs in animals such as cats and dogs located in the same geographic areas.¹³ Nikkomycin Z appears to be relatively non-toxic³ with low oral availability in dogs (9.4–13.4%).¹⁴ A phase I clinical trial was reported in 2009.¹⁵

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Notes

The authors declare no competing financial interest.

In the past 15 years, various attempts to bring the compound to the market were made.¹⁶ One impediment was developing a reliable and inexpensive manufacturing process. The primary obstacle was co-expression of structurally isomeric Nikkomycin X (**2**) during fermentation. Separation of Nikkomycin Z and Nikkomycin X was very challenging due to their structural similarity. Several approaches were reported.^{17,18} Synthetic routes to Nikkomycin Z^{19–21} were too complex for implementation on a manufacturing scale.

Deletion of *nikQ*, a gene responsible for the Nikkomycin X synthetic pathway was initially reported by Lauer and co-workers²² improving the bioprocessing selectivity toward the formation of Nikkomycin Z. This approach was further advanced in the recent years.^{23–27} However, a practical and scalable manufacturing process for Nikkomycin Z has not been published. Herein, we report a reliable scalable approach to manufacturing and purification of Nikkomycin Z hydrochloride on a multi-hundred gram scale with high purity and yield.

Results and Discussion

Our initial focus was mutagenesis and selection of an improved Nikkomycin Z overproducer from the *Streptomyces tendae* ΔNikQ 25-2 strain provided by the University of Arizona. Comparison of the wild type *S. tendae* ATCC 31160 and the ΔNikQ 25-2 strain for Nikkomycin Z production in 5L bioreactors showed that the wild type strain made 0.13 g/L while the ΔNikQ 25-2 strain only made 0.11 g/L. No Nikkomycin X was observed in the fermentation broth from the ΔNikQ 25-2 strain, while the wild type ATCC strain made both Nikkomycin Z and Nikkomycin X. Rational screening for strain improvement has proved useful for antibiotic production.^{28,29} The toxic analog, 5-fluorouracil, was chosen for the first selection since the literature reports that feeding uracil resulted in improved Nikkomycin Z production³⁰ and selection of 5-fluorouracil resistant mutants also improved Nikkomycin Z production.²² From the first round of mutagenesis an improved strain *Streptomyces tendae* ΔNikQ 25-2 UMN01E7, was obtained. This mutant produced 1.6 g/L of Nikkomycin Z in 5 L fermentation. This is a 16 fold increase over the starting mutant ΔNikQ 25-2 which only produced 0.1 g/L of Nikkomycin Z on the same scale.

Isolation of ribosomal mutants resistant to streptomycin or rifampicin have also resulted in increased antibiotic overproduction.³¹ Isolation of the streptomycin resistant mutant, *Streptomyces tendae* ΔNikQ 25-2 UMN033G2, did not result in an increase in Nikkomycin titer. This strain still yielded 1.6 g/L Nikkomycin Z in the pilot scale reactor. However, this strain was superior to the UMN01E7 strain due to the increased purity of the Nikkomycin Z (48% vs. 17%) in the broth at the end of fermentation. This production mutant appeared stable since no decrease in Nikkomycin Z titer from fermentations run over a period of 2 years was observed.

A series of fermentation experiments were carried out to further understand the Nikkomycin Z production media. Dissolved oxygen was important for Nikkomycin production. In reactors where the dissolved oxygen was not controlled, the Nikkomycin levels only reached 0.35 g/L. It was found that the amount of glucose and soy grits determined the final cell mass in the reactor, while the formation of Nikkomycin Z was related to final cell mass and the level of mannitol in the medium. Due to the low solubility of mannitol, feeding did not improve the titer. These experiments resulted in a new media formulation. Using the UMN33G2 strain in a 550 L bioreactor, the optimized fermentation process resulted in a final broth that contained 2.3 g/L Nikkomycin Z with 48% purity as determined by HPLC analysis (Figure 2). The improved purity and slight increase in titer made the UMN33G2 strain the obvious choice for the production of Nikkomycin Z. For comparison, recently reported processes resulted in producing 0.3 g/L²⁷, 0.8 g/L²⁴, and 0.88 g/L²⁶ of Nikkomycin Z.

The chromatographic purification consists of five sequential ion exchange and hydrophobic interaction columns followed by acidification, concentration by rotary evaporation, and freeze drying. The process is schematically depicted in Figure 3. The fractions were collected based on the UV absorption at 254 nm.

The goal at this stage was to isolate Nikkomycin Z from the clarified fermentation harvest by developing a scalable process that would result in high purity and yield. Previously published preparative scale purification processes^{17,18} utilized ion exchange chromatography and reversed phase resin and provided initial guidance for this work. Binding and elution was controlled by solution pH for both ion exchange resins and isocratic reversed phase media purifications. The clarified fermentation harvest contained media components such as proteins, mannitol, salts and other compounds, and fermentation/metabolic products including Nikkomycin Z, related Nikkomycins and highly colored pigments. The fermentation harvest was acidic and below the pI of Nikkomycin Z which would allow the product to easily bind to cation exchange resin. Moreover, while binding Nikkomycin Z, cation exchange resin would not bind most other biomolecules in the harvest. These considerations made cation exchange chromatography (Column A) the first logical capture step of purification. It resulted in the removal of protein residue and pigments while recovering 84% of Nikkomycin Z.

To elute Nikkomycin Z from the cation exchange resin, alkaline solutions are required. Our initial experiments demonstrated that ammonium hydroxide produced the yields higher than sodium hydroxide. However, achieving near quantitative yields using ammonium hydroxide would require 10–20 CV. That may represent a scale up challenge by requiring a larger receiving tank to collect the eluate. To avoid that we took advantage of the observation that the elution conditions on the cation exchange column are identical to those that promote binding to anion exchange column, thus allowing coupling the two columns together. Initial testing of this approach on a small scale using two-column tandem was successful. It demonstrated several operating advantages as opposed to isolating the material from the cation exchange column prior to loading it onto the anion exchange column. This approach allows avoiding large intermediate volumes and collecting tanks, adjusting the solution to acidic pH to stabilize Nikkomycin Z during the transition period, followed by pH adjustment back to alkaline conditions to ensure the anion exchange resin binding. Using the anion exchange column (Column B) allows Nikkomycin Z to be eluted sharply with dilute HCl solutions while significantly reducing the intermediate volume. This approach resulted in decreasing the volume from ~100 L to ~20 L while recovering over 99% of Nikkomycin Z.

The coupled ion exchange columns concentrated Nikkomycin Z and other Nikkomycins but did not effectively remove the pigments. Our attempts to use activated charcoal were successful in removing the pigments but resulted in a significant loss of the product. Various chromatographic approaches, such as reverse phase and size exclusion, were considered. Our observations suggested that pigments can be separated from the product over non-functionalized styrenic resins, such as AMBERCHROM™, in an isocratic mode with aqueous based solutions. By loading the anion exchange eluate in volumes equal to or less than the column volume of the AMBERCHROM™ column, isolation of highly pure Nikkomycin Z was possible. This approach required the AMBERCHROM™ load pH to be adjusted to the pI of Nikkomycin Z (pH 6.2) to maximize its interaction with the resin. Our initial experiments revealed that the highly colored pigments initially stained the AMBERCHROM™ column and then slowly eluted during subsequent runs. Cleaning procedures were investigated testing the relative affinity of the target molecule and impurity pigments for AMBERCHROM™ resin. It was found that under acidic conditions, the Nikkomycins flowed through while the pigments tightly bound to AMBERCHROM™. The pigments then could be almost completely removed from AMBERCHROM™ under basic

conditions thus effectively regenerating the flow through column. This allowed for the acidic anion exchange eluate to be filtered through a small volume AMBERCHROM™ column (Column C) to yield a virtually pigment-free solution consisting primarily of Nikkomycins. Our further experiments demonstrated that application of the resulted filtered solution adjusted to pH 6.2 to a larger AMBERCHROM™ fine purification column achieved a very high purity of the recovered Nikkomycin Z. Since the AMBERCHROM™ column required small loading volumes, the filtrate from Column C was first concentrated using another anion exchange column (Column D) with a small bed volume. Then, the eluate was adjusted to pH 6.2 and loaded onto Column E. Color bearing materials bound at the top of the column while a yellow band ran down the column while eluting with deionized water. The fine purification yielded four fractions with purities ranging from 95.8% to 100% as determined by HPLC analysis (Table 1). This solution was carefully acidified to create the monohydrochloride salt of Nikkomycin Z, the volume was significantly reduced by rotary evaporation, followed by the final freeze drying.

The robustness and scalability of the process was demonstrated by similarity of the yields and purities of the target material on two different scales (see Table 2). Both parameters were moderately higher on a 250 L scale as compared to the 50 L scale.

Our study revealed that zwitterionic Nikkomycin Z is marginally stable in neutral aqueous solutions but highly unstable in basic solutions. To avoid decomposition, the fractions selected for further development were acidified with one molar equivalent of 1.0 N hydrochloric acid solution immediately following collection from column E (see Table 1). The aqueous solution was then concentrated by rotary evaporation and freeze dried (see Figure 4).

The material was stable during the concentration and drying processes; its purity, as determined by HPLC, did not change (see Figure 5). The small peaks in Figure 5 at 2.6 and 3.7 min are Nikkomycin Z hydrolysis products (data not shown). The composition of the resultant powder, Nikkomycin Z HCl, also included two molecules of crystalline water. The water content remained stable after attempts to dry the solid for 18 h in an Abderhalden drying pistol over sodium hydroxide pellets at 40 °C under reduced pressure (1.3 mbar).

Conclusion

The described study resulted in a scalable and reliable manufacturing process for Nikkomycin Z HCl on 170 g scale. This process is characterized by a 2.3 g/L fermentation yield, 79% purification yield, and >98% relative purity of the final product. This method is suitable for further scale up and cGMP production. The *Streptomyces tendae* ΔNikQ strain developed during the course of this study is superior to any previously reported strain in terms of higher yield and purity of Nikkomycin Z.

Experimental Section

General

Purification of the final material was carried out using GE Healthcare BioProcess Purification System. Evaporation was performed on Büchi Industrial Evaporator R-220R. Freeze drying was carried out using LyoStar II freeze drying system. The *Streptomyces tendae* ΔNikQ 25-2 strain was supplied by the University of Arizona.

Column and resin specifications

Cation exchange (Column A): Dowex 50w×4 resin, 28 L bed volume (450×500, 17 cm bed height). Anion exchange (Column B): Dowex 1×4 resin, 8 L bed volume (200×500, 25 cm

bed height). AMBERCHROM™ (Column C): AMBERCHROM™ CG161M, 2.5 L bed volume (100×500, 31.8 cm bed height). Anion exchange (Column D): Dowex 1×4 resin, 7.0 L bed volume (140×500, 45 cm bed height). AMBERCHROM™ fine purification (Column E): AMBERCHROM™ CG161M resin, 20 L bed volume (300×500, 28 cm bed height).

Analytical conditions

HPLC was performed on Waters 2695 Alliance Separations Module using Waters Waters 2996 Photodiode Array Detector and Phenomenex Luna C₁₈ Column (100 Å, 150×4.6 mm, 5 µm) using the following conditions: flow rate 1.0 ml/min, injection Volume 10 µL, detection: 280 nm, run time: 18 min, column temperature 35 °C, sample temperature: 4 °C, Solvent A (0.1% trifluoroacetic acid aqueous solution), Solvent B (methanol), gradient t= 0 min 100% Solvent A – 0% Solvent B, t= 13 min 80% Solvent A – 20% Solvent B, t= 13.1 min 100% Solvent A – 0% Solvent B.

Mutagenesis of *Streptomyces tendae* ΔNikQ 25-2

The *Streptomyces tendae* ΔNikQ 25-2 was grown in liquid medium and then spread onto sporulation agar plates. The plates were incubated for 6 d, then the spores harvested from the lawn that covered the plates. The spores were washed from the plates by scraping with a sterile spreader and transferred into a sterile bottle. To collect individual spores, the suspension was then filtered through sterile glass wool, centrifuged, then re-suspended in 20% sterile glycerol and frozen at –80 °C for storage. The spores were tittered by plate counts with concentrations around $2\text{--}5 \times 10^8$ spores/mL generally obtained. After testing several potential antimetabolites, 5-fluorouracil was chosen as the first selection antimetabolite for screening of mutants. The *S. tendae* ΔNikQ 25-2 spores were plated onto bioassay trays containing 7 µg/mL of 5-fluorouracil, treated with UV light, then incubated at 35 °C for 4 d. After the incubation the plates had 300–400 colonies/plate. Colonies were picked and patched onto grids on 150mm agar plates with 5-fluorouracil agar plates, grown and stored for screening. Colonies were screened for Nikkomycin Z production in shake flasks by inoculation into NL1 seed media and incubation at 35 °C and 200 rpm for two days. They were then transferred into production medium and shaken at 27 °C and 200 rpm for five days. After the incubation, the production media flasks were removed from the incubator and sampled. The Nikkomycin Z levels were determined by HPLC. A total of 244 colonies from this UV mutagenesis were screened by this method. A histogram showing the frequency vs. the Nikkomycin Z formation is shown in Figure 6. The wild type *S. tendae* ΔNikQ gave 0.11 mg/mL of Nikkomycin Z in this shake flask screen.

The top three isolates were screened in 5 L bioreactors and compared to the *Streptomyces tendae* ΔNikQ 25-2 strain and the ATCC 31160 strain. The results are shown in the Table 3 below.

Selection of Streptomycin resistant *S. tendae* ΔNikQ 25-2 UMN01E7

A vial of UMN01E7 was removed from the –80 °C freezer and used to inoculate 100 mL SM2 culture in a 500 mL baffled shake flask. The culture was grown overnight at 30 °C and 200 RPM. The culture was then spread onto 150 mm petri dishes containing SM2 +streptomycin 200 µg/mL and allowed to dry. The dry plates were placed at 35 °C for 5 d. The colonies that grew were transferred onto gridded SM2+streptomycin 200 µg/mL petri dishes and placed in the 35 °C incubator for 2 d. Fifty three mutants were screened following the previously established method (Figure 7). The control *S. tendae* ΔNikQ UMN01E7 gave 0.33 g/L of Nikkomycin Z in this shake flask screen.

Production of Nikkomycin Z Cell Bank

A cell bank of the *S. tendae* UMN033G2 was derived from a single colony of UMN033G2 growing on SM2 plus streptomycin plates. The colony was grown in NL1 medium at 35 °C and 200 rpm for 48 h. The dark pigmented culture was centrifuged and supernatant discarded. The pellet was re-suspended in 25 mL of sterile glycerol and distributed into cryovials. The vials were quick frozen in liquid nitrogen for 2–4 min, then stored in a –80 °C freezer.

Seed Flask Preparation, Inoculation and Growth

A 4 L baffled shake flask was filled with 750 mL of seed production medium (yeast extract 20.0 g/L, soluble starch 10.0 g/L, mannitol 30.0 g/L, soy flour 20.0 g/L). The flask was fitted with a foam plug covered with sterilization wrap and sterilized for 60 min in an autoclave at 121 °C. A working cell bank freezer vial containing *Streptomyces tendae* strain UMN033G2, was removed from the –80 °C freezer and allowed to thaw in the biosafety cabinet. Once thawed, the contents were aseptically transferred into 750 mL of sterile seed flask media in a 4 L baffled shake flask. The inoculated culture was incubated at 27 °C and 200 rpm for 35 h. A sample was aseptically removed from the flask and used for purity testing.

Seed Reactor Preparation, Inoculation and Growth

A pH and dissolved oxygen probes were calibrated and placed into the clean 75 L New Brunswick Scientific bioreactor. The vessel was then batched to 50 L with the following ingredients: yeast extract (20.0 g/L), soluble starch (10.0 g/L), mannitol (30.0 g/L), soy flour (20.0 g/L), antifoam KFO 205 (0.8 g/L). The reactor was steam sterilized at 121 °C for 45 min followed by cooling. Quality control samples were taken aseptically to check for sterility. The reactor was then set to and allowed to reach the following run conditions: air flow 0.5 vvm, vessel pressure 5.0 psi, temperature 28.0 °C, agitation rate 300 rpm. The levels of pH and dissolved oxygen were monitored. The bioreactor containing 50 L of the sterile medium was inoculated using a 1.5% (v/v) inoculum from the seed flask culture. The bioreactor culture was allowed to grow for 14.5 h. To achieve the best Nikkomycin titers, the seed reactor culture must be transferred to the production reactor while still actively growing, before the dissolved oxygen begins to rise. A quality control sample was aseptically taken to test for culture purity and wet cell weight. The wet cell weight of the seed reactor culture was about 0.20 g/g.

Production by Fermentation

A pH probe and dissolved oxygen probe were calibrated and placed into the clean empty 550 L DCI-Biolafitte production bioreactor. The vessel was then batched to 390 L with the following ingredients: yeast extract (5.0 g/L), mannitol (100.0 g/L), soy grits (30.0 g/L), soy peptone (5.0 g/L), and antifoam KFO 205 (0.3 g/L). The reactor was steam sterilized at 122 °C for 45 min followed by cooling. A sterile glucose solution (50%, v/v) was added to set a final glucose concentration at 9.6 g/L. Quality control samples were taken aseptically to check sterility. The reactor was then set and allowed to reach the initial run conditions as follows: air flow 0.5 vvm, vessel pressure 5.0 psi, temperature: 27.0 °C, agitation rate 250 rpm, dissolved oxygen 30% (controlled with oxygen supplementation), pH 6.0 (controlled with 4 N sulfuric acid). The sterile medium in the bioreactor was inoculated by aseptically transferring the contents from the seed vessel into the production vessel. An inoculum volume of 11% (v/v) was used. The bioreactor culture was allowed to grow with the pH initially controlled at 6.0 using 4 N sulfuric acid. The dissolved oxygen level was maintained at 30% or higher by supplementation of the air with oxygen. After the initial growth period (9–11 h), the pH begins to decline and the setpoint is changed to 5.2. Once

the production begins, sodium hydroxide (5 N) was used to maintain the pH at 5.2 for the remainder of the fermentation. The fermentation was run for 110 hours. Once the mannitol concentration reached < 10 g/L, the reactor was cooled for harvest. Quality control samples were periodically taken aseptically to check culture purity, wet cell weight, as well as Nikkomycin Z and mannitol levels. The culture was diluted with 150 L of RO water and centrifuged with a CSA8 disc stack centrifuge. The broth was further diluted with RO water washing the bowl prior to ejection resulting in a near 1:1 dilution with RO water. The broth was filtered through 10, 5, and 1 micron spiral wound filters and frozen at -20 °C. The material was then sterile filtered through 0.2 micron filter into sterile bags for shipping. An aseptic sample of the final material was taken for testing. The material contained Nikkomycin Z at 0.8 g/L level and 48% purity.

Cation/Anion Coupled Isolation (Columns A/B)

The cation exchange column was washed with NaOH (1 N; 5 CV). The column was then washed with RO water until the conductivity decreased to 1 mS/cm. The column was then washed with 1 N HCl to condition the column to the acid state followed by washing with RO water until the conductivity fell below 1 mS/cm. The anion column was washed with NaOH (1 N; 10 CV) following with washing with RO water until the conductivity decreased to 1 mS/cm. The cation exchange column was connected to the chromatography system on the standard column skid ports and the anion exchange column was connected to an outlet port. The frozen broth was removed from the -20 °C freezer and allowed to thaw for 30 h. The resulting broth was diluted with RO water (1:1) to reduce viscosity and the resultant fermentation broth (249 L) was centrifuged (1.8 L/min), the supernatant was filtered through a 22µ filter and loaded on the cation exchange column in a down flow orientation at a flow rate of 40 L/h, with the flowthrough going to waste. The cation column was washed (5 CV) in down flow with the wash going to waste. The elution was performed with aqueous ammonium hydroxide (0.2 N; 40–60 L/h) monitored by UV absorbance at 254 nm and the output routed through the anion column to bind the Nikkomycins. When the elution of the cation exchange column was completed, the system was paused and the cation column was disconnected and the anion column put in its place. The anion column was then washed with RO water (3 CV). The elution was continued using 0.2 N HCl (60 L/h). The eluate (86 L) with the UV absorbance at 254 nm was collected, analyzed, and stored overnight at room temperature.

AMBERCHROM™ Filtration (Column C)

The AMBERCHROM™ column was washed with HCl (0.2 N; 3 column volumes) following by washing with RO water (5 CV). The eluate from the previous step was loaded. The column was eluted with RO water (10 CV) and the eluate with the absorption at 254 nm was collected and used for further purification.

Anion Exchange Concentration (Column D)

The second anion exchange column was washed with NaOH (1 N; 10 CV) and then washed with RO water (20 L/h) until the conductivity dropped below 1 mS/cm. The material collected from the previous column was adjusted to pH 8.28 using NaOH solution (5 N) and loaded on the ion exchange column in down flow orientation. The column was eluted with HCl (0.2 N; 60 L/h), two fractions with 254 nm absorbance were collected, analyzed by HPLC and stored at 6 °C. Fraction 1 that contained 3.55 mmol or 99.5% of the collected material, was used for further purification.

AMBERCHROM™ Fine Purification (Column E)

The column was washed 0.5 N NaOH at 38.8 L/h in a down flow orientation. The column was then washed with RO water until the conductivity dropped below 1 mS/cm. The resin was then conditioned with sodium phosphate (10 mM; pH 6.0; 1.5 CV) following by washing the column with RO water until conductivity dropped below 1 mS/cm. The eluate from the previous step (19.75 L) was adjusted to pH 6.2 with NaOH (5 N; 739 mL), and loaded directly onto the AMBERCHROM™ column. The material was eluted with RO water (7.25 CV; 38.8 L/h) in a down flow orientation. Four fractions, each equal to approximately one column volume, were collected, sparged with nitrogen for 30 min, and stored at 6 °C. Each fraction was analyzed by HPLC for the Nikkomycin Z content and acidified with equimolar amount of 1 N HCl (see Table 1).

Concentrating and Drying

The fractions (73.5 L total) were concentrated under reduced pressure on a rotary evaporator (bath temperature 32 °C, condenser temperature −4 °C, temperature of vapor 18–22 °C, vacuum 16–18 mbar). The residues were combined (1.27 L total), the concentrated solution was transferred into two Pyrex pans (9×13) and placed into the lyophilizer for freezing and subsequent drying. The product temperature and the vapor pressure were continuously monitored (see Figure 4). The resulted Nikkomycin Z hydrochloride dihydrate (171 g) was obtained with 98.29% relative purity by HPLC. M.p. 170 °C (dec.). ¹H NMR (400 MHz, D₂O) TM 8.24 (d, 1H, *J* = 2.64 Hz), 7.96 (dd, 1H, *J*₁ = 8.94 Hz, *J*₂ = 2.64 Hz), 7.81 (d, 1H, *J* = 8.96 Hz), 7.67 (d, 1H, *J* = 8.08 Hz), 5.87 (m, 1H), 5.45 (d, 1H, *J* = 2.36 Hz), 4.65 (d, 1H, *J* = 3.72 Hz), 4.53 (t, 1H, *J* = 5.60 Hz), 4.44 (d, 1H, *J* = 4.64 Hz), 4.36 (t, 1H, *J* = 5.36 Hz), 4.31 (t, 1H, *J* = 3.92 Hz), 2.77 (m, 1H), 0.81 (d, 1H, *J* = 7.12 Hz). ¹³C NMR (100 MHz, D₂O) TM 173.3, 168.2, 166.0, 144.1, 151.6, 146.9, 142.1, 132.8, 129.2, 124.9, 102.5, 89.5, 83.6, 72.8, 71.0, 70.0, 56.6, 56.2, 39.5, 6.3. Found C 42.82%; H 5.41%; N 12.48%; Cl 6.52%. C₂₀H₃₀ClN₂O₁₂ requires C 42.30%; H 5.32%; N 12.33%; Cl 6.24%. HRMS (ESI) calc for C₂₀H₂₅N₅O₁₀ 496.1601 (M+H)⁺, found 496.1671. KF analysis – 4.84% of water.

Acknowledgments

We gratefully acknowledge the National Center on Minority Health and Health Disparities and the Office of Director, National Institutes of Health (1RC3MD005194-01), and Valley Fever Solutions, Inc. for providing financial support for this work. We are thankful to Professor Michael J. Sadowsky, University of Minnesota, and Professor John N. Galgiani, University of Arizona, for valuable assistance, and Professor Raj Suryanarayanan, University of Minnesota, for helpful discussions in the course of this study. We are grateful to Professor Galgiani for providing the *Streptomyces tendae* ΔNikQ 25-2 strain.

ABBREVIATIONS

CV	column volume
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectroscopy
KF	Karl Fischer
NMR	Nuclear Magnetic Resonance
pI	isoelectric point
RO	reverse osmosis

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1; Nikkomycin Z

2; Nikkomycin X

Figure 1.
Nikkomycin Z and Nikkomycin X

Figure 2.
HPLC Chromatogram of Nikkomycin Z Fermentation Broth

Figure 3.
Schematic Representation of the Purification, Concentration and Drying Process

Figure 4.
Freeze Drying Profile

Figure 5.
HPLC Chromatogram of the Final Product Nikkomycin Z

Figure 6.
Frequency of Nikkomycin Z Producing Mutants

Figure 7.
Frequency of Nikkomycin Z Producing Mutants

Table 1

Fractions Collected from Column E and Acidification Data

Fraction	Amount (L)	Conc. (mmol/L)	Nik-Z Total (mmol)	Purity (%)	Amount of 1 N HCl (mL)	pH after acidification
Fraction 1	19.3	2.607	50.328	95.78	50.33	3.52
Fraction 2	17.6	10.204	179.135	99.62	179.14	3.25
Fraction 3	19.0	4.683	88.604	99.84	88.61	3.32
Fraction 4	17.6	0.277	4.878	100.00	4.88	3.94

Table 2

Comparison of the Process Yield/Purity on Different Scales

	50 L (25 g) scale		250 L (171 g) scale	
	Yield	Purity	Yield	Purity
Column A/B	83%	50.0%	84%	49.7%
Column C	98%	51.1%	100%	50.8%
Column D	93%	68.8%	100%	69.4%
Column E	99%	97.9%	94%	98.3%
Total/Final	75%	97.9%	79%	98.3%

Table 3

Nikkomycin Z Production in Bioreactors

Strain Designation	Run#	Nikkomycin Z (g/L)
ATCC 31160	Nik031109#1	0.17
Δ NikQ 25-2	Nik031109#2	0.1
UMN04D10	Nik081409#2	1.0
UMN01E7	Nik082409#1	1.6
UMN08G1	Nik091109#2	0.5