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An improved method for culturing *Streptomyces sahachiroi*: Biosynthetic origin of the enol fragment of azinomycin B

Gilbert T. Kelly [1], Vasudha Sharma [1], Coran M.H. Watanabe [*,2]

Department of Chemistry, Texas A&M University, P.O. Box 30012, College Station, TX 77843, USA

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Abstract

Azinomycin B is an environmental DNA crosslinking agent produced by the soil microorganism *Streptomyces sahachiroi*. While the agent displays potent cytotoxic activities against leukemic cell lines and animal mouse models, the lack of a consistent supply of the natural product has hampered detailed biological investigations on the compound, including its mode of action and biosynthesis. We report here a significant methodological improvement in the culturing of the bacterium, which allows reliable and steady production of the natural product in good yields. The key experimental step involves the culturing of the strain on dehydrated plates, followed by the generation of a two-stage starter culture and subsequent fermentation of the strain under nutrient-starved conditions. We illustrate use of this culture system by investigating the formation of the enol fragment of the molecule in isotopic labeling experiments with threonine and several advanced precursors (β-ketoamino acid **3**, β-hydroxyamino aldehyde **4**, and β-ketoaminoaldehyde **5**). The results unequivocally show that threonine is the most advanced precursor accepted by the NRPS (non-ribosomal peptidyl synthetase) machinery for final processing and construction of the enol moiety of the natural product.

Keywords: Azinomycin; Biosynthesis; *Streptomyces*; PKS/NRPS

1. Introduction

The azinomycins (A and B, Scheme 1) comprise a family of aziridine-containing natural products produced by *Streptomyces* sp. that possess potent anti-tumor activity [1]. Both compounds exhibit cytotoxicity in the nanomolar range against the leukemic cell line L5178 [2]. Likewise, *in vivo* studies against P388 leukemic mice indicate that azinomycin B exhibits an ILS (increased lifespan) of 193% at 16 μg/kg/d and an ILS of 161% at 32 μg/kg/d for Ehrlich carcinoma comparable to the clinical drug, mitomycin C (204% at 1 mg/kg/d, the intraperitoneal dosage of mitomycin C was ~62-fold that of azinomycin B) [3]. In contrast, azinomycin A gave a comparably lower ILS (76% at 16 μg/kg/d), and a much narrower therapeutic index suggesting some degree of selectivity between these two agents.

In vitro experiments reveal the inherent ability of azinomycin B to bind within the major groove of DNA. The electrophilic C-10 and C-21 carbons contained within the aziridino[1,2*a*]pyrrolidine (1-azabicyclo[3.1.0]hexane) and epoxide fragments impart the natural product to form interstrand crosslinks with the N7 positions of suitably disposed purine bases of DNA [4]. Recent studies with DNA microarrays and fluorescence imaging with the natural product provide the first demonstration of DNA damage caused by azinomycin B in whole cells, correlating *in vitro* DNA cross-linking observed with the metabolite with an *in vivo* cellular response [5].

* Corresponding author. Fax: +1 979 458 8095.
E-mail address: watanabe@mail.chem.tamu.edu (C.M.H. Watanabe).
[1] These authors contributed equally to the experimental portion of this manuscript.
[2] This manuscript is dedicated in memory of my academic grandfather, Distinguished Professor Ian Scott, whose pioneering efforts in natural product biosynthesis and passion for science will continue to inspire many of us.

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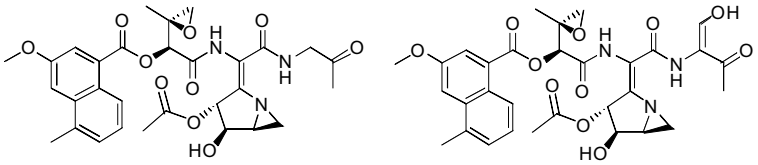


Azinomycin A (**1a**) Azinomycin B (**1b**)

Scheme 1. Structures of azinomycin A and B.

The unusual architecture of the azinomycins coupled with its potent anti-tumor activity has captured the attention of both the biosynthetic and synthetic communities. The total synthesis of azinomycin A was achieved in 2001 by Coleman and co-workers and a number of synthetic analogues have also been reported [6]. Notwithstanding these gains, even after 50 years beyond its initial isolation [7] and more than two decades of structure correction [1], there is no total synthesis yet reported for azinomycin B which is a major barrier to in-depth studies involving biological activity of the natural product. In addition, biosynthetic investigations on the compound have lagged considerably. While some gains have been made to establish the polyketide origin of the naphthoate moiety [8] and a cell-free system developed to support synthesis of azinomycin B *in vitro* [9], progress in this area has been largely impeded by difficulties with the culture method and securing a consistent source of the natural product. Following literature protocols [1,8], we found production of the natural product by *Streptomyces sahachiroi* to be highly erratic (production would be observed once then not seen for 3-weeks or more). As a great majority of biosynthetic studies hinge upon having reliable production of the natural product including isotopic labeling studies and gene disruption experiments, this necessitated the development of a new culture method. We detail here, experiments performed to achieve these optimized growth conditions and utilize these refined culture conditions to investigate the biosynthetic route to the enol fragment of azinomycin B.

2. Materials and methods

2.1. Instrumentation and general methods

Reactions were carried out in flame-dried glassware under nitrogen or an argon atmosphere, unless otherwise noted. Commercial solvents and reagents were used as received from Sigma–Aldrich. All isotopically labeled material was purchased from Cambridge Isotope Laboratories, Inc. Anhydrous solvents were dried over neutral alumina (MBraun system). All reactions were magnetically stirred and monitored by thin layer chromatography (TLC), utilizing glass-backed silica gel plates from Analtech (#47011). Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Flash column chromatography was performed using 60 Å Silica Gel (Silacycle, 230–400 mesh) as a stationary phase. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Inova 500 or Varian Inova 300. ^{1}H NMR chemical shifts are reported as δ values in ppm relative to $CDCl_3$ (7.26 ppm) and coupling constants (J) are reported in Hertz (Hz). Infrared spectra were recorded on a Bruker Tensor 27 spectrometer. Unless otherwise indicated, deuterochloroform ($CDCl_3$) served as an internal standard (77.0 ppm) for all ^{13}C spectra. Mass spectra (ESI) were obtained at the Laboratory for Biological Mass Spectrometry at the Department of Chemistry, Texas A&M University, with API QStar Pulsar, MDS Sciex (Toronto, ON, Canada) Quadrupole-TOF hybrid spectrometer. Gas chromatography/low resolution mass spectra were recorded on a Trace DSQ GCMS spectrometer, ThermoElectron Corporation (Austin, TX, USA). APCI was recorded on a Thermofinnigan LC-Q DECA mass spectrometer. Fermentations were run on Fermentation Design Inc. Model # MS21 (Allentown, PA, USA). The total capacity of the fermentation system is 15 L.

2.2. Organism

Streptomyces sahachiroi (NRRL 2485) was obtained from the American Type Culture Collection (ATCC).

2.3. Culture conditions

Spore stocks: *Streptomyces sahachiroi* spores from dehydrated glucose, yeast extract, and maltose extract (GYM) plates (per liter of medium: glucose monohydrate, 4 g yeast extract, 4 g malt extract, 10 g $CaCO_3$, 2 g and tap water adjusted to pH 6.8 with 1 M NaOH prior to sterilization) were streaked onto large MS (Mannitol Soya flour, per liter medium: Mannitol, 20 g Soya flour, 20 g and deionized water) plates and allowed to incubate at 30 °C for 15 days. At this time the grey spores were removed with sterile water and agitation. The spores were then filtered through sterile cotton, washed three times with sterile water, centrifuged at 3000 rpm, re-suspended in a minimal amount of 10% glycerol solution, flash frozen, and stored at −80 °C.

(i) *Solid plates*. Solid media formulations (See Supporting Information A) were evaluated by streaking a loop full of *S. sahachiroi* spore stock onto prepared plates. The plates were grown at 30 °C in a Fisher Scientific Isotemp incubator for 5–7 days. Once grown, 1/4 of the plate was inoculated into Erlenmeyer flasks containing 100 mL PS5 medium and shaken at 250 rpm at 30 °C. After 72 h of growth, the cultures

were extracted with CH_2Cl_2, concentrated and analyzed by TLC and MS. First and second stage cultures were prepared by streaking an aliquot of the *S. sahachiroi* spore stock suspension onto the surface of the GYM plates and storing them at room temperature to dryness.

(ii) *First stage culture. Streptomyces sahachiroi* (inoculated from dehydrated plates) was grown on GYM plates for 5–7 days. A 1 cm^2 piece of the GYM plate was used to inoculate 100 mL of PS5 medium in a 250 mL Erlenmeyer flask. The culture was incubated at 30 °C for 24 h at 250 rpm.

(iii) *Second stage culture.* The second stage culture was prepared by inoculating 2 L Erlenmeyer baffled flasks (Fernbach containing 600 mL of PS5 medium) with 25 mL of the first stage culture. The culture was incubated at 30 °C for 24 h at 250 rpm.

(iv) *Fermentation.*The fermenter containing 10 L of the reduced PS5 (Pharmamedia/Starch plus additives, per liter of medium: Pharmamedia (yellow cotton seed flour), 5 g soluble starch, 5 g glucose, 2 g casein hydrolysate, 2 g NH_4SO_4, 0.5 g lysine, 0.5 g ornithine, 0.5 g glycine, 0.5 g and deionized water adjusted to pH 7.0 prior to sterilization) medium (reduced by 75%) was autoclaved at 121 °C for 20 min. Following inoculation (with two 600 mL second stage cultures) the fermenter was agitated at ~300 rpm and aerated with sterile filtered air (8 L/min) for 72 h.

2.4. General feeding conditions

The labeled material was weighed in equal portions and solubilized in autoclaved distilled water. The first aliquot was administered after 24 h, followed by addition of the second provided 24 h later. The culture was harvested 72 h post-induction (inoculation of the second stage culture into the fermenter). *Note.* Compound **4** and **5** were administered in D_2O immediately after synthesis. Table 3 (supporting information) provides details on the amounts of each compound fed.

2.5. Isolation and purification of azinomycin B

Following fermentation, the cultures were centrifuged at 7000 rpm at 4 °C. The cell pellets were discarded and the medium extracted with an equal volume of methylene chloride (1X). The organic layer was collected, dried over anhydrous magnesium sulfate, and concentrated *in vacuo*. The resulting crude extract was stored under diethyl ether at −80 °C. The solid was dissolved in a minimal amount of dichloromethane and precipitated with the addition of hexane to give a ratio of 1:29 CH_2Cl_2/hexane. The resulting suspension was centrifuged at 1500 rpm and the supernatant discarded. Diethyl ether (2 mL) was added to the pellet, which was subsequently agitated, centrifuged at 3000 rpm and the supernatant discarded. The resulting residue was dissolved in dichloromethane (600 μL) to which hexanes (2 mL) was added. The heterogeneous mixture was centrifuged at 3000 rpm and the supernatant retained. To the solution was added hexanes (4 mL) and the suspension centrifuged at 3000 rpm to give azinomycin B as a solid.

If full purification is not achieved, azinomycin B can be further purified by flash column chromatography (95:5 CH_2Cl_2:methanol). By TLC azinomycin exhibits an R_f of 0.23. A short column should be utilized to minimize overall contact with the silica gel and degradation by hydrolysis. The process can be repeated if necessary. The compound can be safely stored at −80 °C under anhydrous diethyl ether. The azinomycin B isolated by us matched the NMR spectrum provided by Yokoi et al. [1b].

2.5.1. Characterization of azinomycin B

Pale-white amorphous powder (1:9 CH_2Cl_2:hexane) IR (neat) ν_{max} 3338.4(br), 2957.1, 2925.3, 2872.8, 1725.92(br), 1619.3, 1601.7, 1511.2, 1417.6 cm^{-11} H NMR (300 MHz, $CDCl_3$) δ 12.40 (1H, br), 12.32 (1H, s), 8.54 (1H, dd, $J = 3.6$, 7.0 Hz,), 8.20 (1H, br), 7.94 (1H, d, $J = 2.9$ Hz), 7.46 (1H, d, $J = 2.9$ Hz), 7.32 (1H, s), 7.32 (1H, s), 7.32 (1H, s), 5.50 (1H, d, $J = 4.0$ Hz), 5.12 (1H, s), 4.64 (1H, dd, $J = 4.0$, 4.8 Hz), 3.96 (3H, s), 3.96 (1H,br), 3.36 (1H, m), 2.98 (1H, d, $J = 4.3$ Hz), 2.80 (1H, d, $J = 4.3$ Hz), 2.70 (1H, s), 2.66 (3H, s), 2.30 (1H, s), 2.24 (1H, s), 2.18 (1H, s), 1.52 (1H, s) ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.5, 173.0, 165.7, 164.0, 162.0, 156.0, 153.0, 150.8, 134.5, 133.3, 128.1, 127.9, 127.0, 125.4, 123.9, 122.3, 119.3, 118.6, 108.5, 84.4, 77.4*, 77.1*, 56.2, 55.7, 53.9, 46.4, 36.7, 24.5, 21.0, 20.3, 17.2. APCI-MS (LRMS) 624.2. Found: 624.2 (*obscured by $CDCl_3$ solvent peak).

2.6. Synthesis of isotopically labeled compounds (*3*), (*4*) and (*5*)

2.6.1. Threonine methyl ester hydrochloride (*6*)

Thionyl chloride (398.5 mg, 3.4 mmol) was added dropwise to cold anhydrous methanol (15 mL) at 0 °C. The solution was allowed to stir at 0 °C for 10 min. Threonine, **2** (0.4 g, 3.35 mmol) was added and the reaction mixture allowed to reflux for 1 h. The reaction was allowed to cool and the solvent evaporated *in vacuo*. A 2 N solution of anhydrous HCl in methanol was generated and the reaction was again refluxed for 1 h. The solution was allowed to cool and the solvent evaporated *in vacuo* on a rotary evaporator. A foamy solid was obtained in quantitative yield. IR (NaCl, thin film) ν_{max} 3375.3, 3236.2, 2960.6(br), 1744.1, 1599.0, 1516.1, 1240.9 cm^{-11} H NMR (CD_3OD, 500 MHz) δ 1.35 (d, 3H, $J = 6.5$ Hz), 3.87 (s, 3H), 3.97 (d, 1H, $J = 4.5$ Hz), 4.29 (dq, 1H, $J_1 = 6.5$ Hz, $J_2 = 4.0$ Hz), ^{13}C NMR and DEPT(CD_3OD, 500 MHz) δ 20.5, 53.7, 59.7, 66.3,169.6 HRMS (ESI+): m/z calcd for $C_5H_{12}NO_3Cl$ (M+H), 134.0818. Found: 134.0812. ESI-(LRMS): m/z 34.96, 36.99.

2.6.2. Boc-protected threonine methyl ester (7)

Method A. A flame dried 25 mL three-necked round-bottomed flsk, equipped with a magnetic stirring bar, thermometer, reflux condenser (protected from moisture by a calcium chloride-filled drying tube), and a pressure-equalizing dropping funnel connected to a N_2-line was charged with a solution of di-*tert*-butyl dicarbonate (0.34 g, 1.56 mmol) in THF (2.5 mL). A suspension of methyl threoninate hydrochloride **6** (0.27 g, 1.59 mmol) in THF (5 mL) and triethylamine (0.34 g, 3.4 mmol) was maintained at 0 °C and allowed to stir for 5 min. The solution of di-*tert*-butyl dicarbonate was added dropwise over a period of 1 h at 0 °C. After 10 min of additional stirring, the ice-water bath was removed and the suspension was stirred overnight (14 h) at room temperature, then warmed at 50 °C for a further 3 h. The solvent was removed under reduced pressure and the residue was partitioned between diethyl ether (20 mL) and saturated aqueous bicarbonate solution (25 mL). The aqueous phase was extracted with ether 3× 15 mL. The combined organic phases were dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to give 0.33 g (88% yield) of *N*-Boc-L-threonine methyl ester, **7** as a thick colorless oil that was used without further purification.

Method B. Threonine methyl ester hydrochloride salt, **6** (3.0 g, 17.7 mmol) and $NaHCO_3$ (4.6 g, 53.2 mmol) were dissolved in a 1:1 (v/v) mixture of water and methanol (36 mL). $(Boc)_2O$ (5.8 g, 26.6 mmol) was added drop-wise and the solution was stirred at room temperature for 20 h. The reaction mixture was concentrated under vacuum. The reaction mixture was then acidified with aqueous citric acid (1 M) to pH 4.5. The reaction mixture was extracted with ethyl acetate (30× 4 mL). The organic layers were combined, dried with $MgSO_4$, filtered and concentrated *in vacuo* to obtain the product **7** in quantitative yield and was used without further purification.

IR (NaCl, thin film) ν_{max} 3432.1, 2982.4, 2853.2, 1753.4, 1719.9, 1510.4, 1365.2, 1172.4 cm^{-1} ^1H NMR ($CDCl_3$, 500 MHz) and gCOSY δ 1.21 (d, 3H, $J = 7$ Hz), 1.45 (s, 9H), 2.23 (br, 1H), 3.74 (s, 3H), 4.22 (d, 1H, $J = 9$ Hz), 4.26 (d, 1H, $J = 6$ Hz), 5.41 (d, 1H, $J = 7$ Hz). ^{13}C NMR ($CDCl_3$, 500 MHz) and gHMQC δ 19.8, 28.2, 52.5, 58.6, 68.1, 80.1, 156.1, 172.0 HRMS (ESI+): *m/z* calcd for $C_{10}H_{19}NO_5$ (M+Li), 240.1423. Found: 240.1415.

2.6.3. tert-Butyl 1-(methoxycarbonyl)-2-oxopropylcarbamate (Boc-protected β-keto-threonine methyl ester) (8)

A solution of Boc-protected threonine methyl ester, **7** (0.1 g, 0.43 mmol) and Dess–Martin periodinane (0.22 g, 0.52 mmol) in dichloromethane (2 mL), was stirred for 1 h at room temperature. The reaction was then quenched with 15 mL of 1:1 v/v solution of $NaHCO_3$ and 10% $Na_2S_2O_3$ and extracted with ethyl acetate (3× 30 mL). The organic phase was collected, dried over Na_2SO_4 and concentrated *in vacuo* to afford the product (**8**) as a low melting white solid in 91% yield (92 mg). Mp = 61 °C $[\alpha]_D = -3.8$(c = 2.5 ,$CHCl_3$) IR (NaCl, thin film)ν_{max} 3384.2, 2931.2, 2851.3, 1758.9, 1720.4, 1708.6, 1483.6, 1365.1, 1157.9 cm^{-1} ^1H NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 9H), 2.35 (s, 3H), 3.78 (s,3H), 5.02 (d, 1H, $J = 7.2$ Hz), 5.74 (br, 1H). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 7.8, 28.1, 53.2, 64.2, 80.8, 154.9, 166.9, 198.8 HRMS (ESI+): *m/z* calcd for $C_{10}H_{17}NO_5$ (M+H), 232.1185. Found: 232.1178.

2.6.4. 2-Amino 3-oxobutanoic acid TFA salt (3)

A solution of *tert*-butyl 1-(methoxycarbonyl)-2-oxopropylcarbamate, **8** (0.13 g, 0.56 mmol) in 5 mL TFA:H_2O (v/v 1:1) was refluxed for 8 h and allowed to stir for an additional 5 h. The solvent was evaporated *in vacuo* to give the desired trifluoroacetate salt as a light yellow sticky solid, **3**. $[\alpha]_D$ −3.24 (*c* 0.4, MeOH) IR (NaCl, thin film)ν_{max} 3422.7, 3014.1, 2857.2, 1732.2, 1693.4, 1675.9, 1199.0 cm^{-1} ^1H NMR (D_2O, 500 MHz) δ 2.06(s, 3H), 3.89 (s, 1H) ^{13}C NMR (D_2O + 1 drop CD_3OD, 500 MHz) δ 26.6, 47.7, 116.4 (q, $J_{C-F} = 1160$ Hz), 162.8(q, $J_{C-F} = 141$ Hz), 171.6, 204.03 HRMS (ESI+): *m/z* calcd for $^{13}C_4H_7NO_3$((M+H)-H_2O), 104.0. Found: 103.99.

2.6.5. (4S,5R)-3-tert-butyl 4-methyl 2,2,5-trimethyloxazolidine-3,4-dicarboxylate (9)

2-Methoxypropene (0.31 g, 4.29 mmol) and camphor sulphonic acid (6.7 mg, 0.02 mmol) was added to a solution of *N*-Boc-L-threonine methyl ester, **7** (0.1 g, 0.43 mmol) in acetone (2 mL). The resulting orange solution was stirred at room temperature for 3.5 h (TLC analysis indicated completion of reaction). The reaction mixture was quenched with 10 μL of triethylamine and the solvent removed under reduced pressure. The residual brown syrup was partitioned between diethyl ether (20 mL) and saturated aqueous sodium bicarbonate solution (30 mL). The aqueous layer was extracted with diethyl ether (2× 30 mL) and the combined organic phases were dried with anhydrous sodium sulfate and concentrated under reduced pressure to give 105 mg (90%) of oxazolidine methyl ester, **9** as a yellow oil in both rotameric forms (3:1). IR (NaCl, thin film) ν_{max} 2984.5, 2928.3, 2857.2, 1755.9, 1720.4, 1376.9, 1362.2, 1258.6 cm^{-1} ^1H NMR and gCOSY ($CDCl_3$, 300 MHz) δ 1.31, 1.32 (s, 9H), 1.41 (s, 3H), 1.49–1.57 (m, 6H), 3.69 (s, 3H), 3.83, 3.91 (d, 2H, $J = 7.5$ Hz) 4.05–4.09 (m, 1H) ^{13}C NMR ($CDCl_3$, 300 MHz) and DEPT δ 18.7, 18.5, 23.9,24.7, 26.4, 27.7, 28.1, 28.2, 52.1, 52.3, 65.9, 66.1, 73.4,73.7, 80.2, 80.7, 94.4, 95.0, 151.2, 152.3, 171.0, 171.5 HRMS (ESI+): *m/z* calcd for $C_{13}H_{23}NO_5$, (M+H), 274.1654. Found: 274.1645.

2.6.6. (4R,5R)-tert-butyl 4-(hydroxymethyl)-2,2,5-trimethyloxazolidine-3-carboxylate (10)

A 25-mL, two-necked, round-bottomed flask was equipped with a magnetic stirring bar, reflux condenser bearing a drying tube and a pressure-equalizing dropping funnel fitted with a rubber septum. The flask was charged with 10 mL of tetrahydrofuran and 20.9 mg (0.55 mmol) of lithium aluminum hydride. While the suspension in the flask was stirred, a solution of the oxazolidine ester, **9** (100 mg,

0.37 mmol) in tetrahydrofuran (1 mL) was added dropwise over 20 min. The dropping funnel was washed with two 1-mL portions of tetrahydrofuran and the suspension stirred for an additional 20 min, when TLC analysis showed the complete formation of the alcohol. The reaction mixture was cooled with an ice-water bath while 1 mL of a 10% aqueous KOH solution was added drop-wise over 10 min. *Caution! The reaction is exothermic*. The reaction was stirred for another hour at room temperature, and filtered through a Celite pad (1 × 2.5 cm) that was subsequently rinsed with diethyl ether (3× 10 mL). The combined organic filtrates were washed with 25 mL of aqueous phosphate buffer (pH 7.0), and the aqueous layer extracted with diethyl ether (3× 30 mL). The combined organic phases were dried with anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give 85.1 mg (~95%) of the desired product, **10** as a pale yellow oil which was used without further purification. IR (NaCl, thin film) ν_{max} 3437.5, 2978.6, 2928.3, 2875.0, 1696.7, 1670.1, 1456.9, 1406.6, 1255.6 cm^{-1} ^1H NMR and gCOSY ($CDCl_3$ + 1 drop D_2O), 300 MHz) δ 1.32 (d, 3H, $J = 6$ Hz), 1.44 (s, 3H), 1.47 (s, 3H,), 1.55 (s, 3H), 3.49 (m, 1H), 3.62 (d, 2H, $J = 3$ Hz) 3.71 (m, 1H) ^{13}C NMR ($CDCl_3$, 300 MHz) and HMQC δ 18.1, 25.9, 27.8, 28.3, 64.7, 67.2, 71.9, 81.3, 94.1, 154.2 HRMS (ESI+): *m/z* calcd for $C_{12}H_{23}NO_4$, (M+H), 246.1705. Found: 246.1699.

2.6.7. (4S,5R)-tert-Butyl 4-formyl-2,2,5-trimethyloxazolidine-3-carboxylate (11)

Oxazolidine alcohol **10** (85 mg, 0.35 mmol) was dissolved in ethyl acetate (2.5 mL, 0.14 M final concentration), and IBX (307.2 mg, 1.1 mmol) was added. The resulting suspension was refluxed in an oil bath set to 80 °C with vigorous stirring. After 2 h (TLC monitoring), the reaction was cooled to room temperature and filtered through a medium glass frit. The filter cake was washed with ethyl acetate (3× 2 mL), and the combined filtrates washed with pre-chilled $NaHCO_3$ (1×). The organic layer was collected, dried over Na_2SO_4, filtered and concentrated *in vacuo* to yield 82 mg (98% yield) of the desired product **11** as a mixture of rotomers (2:1). *Note: the aldehydes were immediately used for deprotection. Prolonged storage in an organic solvent led to decomposition of the product*. IR (NaCl, thin film) ν_{max} 2963.8, 2860.2, 1717.4, 1684.9, 1374.0, 1264.5 cm^{-1} ^1H NMR and gCOSY ($CDCl_3$), 300 MHz) δ 1.34 (d, 3H, $J = 6$ Hz), 1.41, 1.49 (s, 9H,), 1.55–1.64 (m, 6H), 4.02–4.08, 4.19–4.26 (m, 1H), 3.68, 3.80 (dd, 1H, $J = 2.4$, 2.7 Hz), 9.37, 9.46 (d, 1H, $J = 1.8$ Hz) ^{13}C NMR ($CDCl_3$, 300 MHz) and gHMQC δ 17.6, 17.7, 25.0, 25.8, 26.2, 27.3, 28.1, 28.2, 69.8, 70.0, 70.9, 71.0, 81.4, 81.5, 94.1, 94.9, 150.9, 152.5, 197.5 HRMS (ESI+): *m/z* calcd for $C_{12}H_{21}NO_4$ (M+H), 244.1549. Found: 244.1546.

2.6.8. (R)-2-Aminobutane-1,1,3-triol trifluroacetate salt (4)

A solution of aldehyde **11** (0.14 g, 0.58 mmol) was stirred in deuterated acetone (15% in D_2O) at room temperature. Trifluoroacetic acid (N_2 flushed, Aldrich, 0.21 g, 1.84 mmol) was added drop-wise to this solution. A brown grease-like material separated instantaneously. The slurry was stirred for 20 min to yield the product as a trifluoroacetate salt, **4** in the D_2O layer. *Note: all attempts to concentrate the product led to decomposition of the material. This adduct was used for feeding studies immediately. Stability studies indicated the presence of ~70% of the compound after 48 h in D_2O at room temperature*. IR (NaCl, thin film) ν_{max} 3393.1, 1675.9 cm^{-1} ^1H NMR and gCOSY(D_2O, 500 MHz) δ 1.01 (d, 3H, $J = 6.5$ Hz), 2.76–2.80 (m,1H), 3.76–3.84 (m, 1H), 4.08 (d,1H, $J = 5$ Hz). ^{13}C NMR (D_2O, 500 MHz) δ 19.2, 61.1, 63.9, 86.6, 116.0 (q, $J_{C-F} = 1153$ Hz), 162.2 (q, $J_{C-F} = 139.5$ Hz) HRMS (ESI+) *m/z* calcd for $C_4H_{11}NO_3$(M+H), 121.0739. Found: 122.0819 $C_4H_9NO_2$(M+H) 104.0712. Found: 104.0715.

2.6.9. Methyl (2S,3R)-2-(tert-Butoxycarbonylamino)-3-O-(tert-butyldimethylsilyl) butanoate, (12)

A mixture of Boc-protected methyl ester **7** (1.0 g, 4.29 mmol), triethylamine (0.99 mL, 6.86 mmol), 4-*N*, *N*-(dimethylamino)pyridine (52.3 mg, 0.42 mmol) in anhydrous DMF (16 mL) was cooled at 0 °C. *tert*-Butyldimethylsilyl chloride (0.84 g, 5.57 mmol) was added and the mixture was stirred for 1.5 h. The ice-bath was removed and the reaction mixture was stirred at room temperature for 36 h. The reaction was quenched with methanol (1 mL), stirred for an additional 30 min, diluted with Et_2O (100 mL), and washed with saturated aqueous NH_4Cl (3× 25 mL). The organic phase was dried ($NaSO_4$), filtered and concentrated *in vacuo* to give the corresponding silyl derivative, **12** (1.35 g, 91.2%). $[\alpha]_D = -28.6$ (*c* 0.73, MeOH) IR (NaCl, thin film) ν_{max} 2916.3, 2860.0, 1757.8, 1713.3, 1380.2, 1162.2 cm^{-1} ^1H NMR and gCOSY ($CDCl_3$, 500 MHz) δ 0.01 (s, 3H), 0.06 (s, 3H), 0.87 (s, 9H), 1.21 (d, 3H, $J = 6.5$ Hz), 1.48 (s, 9H), 3.74 (s, 3H), 4.24 (dd, 1H, $J = 2$, 8 Hz), 4.44 (m, 1H), 5.20 (1H, $J = 8$ Hz) ^{13}C NMR ($CDCl_3$, 500 MHz) δ −5.2, −4.3, 17.9, 20.7, 25.5, 28.3, 52.1, 59.5, 68.8, 79.7, 156.2, 171.7 HRMS (ESI+): *m/z* calcd for (M+H) $C_{16}H_{33}NO_5Si$, 348.2206. Found: 348.2208.

2.6.10. (2R,3R)-2-(tert-Butoxycarbonylamino)-3-O-(tert-butyldimethylsilyl)-1,3-butandiol, (13)

A well-stirred suspension of $LiAlH_4$ (0.59 g, 15.5 mmol) in anhydrous THF (24 mL) was maintained at −50 °C. Boc-protected silyl derivative, **12** (1.35 g, 3.88 mmol) in anhydrous THF (2 mL) was added over a period of 20 min. The mixture was stirred at −50 °C for an additional 30 min, diluted with 1 M phosphate buffer at pH 7 (3 mL) and EtOAc (30 mL), warmed to room temperature, and filtered through a pad of Celite. The organic layer was collected and concentrated. The residue was eluted from a column of silica gel with 4:1 cyclohexane–AcOEt to give product **13** (0.99 g, 80%) as a thick colorless oil. $[\alpha]_D = -6.9$ (*c* 0.8, $CHCl_3$) lit. $[\alpha]_D = -7.5$ (*c* 0.8, $CHCl_3$) IR (NaCl, thin film)ν_{max} 3446.3, 2929.1, 2850.6, 1702.7, 1504.2, 1169.2 cm^{-1} ^1H NMR and gCOSY ($CDCl_3$, 500 MHz) δ 0.09 (s, 3H, Si–CH_3), 0.1 (s, 3H, Si–CH_3), 0.90 (s, 9H, Si–C(CH_3)$_3$), 1.19 (d, 3H, $J = 6$ Hz), 1.47 (s, 9H), 3.52–3.70 (m, 3H), 4.06–

4.11 (m, 1H), 4.91 (1H, $J = 8$ Hz) ^{13}C NMR ($CDCl_3$, 500 MHz) δ −5.3, −4.3, 17.8, 20.8, 26.0 , 28.3, 57.3, 64.2, 67.7,79.7, 156.8 HRMS (ESI+): *m/z* calcd for (M+H) $C_{15}H_{33}NO_4Si$, 320.2257. Found: 320.2260.

2.6.11. (4R)-4-[(R)-1-O-(tert-butyldimethylsilyl)ethyl]-2,2-dimethyl-N-(tert-butoxycarbonyl)-1,3-oxazolidine, (14)

A solution of Boc-protected silyl alcohol, **13** (0.85 g, 2.66 mmol) and 2-methoxypropene (1.73 g, 23.98 mmol) in acetone (10 mL) was maintained at 0 °C. 10-Camphorsulfonic acid (61.79 mg, 0.26 mmol) was added and the mixture stirred for 1 h at 0 °C, followed by 30 min at room temperature. The reaction was quenched with Et_3N (0.2 mL, color changes from dark red to yellow) and the solvent was removed under reduced pressure. The residual brown syrup was partitioned between diethyl ether (50 mL) and saturated aqueous sodium bicarbonate solution (4× 30 mL). The organic layer was collected and washed once with brine. The organic phase was dried (Na_2SO_4) and concentrated to give the desired product **14** as a mixture of rotamers (2:1) (0.9 g, 95%). IR (NaCl, thin film) ν_{max} 2929.3, 2856.7, 1705.9, 1471.3, 1381.9, 1256.2 cm^{-1} ^{1}H NMR and gCOSY ($CDCl_3$, 500 MHz) δ 0.07 (m, 6H), 0.88 (s, 9H), 1.09 (d, 3H, $J = 6.5$ Hz), 1.27, 1.34 (s, 3H), 1.47, 1.49 (s, 9H), 1.56, 1.62 (s, 3H), 3.78–3.82, 3.92–3.96 (m, 1H), 3.87–3.92, 4.14–4.19 (m, 1H) 4.22–4.28, 4.31–4.37 (m, 1H) ^{13}C NMR ($CDCl_3$, 500 MHz) δ −4.8, −4.7, 17.8, 22.6, 22.7, 25.7, 28.1, 28.3, 28.6, 28.6, 29.7, 60.6, 61.2, 62.8, 63.0, 66.2, 66.9, 79.7, 79.9, 94.6, 93.9, 152.3, 152.7 HRMS (ESI+): *m/z* calcd for (M+H) $C_{18}H_{37}NO_4Si$, 360.2570. Found: 360.2580.

2.6.12. (4R)-4-[(R)-1-Hydroxyethyl]-2,2-dimethyl-N-(tert-butoxycarbonyl)-1,3-oxazolidine (15)

A solution of the silyl ether derivative (0.9 g, 2.82 mmol) in anhydrous THF (10 mL) was treated with *n*-$Bu_4N^+F^-{\cdot}3H_2O$ (3.38 mL (1 M in THF), 3.39 mmol) at room temperature for 8 h and concentrated. The residue was dissolved in CH_2Cl_2 (20 mL), washed with H_2O (2× 20 mL), dried (Na_2SO_4), and concentrated to give the crude product as a dark yellow oil. The residue was eluted from a plug of silica gel with 4:1 hexane–AcOEt (containing 0.3% of Et_3N) to afford the product (**15**) as a white solid (0.55 g, 88%). IR (NaCl, thin film) ν_{max} 3443.4, 2972.7, 2827.3, 1696.7, 1379.9 cm^{-1} ^{1}H NMR($CDCl_3$, 500 MHz) δ 1.14 (d, 3H, $J = 6.5$ Hz), 1.46 (s, 3H),1.47 (s, 9H), 1.55 (s, 3H), 3.78–3.98, 4.11–4.19 (2m, 4H) ^{13}C NMR ($CDCl_3$, 500 MHz) δ 24.4, 25.9, 28.2, 28.3, 28.4, 29.8, 63.2, 64.7, 70.1, 81.3, 94.0,152.3 HRMS (ESI+): *m/z* calcd for (M+H) $C_{12}H_{23}NO_4$, 246.1705. Found: 246.1697.

2.6.13. (4R)-4-Acetyl-2,2-dimethyl-N-(tert-butoxycarbonyl)-1,3-oxazolidine, (16)

A mixture of alcohol **15** (0.19 g, 0.79 mmol), and Dess–Martin reagent (0.37 g, 0.88 mmol) in anhydrous CH_2Cl_2 (10 mL) was stirred at room temperature for 2 h away from light. The reaction was quenched with a 1:1 solution of cold $Na_2S_2O_3$ and $NaHCO_3$(v/v) and extracted in ether (3× 30 mL). The organic layer was collected, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give crude product **16** as a thick yellow oil and a mixture of rotamers as a thick yellow oil (0.17 mg, 90%). IR (NaCl, thin film) ν_{max} 2917.2, 2847.7, 1732.6, 1697.8, 1366.3 cm^{-1} ^{1}H NMR($CDCl_3$, 500 MHz) δ 1.43, 1.51(s, 9H),1.48, 1.54 (s, 3H), 1.66, 1.72 (s,3H), 2.20, 2.22 (s, 3H), 3.93, 3.98 (dd, 1H, $J = 3.0$, 6.5 Hz), 4.10–4.18 (m,1H), 4.28, 4.42 (dd, 1H, $J = 3.0$, 5.5 Hz) ^{13}C NMR ($CDCl_3$, 500 MHz) δ 24.7, 25.6, 25.4, 25.9, 26.3, 26.5, 28.3, 28.2, 65.1, 65.5, 65.5, 65.6, 81.1, 80.7, 94.5, 95.2, 151.4, 152.4, 206.5, 207 HRMS (ESI+): *m/z* calcd for (M+H) $C_{12}H_{21}NO_4$, 244.1549. Found: 244.1537.

2.6.14. tert-Butyl (S)-1-hydroxy-3-oxobutan-2-ylcarbamate (17)

A solution of ketone **16** (0.17 g, 0.71 mmol) in CH_2Cl_2 (1 mL) was mixed with 0.5 M TFA in anhydrous CH_2Cl_2 (15 mL) and stirred at room temperature for 15 min. The reaction was quenched with cold $NaHCO_3$ (30 mL) and extracted in ether (3× 30 mL). The organic layer was collected, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give the crude product as a thick yellow oil. The crude material was then purified by column chromatography by eluting with ethyl acetate:hexane (1:3) $R_f = 0.25$ to yield acetyl glycinol **17** (0.12 g, 85%). IR (NaCl, thin film) ν_{max} 3418.4, 2919.2, 2851.1, 1711.9, 1687.7, 1366.7 cm^{-1} ^{1}H NMR($CDCl_3$, 500 MHz) δ 1.46 (s, 9H), 2.29 (s, 3H), 3.96 (ddd, $J = 4$, 8, 25 Hz), 4.32 (m, 1H), 5.68 (br,1H) ^{13}C NMR ($CDCl_3$, 500 MHz) δ 27.3, 28.2, 62.1,62.9, 80.2, 155.9, 205.6 HRMS (ESI+): *m/z* calcd for (M+H) $C_9H_{17}NO_4$, 204.1236. Found: 204.1239.

2.6.15. 3-Amino-4-hydroxy but-3-en-2-one trifluoroacetate salt (5)

A suspension of ketone **17** (0.08 g, 0.4 mmol) in dichloroethane (2 mL) was refluxed gently with IBX (0.7 g, 1.2 mmol, 45%) for 3 h. The suspension was allowed to cool to room temperature and further cooled at −20 °C for 20 min. The resulting white precipitate was filtered. The filtrate was mixed with aqueous TFA (50 mg in 1 mL water) and allowed to stir for 25 min at room temperature. The aqueous layer was collected and characterized. *Note: the keto-amino aldehyde was found to be unstable to isolation conditions. Any attempts to concentrate this material led to extensive decomposition of the product. This precursor was used for feeding studies immediately. Stability studies indicated the presence of ~35% of the compound after 48 h in D_2O at room temperature.* ^{1}H NMR (D_2O, 500 MHz) δ 1.69(s,3H), 1.83 (s, 3H), 4.69 (s, 1H), 5.06 (s, 1H). HRMS (ESI+): *m/z* calcd for (M+H) $C_4H_7NO_2$, 102.0555. Found: 102.0551.

3. Results and discussion

3.1. Development of the culture system

As the production of secondary metabolites from *Streptomycetes* frequently coincides or precedes the formation of

aerial hyphae on solid media [10], we initially examined a variety of media conditions including LB, ISP+ [11], NB+, YPD [12], TO [13], GYM [4], PS5 [4], MS [14], YEME [14], and R2YE [14]. Several solid media conditions were evaluated by streaking a loop full of *S. sahachiroi* spore stock upon prepared plates. The growth was evaluated at regular intervals. All plates showed growth of *Streptomyces*, however, only GYM, MS, PS5 and PS5+ plates exhibited sporulation (see Supporting information A, Table 1). As azinomycin B production was undetectable in organic extracts of the spores, the plates were inoculated into Erlenmeyer flasks containing 100 mL of PS5 medium. After a 72 h post-inoculation period at 30 °C, the cultures were extracted with dichloromethane, concentrated, and analyzed by TLC and mass spectrometry. Results revealed that while the MS plates showed traces of azinomycin B production, the GYM plates produced the most robust and productive spores at 5–7 days growth when inoculated from spores stored on dehydrated GYM plates (see Supporting information A, Table 1).

Proceeding to the evaluation of liquid culture conditions, we examined a variety of media formulations (YPD, R2YE, PS5, PS5+, LB, TO, GYM, MS, YEME, NB+, and ISP+) [11–14] (see Supporting Information A for media formulations and abbreviations) to support azinomycin production by *S. sahachiroi* in shake flasks and by fermentation (as summarized in Table 1 and Supporting information A, Table 2). Since natural product production is generally confined to stationary phase of the growth curve, in shake culture (Erlenmeyer flasks or baffled flasks) we evaluated each medium composition by generating a first stage culture (from GYM plates), which was used to inoculate and produce the culture in large scale (in 2 L baffled flasks). The cells were harvested at a fixed time of 72 h. The cultures were centrifuged and extracted with dichloromethane. Under these growth conditions, azinomycin B production was observed in YPD, R2YE, PS5, and PS5+, but not in LB, TO, GYM, MS, YEME, NB+, and ISP+ as determined by TLC, mass spectrometry, ^{1}H NMR and ^{13}C NMR spectroscopy. Azinomycin B production, however, remained erratic, with the final pH of the culture varying from pH 4.25 to as high as pH 8.75.

As the highest levels of azinomycin B production typically correlated with an ending pH value of 8, we attempted to adjust the pH throughout the culturing period (using PS5 medium) by the addition of base (NaOH) or use of phosphate buffer but improvement in either case was not observed (see Supporting information Table 2 entry 9 and 10). Nutrient limitation was also investigated as well as varying the culture period from 1–6 days. In all cases, azinomycin production was low and/or inconsistent. As modifying liquid shake culture protocols did not give much improvement, we decided to explore usage of a large scale fermentation system (Fig. 1). Such an approach would afford more control over several factors not easily controlled with shake flasks such as degree of aeration and foaming. Initially, the fermenter (15 L capacity) was inoculated with two 24 h first stage cultures (100 mL, generated from GYM plates), agitated at 250 rpm, and aerated at 6 L/min with sterile filtered air for 72 h. The experiment resulted in considerable foaming leading to loss of culture and low production of the azinomycins. We, therefore, decreased the volume of medium to 10 L and repeated the experiment inoculating with two 24 h second stage cultures (600 mL each) that were generated by inoculating 2 L baffled flasks with 25 mL of a 24 h first stage culture. As azinomycin production was minimal, nutrient starvation was explored (see Table 1, entry 4). The component composition of the PS5 medium (10 L) was reduced by 50% and inoculated with two 600 mL second stage cultures. After 72 h, the cells were harvested and ~30 mg of azinomycin B was obtained from 10 L. To further investigate the stress imposed by medium deprivation, the medium composition was reduced by 75%, inoculated with two second stage cultures (600 mL), and harvested after 72 h. This sequence yielded approximately 40 mg of azinomycin B from 10 L of culture. Further reduction of the PS5 medium composition resulted in lower levels of azinomycin production (Fig. 1c) [15]. By increasing the two second stage cultures to 1 L and the aeration rate to 8 L/min, we can now reproducibly obtain about 60 mg of azinomycin from 10 L. Interestingly, the pH of the cell culture steadily increased to ~8.0 over a period of 4 days. Fig. 1b and d show the pH profile and amount of azinomycin B produced as functions of time. Optimal production of the natural product was observed between 64–72 h (Fig. 1d) corresponding to pH 7.4–7.8 (Fig. 1b), probably due to instability of the molecule under strongly acidic or alkaline conditions.

Manual adjustment of the pH throughout the culture period with bicarbonate did not have a dramatic effect on production levels, 40–60 mg of azinomycin was obtained.

3.2. Whole cell feeding studies: biosynthetic route to the enol fragment

With a reliable culture system in hand, our aim was to employ these new growth conditions to investigate the formation of the enol fragment of azinomycin B. Initially, to examine our feeding conditions, we fed [1-^{13}C] acetate and [methyl-^{13}C]methionine to cell suspensions of *S. sahachiroi*. The isotopically labeled compounds were provided to the cultures in two separate aliquots (in equal portions), the first after 24 h of incubation and the second 24 h later. As expected, whole cell feeding of [1-^{13}C] acetate gave rise to an alternate labeling pattern within the naphthoate fragment owing to its PKS origin and confirming the results reported by Lowden [8] (Fig. 2a and 2b, blue C2′-C8a′, 7.9–11.4% incorporation). Furthermore, both carbons C1 and C4 revealed moderate incorporation (Fig. 2a and b, blue, C1, 2.8% C4, 2.9%) supporting the hypothesis that scrambling of label could occur to give threonine via oxaloacetate in the Kreb's cycle [16]. The results further suggest that C-14 (Fig. 2a and b, blue, 8.2%) of the molecule is derived from acetate and does not arise from rearrange-

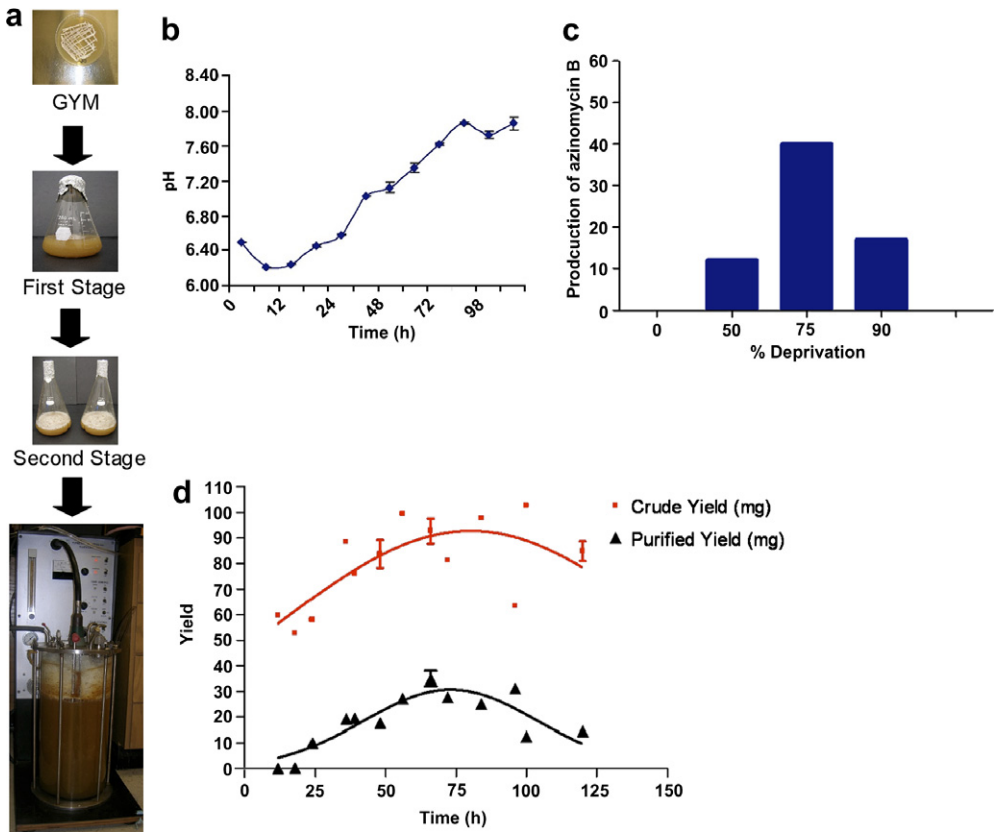


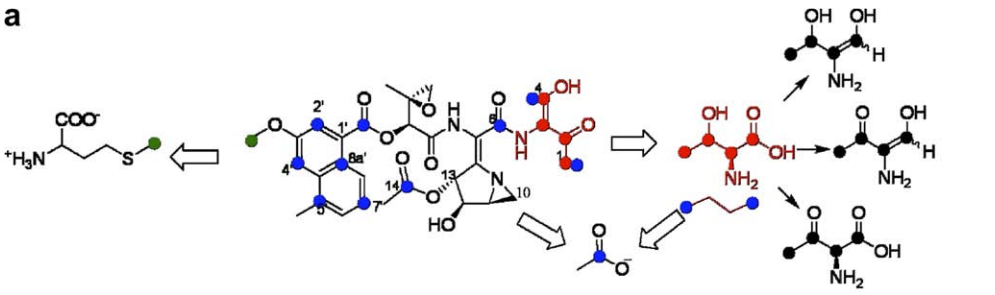
Fig. 1. (a) Sequence of fermentation system: culture of *S. sahachiroi* (b) pH profile as a time course. (c) Effect of nutrient deprivation on production of azinomycin B. (d) Time course production of azinomycin B with 75% nutrient deprivation (second stage culture, 600 mL baffled flask). Crude yield of azinomycins and purified azinomycin B are shown as a function of time.

Table 1
Examination of culture conditions for azinomycin production in *Streptomyces sahachiroi*

Entry	Culture conditions	Result
1	Solid media, culture flasks, 72 h	Little or no production
2	Solid media, first stage culture, baffled flasks, 72 h	Little production, erratic
3	Solid media, first stage culture, fermenter (300 rpm aerated at 6 L/min), 72 h	Little production, erratic
4	Solid media, first stage culture, second stage culture (600 mL baffled flask), fermenter (250 rpm aerated at 6 L/min), 72 h	Traces of azinomycin B observed
5	Solid media, first stage culture, second stage culture (600 mL baffled flask), 50% nutrient deprivation (300 rpm aerated at 8 L/min), 72 h	~15 mg azinomycin B from 10 L of culture
6	Solid media, first stage culture, second stage culture (600 mL baffled flask), 75% nutrient deprivation (300 rpm aerated at 8 L/min), 72 h	~30–40 mg azinomycin B from 10 L of culture
7	Solid media, first stage culture, second stage culture (1 L baffled flask), 75% nutrient deprivation (300 rpm aerated at 8 L/min), 72 h	~60 mg azinomycin B from 10 L of culture

ment or Baeyer–Villiger oxidation of a more advanced precursor. Likewise, feeding of [methyl-^{13}C]methionine to cultures of *S. sahachiroi* gave an unambiguous clear enhancement of signal (33.8% incorporation, Fig. 2b) at the methoxy carbon of the molecule (Fig. 2a, green). The finding not only supports the involvement of the co-factor SAM (*S*-adenosylmethionine) in the biosynthesis of the methoxy group of the naphthoate, but also excludes the involvement of SAM in the formation of the aziridinopyrrolidine ring system (specifically, the electrophilic C-10 carbon does not arise from SAM).

Having established a proper feeding regimen with our new culture system, our focus shifted to utilizing our new fermentation strategy to probe the biosynthesis of the late stages of the pathway, in the construction of the enol fragment of azinomycin B. The structure of the enol fragment suggests that it might originate from L-threonine the β-alcohol of the amino acid would necessitate oxidation and the terminal carboxylate reduction (Scheme 2). Our earlier results from cell-free extract studies [9] and acetate labeling experiments support such an argument. Neither approach has shown, however, that the amino acid is



b

Compound		C % incorporation
[methyl-^{13}C]-Methionine	-OCH_3	33.8
[1-^{13}C]Sodium acetate	C_1	2.8
	C_4	2.9
	C_6	9.2
	C_{14}	8.2
	C_1'CO	1.6
	C_2'	11.4
	C_4'	9.6
	C_5'	7.9
	C_7'	9.4
	C_{8a}'	9.7
[U-^{13}C]Threonine	C_1	7.9
	C_2	11.7
	C_3	10.3
	C_4	8.6
[U-^{13}C] **3**		n.d.
[U-^{13}C] **4**		n.d.
[U-^{13}C] **5**		n.d.

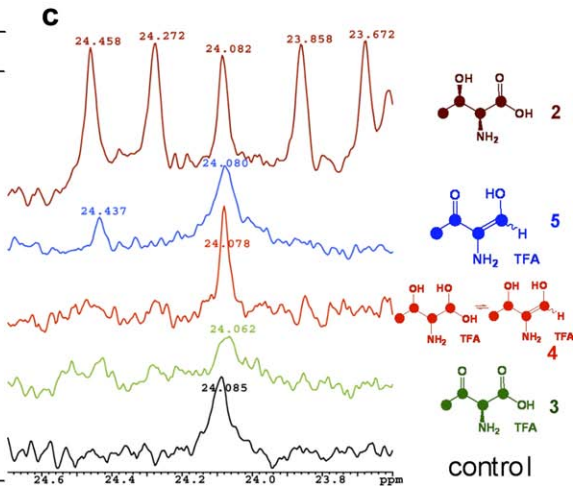
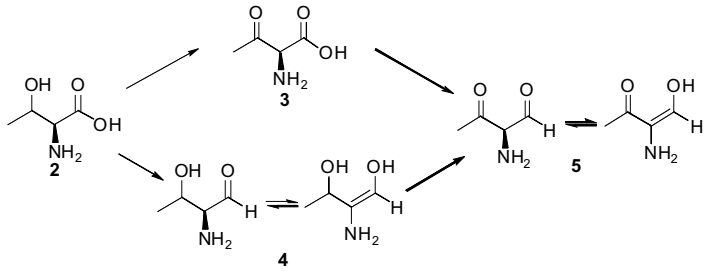


Fig. 2. (a) Summary of feeding studies: [methyl-^{13}C]methionine incorporates at –OMe [1-^{13}C]sodium acetate incorporates at C1, C4, C6, C14, C1′CO–, C2′, C4′, C5′, C7′, C8a′ [U–^{13}C]threonine incorporates C1–C4. (b) % incorporation = $[(A - B)/B] \times 1.07$ where A, intensity of labeled carbon; B, intensity of unlabeled carbon; 1.07, is the natural abundance of ^{13}C; n.d., not detectable by ^{13}C NMR. (c) Representative comparison of C1 of azinomycin B via feeding of [U–^{13}C]-labeled threonine, **3**, **4** and **5** to that of the negative control.



Scheme 2. Proposed biosynthetic routes to the enol fragment of azinomycin B.

site-specifically incorporated into the enol fragment of the natural product. We, therefore, examined uptake of [U–^{13}C]- L-threonine (**2**, Scheme 2). The experiment resulted in labeling of all four carbons (C1–C4) of the enol fragment (Fig. 2a, red). This 'tail-to-tail' incorporation clearly suggested that threonine was site-specifically incorporated. Close inspection of the ^{13}C NMR indicated intact incorporation (C1–C4, 7.9–11.7% incorporation) of the [U–^{13}C]-labeled threonine. Isotopic labeling was evident at C1 as a doublet of a doublet ($J_1 = 55.8$, $J_2 = 180.0$ Hz) flanking the natural abundance peak at 24.1 ppm. Similarly, C4 gave a doublet of doublet ($J_1 = 38.7$, $J_2 = 324.6$ Hz) flanking the natural abundance peak at 149.5 ppm. Multiplets were observed for carbons C2 and C3 probably owing to extensive ^{13}C–^{13}C coupling. To address the order of events, the timing of the biosynthesis in the oxidation of the alcohol and reduction of the carboxylate of **2**, we synthesized β-ketoamino acid **3**, β-hydroxyamino aldehyde **4**, and *β*-ketoaminoaldehyde **5** in labeled form. Synthesis of such precursors is important to establish whether the enol fragment or its respective intermediates are pre-formed prior to loading onto the NRPS or generated at a later stage in the biosynthesis.

3.3. Synthesis

The synthetic routes were designed to allow easy and efficient preparation in high yields, selective deprotection of *O*- and *N*-functional groups and minimal chromatographic separations to afford synthesis of the molecules in a cost effective manner for the implementation of stable isotopes. The availability of synthetic routes to α-amino-

compounds (**8**, Scheme 3), oxazolidine-aldehyde (**11**, Scheme 3), and amino alcohol (**17**, Scheme 4) is of additional importance as they can serve as models for stereochemical studies [17] as well as serve as building blocks in the synthesis of amino-sugars [18], aza-sugars [19], sphingosines [20], and unnatural amino acids/derivatives [21].

The syntheses of all three precursors **3**, **4** and **5** began from commercially available L-threonine. Threonine, **2** was converted into its methyl ester hydrochloride using $SOCl_2$ in MeOH under reflux conditions. The methyl ester **6** was then protected with Boc-anhydride in THF in the presence of triethylamine to provide the Boc-protected methyl ester of threonine **7** in ~88% yield. This Boc-protected methyl ester **7** served as the common substrate for all precursors (Scheme 3).

3.3.1. β-Keto amino acid *3*

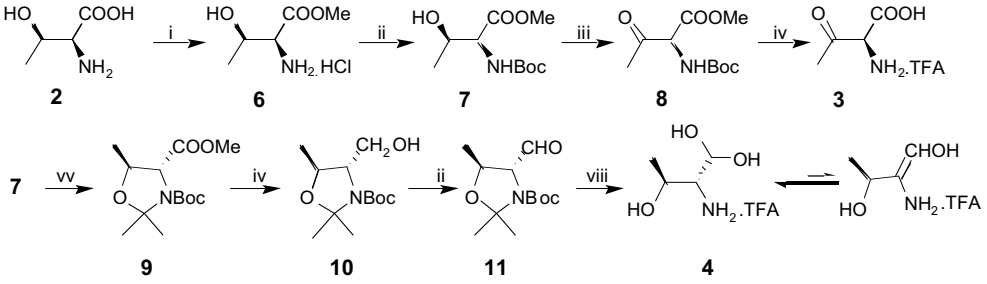
Upon oxidation with Dess–Martin periodinane, Boc-protected threonine methyl ester was converted into the corresponding Boc-protected keto-ester **8** in 91%, which when hydrolyzed with TFA/H_2O under reflux afforded the desired compound **3** in an overall 72% yield (Scheme 3).

3.3.2. Hydroxy-aldehyde *4*

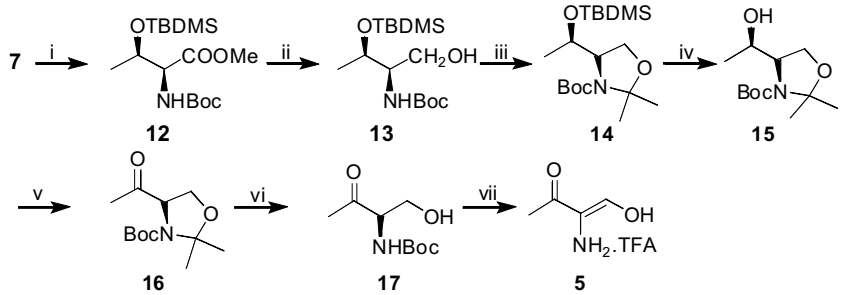
The hydroxy-aldehyde **4** (Scheme 3) was synthesized over six steps by modification of the synthesis of Garner's aldehyde [22]. The *N*-Boc 2,2-dimethyl oxazolidine ring constituted a convenient masked modified amino acid that was expected to tolerate synthetic elaboration. Thus, Boc-protected amino acid methyl ester, **7** was protected completely as an oxazolidine **9** which when subjected to reduction with $LiAlH_4$ afforded the protected alcohol **10** in excellent yields. The alcohol was further oxidized to the threonine analog of Garner's aldehyde **11** in 98% yield. The protected amino-aldehyde was not stable over long periods of time and was immediately subjected to deprotection by treatment with TFA/D_2O. This TFA salt of the amino aldehyde hydrate system generated was unstable to isolation and was characterized and directly utilized in feeding experiments.

3.3.3. β-Ketoaminoaldehyde *5*

We next examined a synthetic route for the preparation of β-ketoaminoaldehyde **5**. Since the β-ketoenamine system is synthetically equivalent to the corresponding ketoaldehyde, we expected it to be directly formed via oxidation of the corresponding 1,3 diol system. Treatment with TPAP/NMO [23], PCC [24], PDC [25], Dess–Martin [26], and IBX(2-iodoxy benzoic acid) [27], however, resulted in a complex mixture of products unstable to column chromatography. Alternatively, access to the Boc-protected aminoaldehyde using reduction of Boc-protected esters or Weinreb amides [28] led to overreduction to the corresponding alcohol or complicated and tedious column chromatographic separations, not suitable for implementation of stable isotopes. Based on synthesis and stability studies of **3**, we envisaged *N*-Boc-protected acetyl glycinol **17** (Scheme 4) as a convenient moiety to undergo oxidation and subsequent deprotection. Thus, chiral acetyl oxazolidine **16** was synthesized from the common precursor *tert*-butoxycarbonyl-protected methyl L-threoninate **7** using a



Scheme 3. Reagents: (i) MeOH, HCl (dry) reflux, 2 h; (ii) Boc_2O, Et_3N, THF, 0°C–r.t. 14 h, then 50 °C 3 h; (iii) Dess–Martin periodinane, CH_2Cl_2, r.t., 1 h; (iv) TFA (aq.), reflux; (v) 2- methoxypropene, acetone, r.t., 3 h; (vi) $LiAlH_4$, THF, 40′; (vii) IBX, EtOAc, reflux, 3 h; (viii) TFA, water, 25′.



Scheme 4. Reagents: (i) TBDMSCl, DMAP, Et_3N, dry DMF, 0 °C–r.t., 36 h; (ii) $LiAlH_4$, THF, −50 °C, 30′; (iii) 2-methoxypropene, acetone, r.t., 2 h; (iv) TBAF, THF, 8 h; (v) Dess–Martin periodinane, CH_2Cl_2, r.t., 1 h; (vi) 0.5 M TFA, CH_2Cl_2, r.t., 15′; (vi) IBX, DCE, 3 h, cool, filter then TFA, H_2O, 15′.

modified procedure employed by Dondoni et al. [29]. This ester was first transformed into the alcohol **13** by silylation (91%) of the secondary hydroxyl group with *tert*-butyldimethylsilyl chloride (TBDMSCl) and subsequent reduction of the ester group with $LiAlH_4$. Our earlier efforts and studies by others [29] revealed that the selective step-wise protection of the two hydroxyl groups was important. Acetonation with 2-methoxypropene (95%) and desilylation (n-$Bu_4N^+F^-$) converted **13** into the (*R*,*R*)-hydroxyethyl oxazolidine **15** (88%) in rotameric forms. The secondary alcohol was then subjected to oxidation with Dess–Martin periodinane to yield **16**. Use of PCC for oxidation gave a complex mixture of products that remained impure even after column chromatography. The oxazolidine ring was cleaved with very dilute TFA (boc group still intact) to yield the desired acetyl Boc-protected glycinol **17**. Alternatively, the acetyl Boc-protected glycinol was also synthesized starting from *N*-Boc-protected serine, which was converted into its Weinreb amide. Further acetonation with 2-methoxypropene, followed by treatment with organolithium (MeLi) in presence of CH_3MgBr as the sacrificial base provided acetylation of the α-C [30]. However, overall yields from this methodology and purification were not found to be cost-effective in our hands. This primary alcohol was now ready for oxidation and subsequent final deprotection. Heterogenous IBX oxidation [27] provided a mild method to form the aldehyde, however, all efforts to isolate the keto-amino aldehyde resulted in rapid decomposition of the product. Hence, the reaction was simply filtered and treated with TFA/H_2O for 25 min at room temperature to afford final deprotection, producing the desired keto-amino aldehyde **5**.

Each of the threonine derivatives (compounds **3**, **4**, and **5**) were synthesized in universally labeled form and fed individually to whole cell suspension cultures as detailed previously. Interestingly, none of these amino acid precursors gave any site-specific incorporation above background (Fig. 2c, supporting information c). As a control for cellular uptake, since the majority of the amino acid derivatives were synthesized as their respective TFA salts, we fed [methyl-^{13}C] methionine as its TFA salt and monitored its incorporation ([methyl-^{13}C]methionine labels specifically the methoxy group of azinomycin B, Fig. 2a, b and supporting information b). Intact incorporation was observed as reported previously, confirming that the TFA salts of amino acids are capable of penetrating the cell membrane of the producer strain, *S. sahachiroi*. As noted previously [31], both of the enol systems (**4** and **5**) were found to be relatively unstable as compared to the corresponding acetylglycine **3**. Fig. 3 illustrates the stability analysis of the compounds. After 48 h at room temperature under aqueous conditions, only 70% of the hydroxyl-amino aldehyde **4** and 35% of the keto-amino aldehyde **5** were detected (Supporting Information B, Table 4).

Despite the relative instability of some of these amino acid derivatives, given our feeding regimen (two separate and equal aliquots fed 24 h apart), had these compounds been precursors in the biosynthesis, intact incorporation into the molecule should have been detectable. The gatekeeper in the biosynthesis of the enol fragment is presumed to be that of the NRPS adenylation domain, where in this case, based upon data reported here, threonine appears to be the most advanced precursor to be accepted by the module. One possibility is that the adenylation domain itself catalyzes (all or in part) the oxidation of the alcohol and the reduction of the carboxylate. In myxochelin biosynthesis, reductase domains have been found to replace the TE (thioesterase) domain in the gene cluster resulting in release of the natural product with concomitant production of an aldehyde moiety [32]. Moreover, adenylation domains have also been found to harbor *N*- and *C*-methyltransferases, decarboxylases, as well as α-ketoreductase domains [33]. Alternatively, the required modifications might be post-NRPS processes. The localization of the azinomycin biosynthetic gene cluster will certainly shed light on these issues.

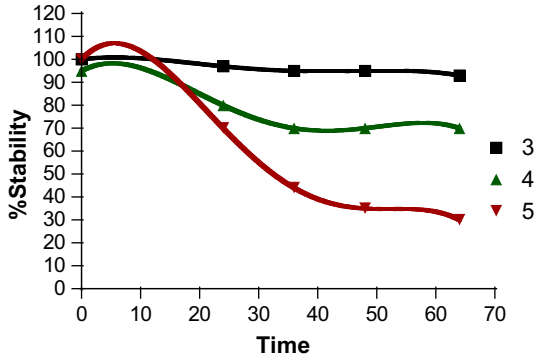


Fig. 3. Stability curve as a function of time for **3**, **4** and **5**.

4. Conclusion

The azinomycins represent a structurally unusual class of DNA crosslinking agents produced by Nature. While the *in vivo* actions of azinomycin B show significant promise, the clinical potential of the agent remains largely unexplored due to dearth of synthetic procedures for the molecule as well as unreliable culture methods for the producing organism. We report here a reliable fermentation procedure for *S. sahachiroi*, the azinomycin producer, where up to 60 mg of purified compound can be obtained from 10 L of culture. We successfully employed this system to investigate the biosynthetic origin of azinomycin B, principally in the late stages of the pathway, the identification of the source of the enol fragment of the molecule. Threonine and advanced precursors (**3**, **4** and **5**) that were synthesized in isotopically labeled form were fed to suspension cultures of the *Streptomyces* strain. The results indicated that threonine is the most advanced precursor accepted by the NRPS module for further processing. Elucidation of the azinomycin biosynthetic gene cluster is currently in progress, which will pave the way for detailed characteriza-

tion and functional analysis of individual biosynthetic steps of the pathway.

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Appendix A. Supplementary data

Details on the culturing of *Streptomyces sahachiroi*, media formulations, ^{1}H and ^{13}C spectra of azinomycin B, details on feeding studies with [1-^{13}C]sodium acetate, [methyl-^{13}C]methionine, [U–^{13}C]threonine, [U–^{13}C]-**3**, [U–^{13}C]-**4**, [U–^{13}C]-**5**, comparison of feeding of all threonine derivatives, ^{1}H and ^{13}C spectra for compounds **3-17** is available via the Internet. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2007.08.002.

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