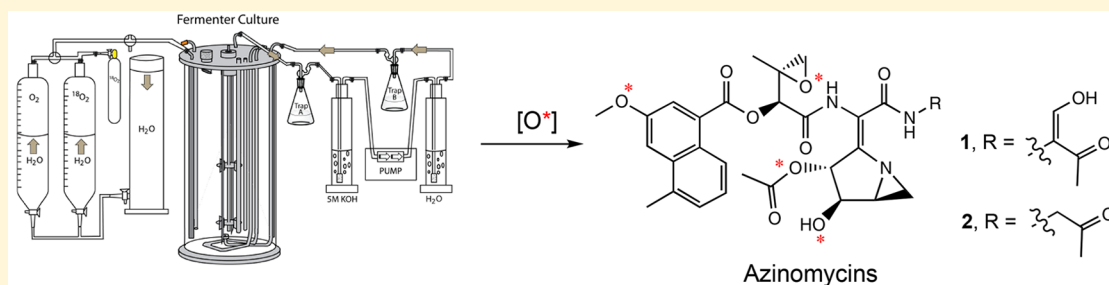


The Fate of Molecular Oxygen in Azinomycin Biosynthesis

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Supporting Information



ABSTRACT: The azinomycins are a family of aziridine-containing antitumor antibiotics and represent a treasure trove of biosynthetic reactions. The formation of the azabicyclo[3.1.0]hexane ring and functionalization of this ring system remain the least understood aspects of the pathway. This study reports the incorporation of ^{18}O -labeled molecular oxygen in azinomycin biosynthesis including both oxygens of the diol that ultimately adorn the aziridino[1,2-*a*]pyrrolidine moiety. Likewise, two other sites of heavy atom incorporation are observed.

The azinomycins, A (1) and B (2) (Figure 1), are powerful antitumor antibiotics produced by the soil dwelling bacterium *Streptomyces sahachiroi*.¹ These unique natural products form interstrand cross-links within the major groove of DNA without prior activation. *In vitro* studies reveal the azinomycins target the duplex DNA sequence 5'-d(PuNPy)-3', enabling covalent linkages to be formed between the electrophilic C10 and C21 carbons of azinomycin and the N7 positions of suitably disposed purine bases.^{2–5} The azinomycins have been shown to be effective in the nanomolar range against the leukemic cell line LS178Y, and azinomycin B has exhibited antitumor activity against murine transplantable tumors (2–3 $\mu\text{g kg}^{-1}$).⁶ In an early phase clinical trial, azinomycin B displayed remarkable efficacy against squamous cell carcinoma, a form of skin cancer.⁷ Additionally, the azinomycins exhibit antimicrobial activity against a range of Gram-negative and Gram-positive bacteria. Against most strains of bacteria, azinomycin A is more effective than azinomycin B; against *S. aureus*, azinomycin A gave an MIC of 6.25 $\mu\text{g mL}^{-1}$ versus that of azinomycin B which gave an MIC of 50 $\mu\text{g mL}^{-1}$.⁷

Considerable effort has been expended toward total synthesis and SAR studies with azinomycin and its derivatives.^{1,8,9} Understanding the mechanism by which the azinomycins are formed will pave the way for synthetic biology efforts in the design of targeted delivery of azinomycin-based anticancer agents.

The biosynthesis of these potent antitumor antibiotics is enabled by the action of an unusual polyketide synthase (PKS) in the formation of the naphthoate moiety (Figure 2). The Type I PKS AziB works in conjunction with a thioesterase AziG, which functions as a chain elongation and cyclization

(CEC) domain. Without this domain, a truncated, single ring shunt metabolite is formed, 2-methyl benzoic acid 4.¹⁰ The P-450 enzyme AziB1 oxidizes the 3' position,¹¹ which is methylated by S-adenosylmethionine (SAM) as demonstrated by feeding studies.¹² The epoxide unit of the molecule is generated from valine 5 (Figure 2),^{13,14} which is converted to 3-methyl-2-oxobutenoate 8 through a sequence of oxidation and transamination events. It is presumed that oxidation of the alkene of 3-methyl-2-oxobutenoate 8 gives the epoxide unit. Generation of the azabicyclo[3.1.0]hexane ring system is complex and believed to involve ca. 14 enzymatic steps, which initiates from glutamic acid (Figures 2 and 6).^{15,16} A protection step ensues as mediated by AziC2. The ϵ -carboxylic acid group of glutamic acid is converted into an acyl phosphate as mediated by AziC3 and subsequently reduced to that of the aldehyde. The remaining steps have yet to be demonstrated but require a 2-carbon extension and a transamination step to install the aziridine nitrogen, followed by a series of oxidative steps, ring forming reactions, and deprotection. The terminal end unit, which distinguishes azinomycins A (1) and B (2) is derived from aminoacetone 10 for azinomycin A (1) and threonine 11 in the case of azinomycin B (2).^{17,18}

In this study, we track the fate of molecular oxygen in azinomycin biosynthesis and demonstrate the incorporation of the heavy isotope $^{18}\text{O}_2$ at C-3', C-19/C-21, C-12, and C-13/C-14. The data provide a molecular fingerprint of oxidative transformations throughout the pathway and places mechanistic constraints on the biosynthesis of the azinomycins 1 and 2.

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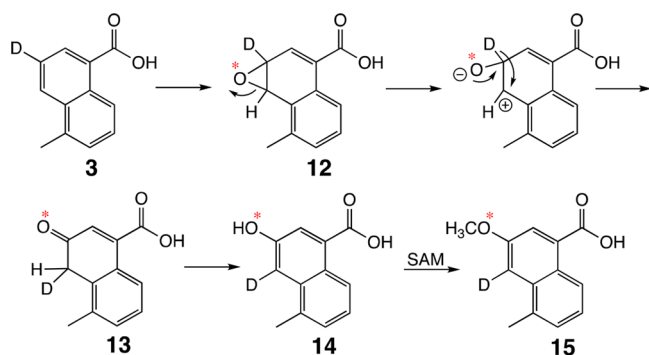


Figure 3. “NIH shift” mechanism in the formation of 3'-methoxy 5-methylnaphthoic acid **15**.

within the azinomycin gene cluster are two cytochrome P-450 enzymes: AziB1, shown previously to be involved in the construction of the functionalized azinomycin naphthoate moiety, and AziC9, which has yet to be functionally characterized.^{1,22} Reannotation of the azinomycin gene cluster has also revealed AziU1, an enzyme with similarity to 2-oxoglutarate Fe-dependent oxygenases.^{23–25} Whole cell feeding experiments with isotopically labeled precursors have shown that the epoxide unit is derived from valine **5** (Figure 4), which is oxidized to give alcohol **6**. Transamination results

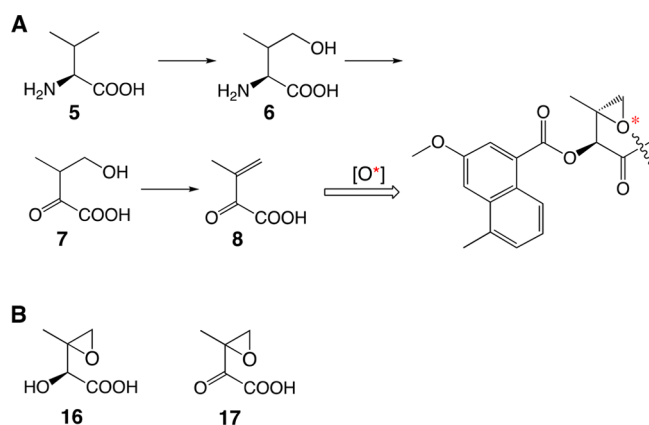


Figure 4. Biosynthetic route to the epoxide unit. (A) Metabolites demonstrated to be intermediates in the pathway. (B) Structures of epoxide compounds.

in the formation of **7**, which upon dehydration gives 3-methyl-2-oxobutenoate **8**. Each of these metabolites were shown to be site-specifically incorporated,¹⁴ with **8** being the penultimate precursor to be incorporated. While epoxide compounds **16** and **17** were evaluated, neither was shown to be incorporated. Both compounds show instability in aqueous medium over time, forming ring-opened products and lactonization, which limits their survival in whole cell experiments. Reconstitution of the enzymes involved in these transformations will ultimately rigorously establish these steps of the pathway.

The most interesting result was the incorporation of molecular oxygen at both positions on the aziridinopyrrolidine ring as reflected by the shift in carbon signals at the C-12 and C-13/C-14 positions (Table 1) and ESI-MS/MS (SI Figures S10 and S11). Taking into account the oxygen labeling pattern and the presence of the P-450 oxidases (AziB1 and AziC9) and Fe-dependent dioxygenase (AziU1) within the gene cluster,^{1,22} one possibility is that diol formation results from oxidation of

an alkene to give an initial epoxide (Figure 5, Route A). Ring opening would then result in the formation of the diol, and the

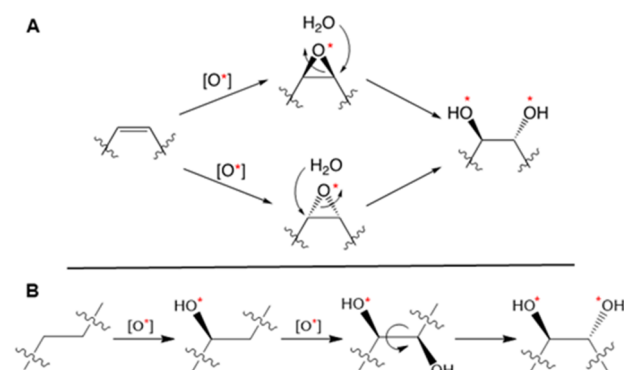


Figure 5. Proposed routes to the formation of the azabicyclic moiety through (A) epoxide formation, (B) syn dihydroxylation.

presence of the heavy oxygen isotope at both the C-12 and C-13 positions. This route requires regioselective addition of water and is limited by stereochemical requirements as well. Alternatively, syn dihydroxylation could give rise to the diol (Route B). The involvement of a dioxygenase could be envisioned, and the starting trans configuration would result in the necessary stereochemistry.

In summary, we have examined the incorporation of molecular oxygen in azinomycin biosynthesis as demonstrated by the isotope-induced chemical shift method. Heavy atom (¹⁸O) labeling was observed giving the 3' oxygen of the naphthoate group, the epoxide moiety, and the two ring oxygens of the aziridinopyrrolidine ring system. The observance of molecular oxygen at the naphthoate position is consistent with an NIH shift mechanism and the recruitment of P-450 hydroxylase AziB1.^{11,21} Likewise, the appearance of the heavy atom at the epoxide moiety is congruent with whole cell feeding experiments and the proposed recruitment of a P-450 or an Fe-dependent oxygenase enzyme in mediating the process.¹⁴ The presence of ¹⁸O-labeling within the azabicyclic system enables the more complex issue on the formation of the functionalized azabicyclic system to be addressed. The initial steps of azabicyclic biosynthesis have been demonstrated through a combination of whole cell feeding studies and biochemical studies (Figure 6, highlighted in blue).^{15,16} The proposed route, taking also into account the findings of these ¹⁸O-labeling studies, is shown in Figure 6. Biosynthesis of the aziridino[1,2-*a*]pyrrolidine ring initiates from glutamic acid **9**. A “protection” step, via acetylation (**18**) or through an amino group carrier protein, prevents cyclization of the amino acid and the formation of the Δ1-pyrrolidine carboxylate.²⁶ Formation of the acyl phosphate (**19**) then facilitates the formation of *N*-acetyl glutamic acid semialdehyde (**20**). A transketolase is proposed to provide the needed two-carbon extension to give product **21**. Transamination would install the amino group, generating **22**. Subsequent dehydration would give **24**, which sets the stage for oxidation. This results in the formation of the diol **25**, which ultimately becomes the two ring oxygens of the azabicyclic ring system as supported by the ¹⁸O-isotopic labeling studies shown in this study.

Formation of the aziridine ring could be facilitated by sulfation (**26**). Experiments with rat liver homogenate demonstrating conversion of ephedrine to aziridine in the

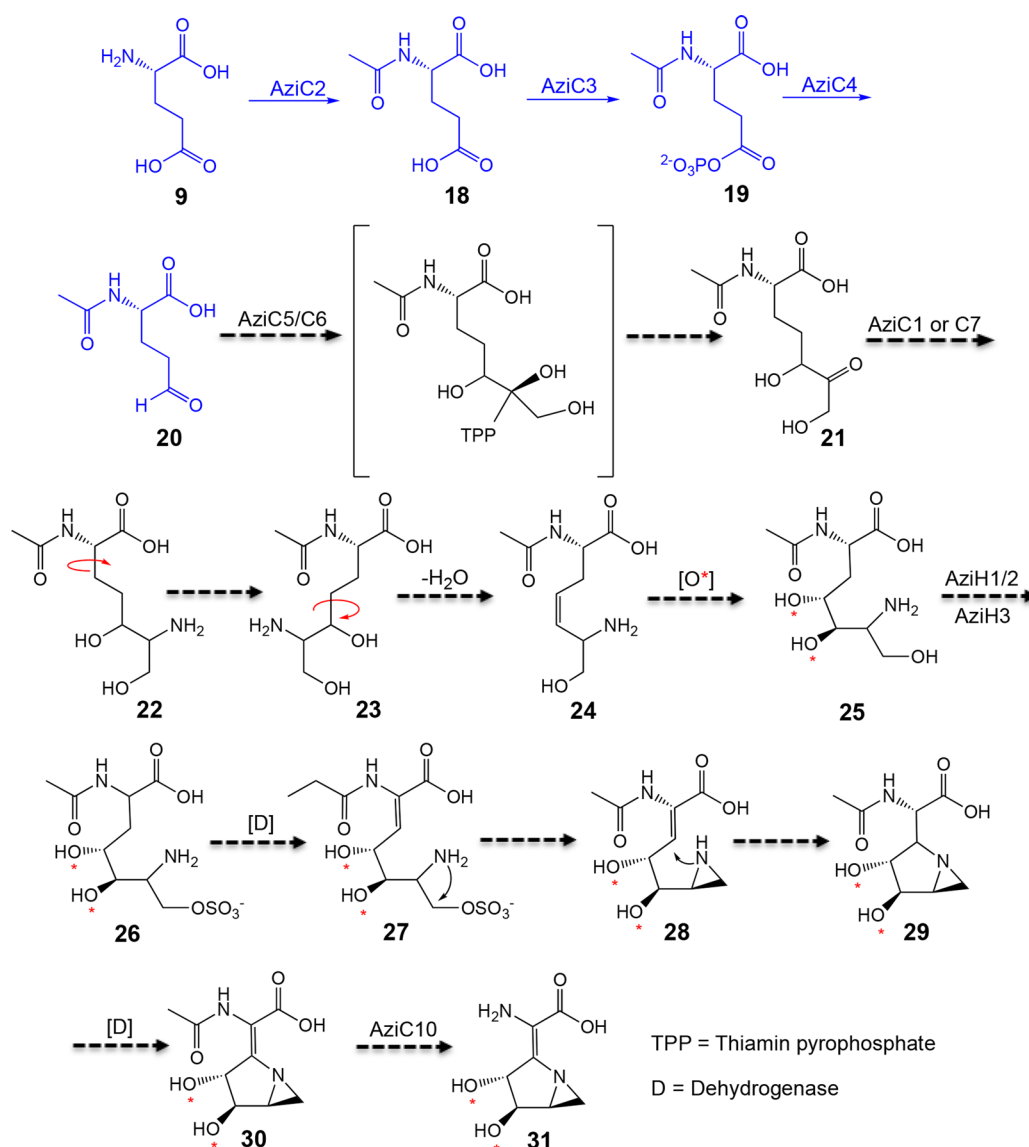


Figure 6. Proposed biosynthetic route to the azabicyclic.

presence of ATP and sulfate provide precedence for such a mechanism.²⁷ While aziridine formation might not necessitate sulfation for ring closure, the additional ring strain imposed by the formation of a bicyclic system might require the involvement of a more facile leaving group. Dehydrogenation would give the alkene **30**, with deacetylation **31** resulting in the formation of the azabicyclic ring.

EXPERIMENTAL SECTION

Instrumentation and General Methods. Azinomycin production was monitored by thin layer chromatography (TLC), performed using glass-backed silica gel plates, Analtech (#47011). Flash column chromatography was performed using 60 Å Silica Gel (Silicycle, 230–400 mesh) as a stationary phase. ¹H and ¹³C{¹H}NMR spectra were recorded on either a Varian Inova 500 or Varian Inova 300. ¹H NMR chemical shifts are reported as δ values in ppm relative to CDCl₃ (7.26 ppm), and coupling constants (*J*) are reported in hertz (Hz). Infrared spectra were recorded on a Bruker Tensor 27 spectrometer. Unless indicated otherwise, deuteriochloroform (CDCl₃) served as an internal standard (77.0 ppm) for all ¹³C{¹H} spectra. Mass spectra (ESI) were obtained at the Laboratory for Biological Mass Spectrometry at the Department of Chemistry, Texas A&M University, with an 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 a Fermentation Design Inc. Model # MS21 (Allentown, PA, USA). The total capacity of the fermentation system is 15 L.

Materials. The ¹⁸O₂ was obtained from ICON Services Summit, NJ 07901.

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

Culture Conditions. Same as reported in Kelly et al. 2008.¹⁸

Spore Stocks. *Streptomyces sahachiroi* spores prepared from dehydrated GYM (glucose, yeast extract, and maltose extract) plates (per liter of medium: glucose monohydrate, 4 g; yeast extract, 4 g; malt extract, 10 g; CaCO₃, 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 gray spores were removed with sterile water and agitated. The spores were then filtered through sterile cotton, washed three times with sterile water, centrifuged at 3000 rpm, resuspended in a minimal amount of 10% glycerol solution, flash frozen, and stored at –80 °C.

Solid Medium. Solid medium cultures were inoculated by streaking a loop full of *S. sahachiroi* spore stock onto prepared GYM plates. The plates were grown at 30 °C in a Fisher Scientific Isotemp incubator for 5–7 days before use in the first stage culture.

First Stage Culture. *Streptomyces sahachiroi* (inoculated from dehydrated plates) was grown on GYM plates for 5–7 days. A 1 cm² piece of the GYM plate was used to inoculate 100 mL of PSS media in a 250 mL Erlenmeyer flask. The culture was incubated at 30 °C for 24 h at 250 rpm.

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

Fermentation. The fermenter contained 10 L of reduced PSS medium [Pharmamedia/Starch plus additives, per liter of medium: Pharmamedia (yellow cotton seed flour), 12.5 g; soluble starch, 12.5 g and deionized water, adjusted to pH 6.5 with 1 M NaOH prior to sterilization]. The reduced PSS medium contains 25% of the concentration of the first and second stage cultures. The 10 L of reduced PSS medium were 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 at room temperature.

General Feeding Conditions. *Oxygen* ¹⁸O₂/¹⁶O₂ Recirculation System. To examine the uptake of dioxygen during the course of azinomycin biosynthesis, the wild-type *Streptomyces sahachiroi* (ATCC 2485) was grown in a closed system described by Vederas^{19,28} to allow replacement of consumed oxygen with a mixture of ¹⁸O₂ and ¹⁶O₂. *Streptomyces sahachiroi* culture was grown under normal atmospheric conditions until major azinomycin B production had begun, at ca. 18 h of growth. It remained under a mixed atmosphere for approximately 50 h until the natural product was isolated as described in Kelly et al.¹⁸ A total of 3.7 L of ¹⁸O₂ was consumed during the course of the experiment.

Extraction and purification of azinomycin B from the media supernatant afforded approximately 35.9 mg of purified natural product. The sample was analyzed by proton decoupled ¹³C NMR spectroscopy, as isotopic substitution with ¹⁸O gives rise to an upfield shift in the position of resonances of directly bound carbon atoms.^{19,28} The ¹³C{¹H} NMR spectrum of ¹⁸O/¹⁶O-labeled azinomycin B revealed that 4 of the 11 oxygen atoms were derived from molecular oxygen corresponding to those at carbon centers C3', C3'OCH₃, C21, C19, C13, C14, and C12.

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 CH₂Cl₂/hexane. The resulting suspension was centrifuged at 1500 rpm, and the supernatant was discarded. Diethyl ether (2 mL) was added to the pellet, which was subsequently agitated and centrifuged at 3000 rpm, and the supernatant was 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 was retained. To the solution was added hexanes (4 mL), and the suspension was 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 CH₂Cl₂/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. For shorter periods of time it may be stored as a precipitant from a solution of 20:1 hexanes–chloroform/dichloromethane. The

azinomycin B isolated matched the NMR spectrum provided by Yokoi et al.^{7,29}

Azinomycin B Characterization. Pale-white amorphous powder (1:9 CH₂Cl₂/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^{–1}; ¹H NMR (300 MHz, CDCl₃) 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); ¹³C{¹H} NMR (75 MHz, 125 MHz, CDCl₃) 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. (* obscured by CDCl₃ solvent peak). ¹³C{¹H} NMR (125 MHz, CD₂Cl₂) δ 191.3, 172.8, 165.8, 164.3, 162.2, 156.2, 154.1, 150.6, 134.6, 133.7, 128.4, 127.9, 126.9, 125.3, 123.8, 122.3, 191.3, 118.6, 108.4, 84.5, 76.9, 77.0, 56.1, 55.8, 53.8, 46.9, 36.9, 24.4, 20.9, 20.1, 17.1. APCI-MS (LRMS) 624.2, found 624.2.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.8b03007.

¹H and ¹³C{¹H} NMR spectra of natural and ¹⁸O labeled azinomycin; APCI and ESI-MS of natural and ¹⁸O labeled azinomycin; Fermenter oxygen recirculation diagram (PDF)

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Notes

The authors declare no competing financial interest.

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