SUPPORTING INFORMATION

Aminoacetone as the Penultimate Precursor to the Antitumor Agent Azinomycin A

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Experimental Section

FramePlot and alignment details:

S. sahachiroi genomic sequence was analyzed using FramePlot 3.0beta and NCBI BLAST. The protein sequences for L-threonine-3-dehydrogenase and 2-amino-3-ketobutyrate CoA ligase from other *Streptomyces* species were obtained through StrepDB, the Streptomyces Annotation Server (http://strepdb.streptomyces.org.uk/cgi-bin/search_by_kegg_pathway.pl), which organizes genes according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.[1-3] Alignments were performed with CLUSTALW.[4]

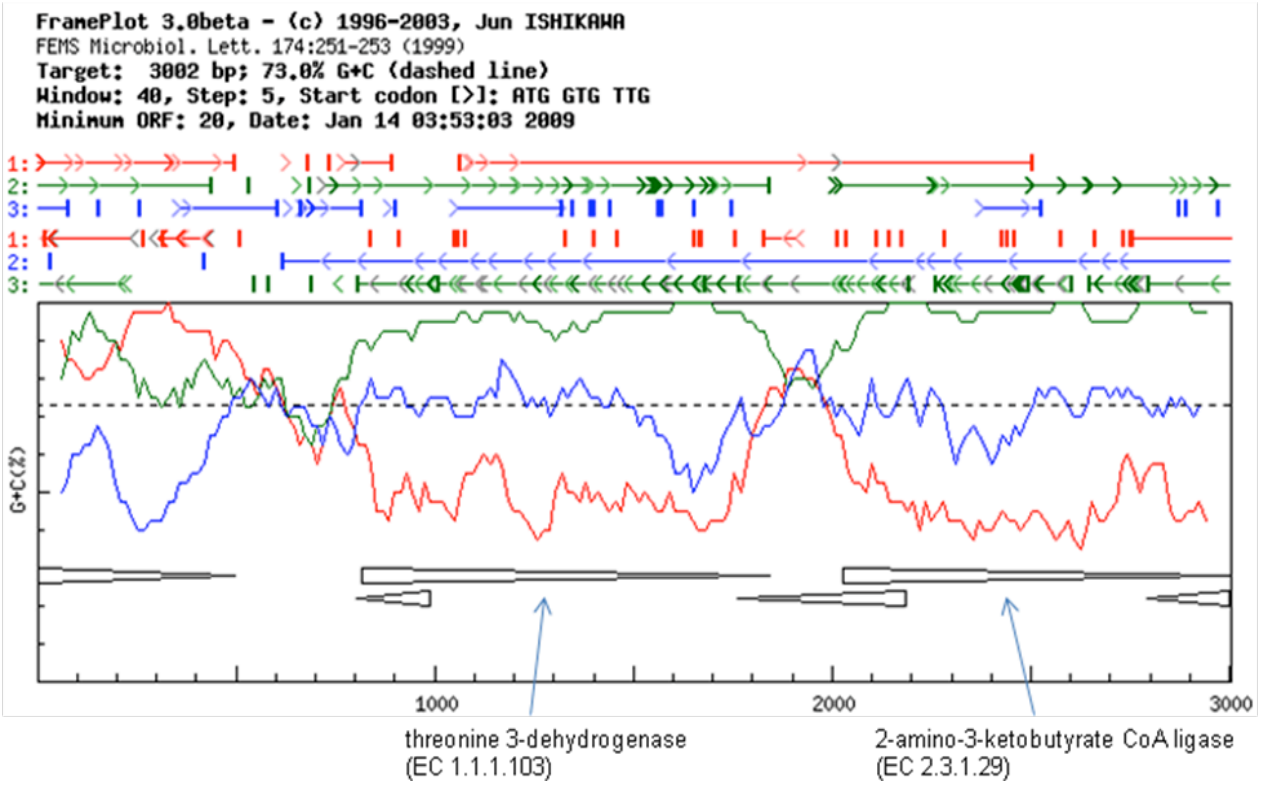


Figure 1. FramePlot analysis of a contig from genomic sequencing of *S. sahachiroi*.

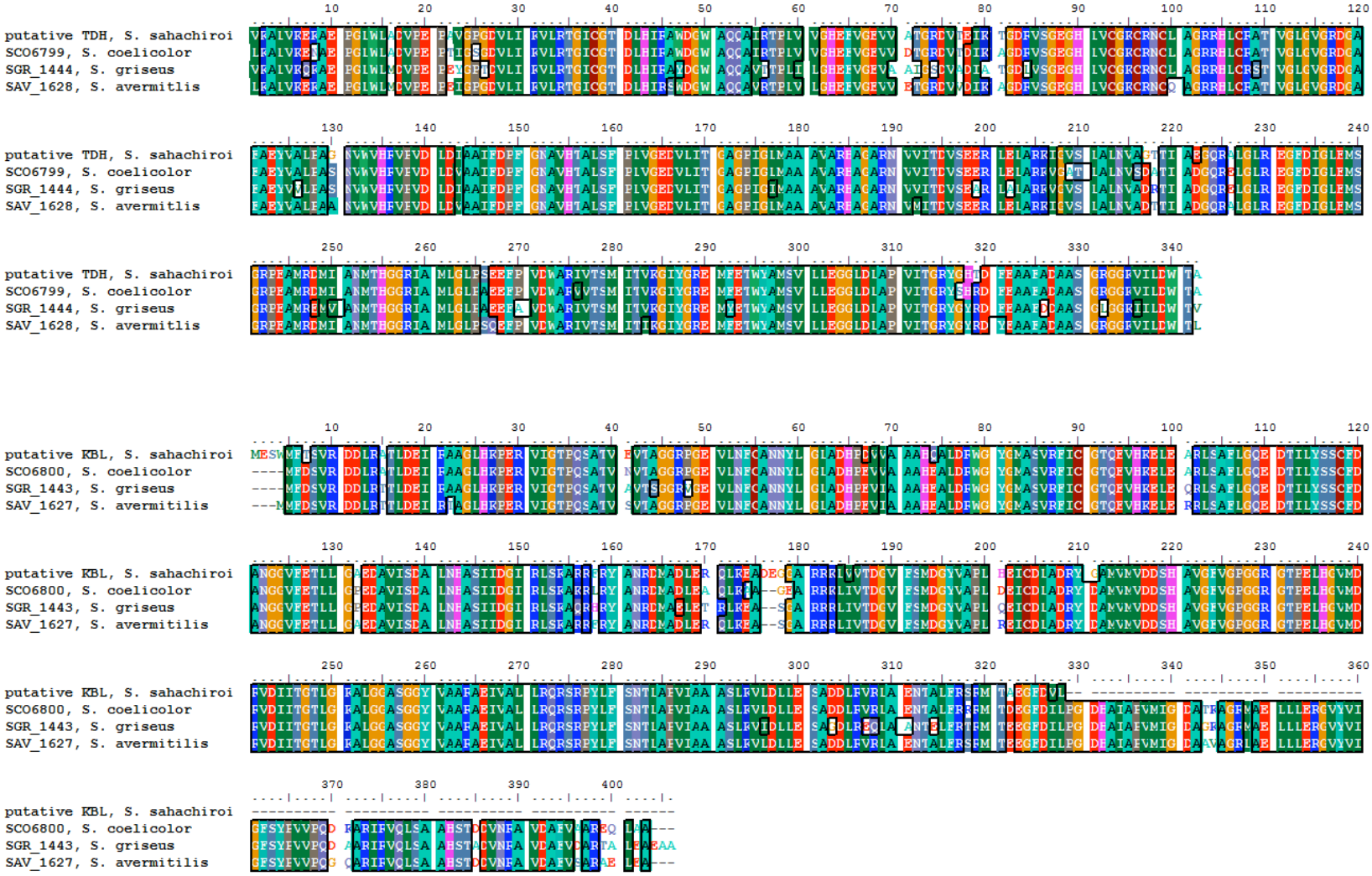


Figure 2. CLUSTALW alignment of threonine-3-dehydrogenases and 2-amino-3-ketobutyrate CoA ligases in *Streptomyces*.

Table 1. Feeding results for [^{15}N]-threonine and [^{15}N]-glycine

Entry	Compound	% incorporation in azinomycin A	% incorporation in azinomycin B
1	[^{15}N]-threonine	0.65	5.5*
2	[^{15}N]-glycine	1.49	1.41

% incorporation =[(A-B)/B] X 1.10 where A, intensity of peak for labeled material; B, intensity of peak for unlabeled material for M+H+1 as normal mass ratios; 1.10, natural abundance of ^{13}C; n.d.= not detected by APCI Mass Spectrometry. *Detected by ^{1}H NMR seen in Supporting Information Figure 3**.**

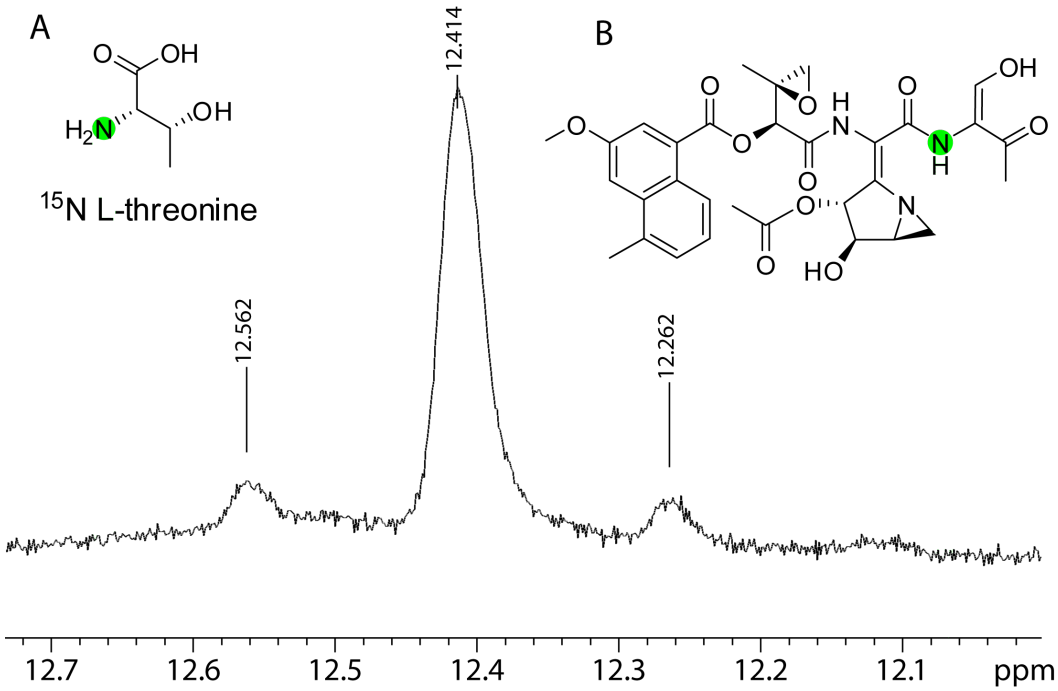


Figure 3. ^{1}H NMR of [^{15}N]-L-threonine incorporation into azinomycin B.
The incorporation resulted in splitting of the proton signal at 12.414 (1H, d, *J*=90Hz)ppm. Spectrum taken on a Varian Inova 500 MHz instrument in $CDCl_3$.

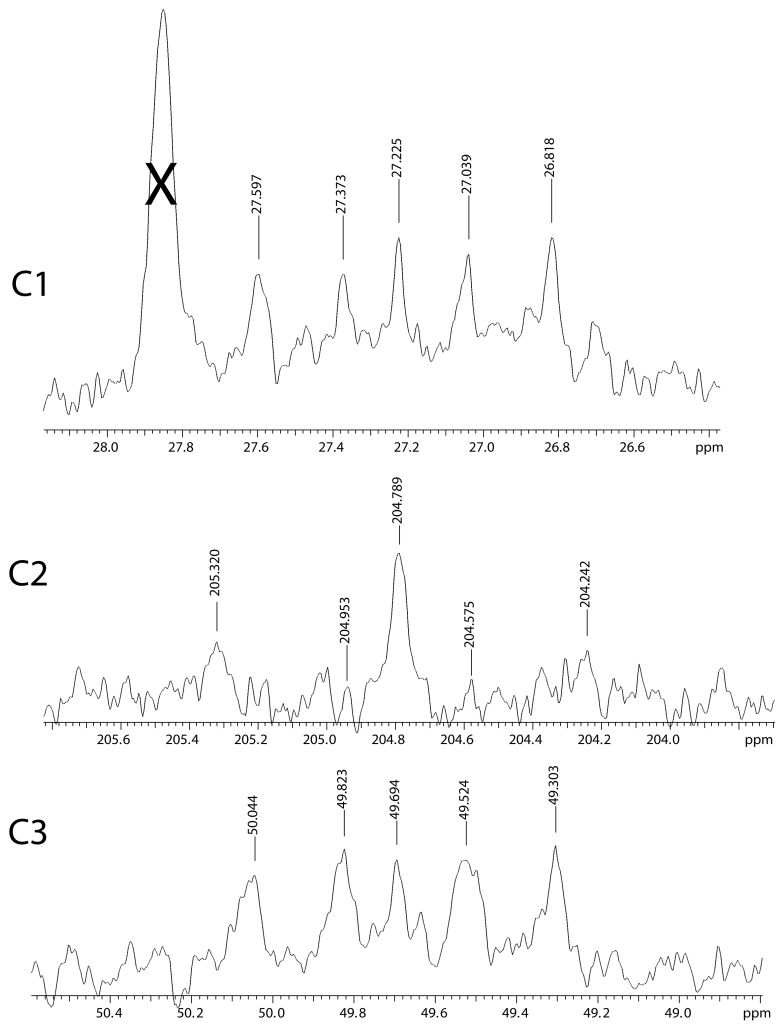


Figure 4. ^{13}C NMR of [U-^{13}C]-L-threonine incorporation into azinomycin A at carbons C1-C3.
Spectra taken on a Varian Inova 300 instrument at 75 MHz in $CDCl_3$.

Experimental Section

a) Synthesis. Reactions were carried out in flame-dried glassware under a nitrogen or argon atmosphere, unless otherwise noted. All ^{2}H (D), ^{13}C, and ^{15}N labeled starting materials were obtained from Cambridge Isotope Laboratories, Inc. Andover, MA 01810-5413. Chemical reagents were purchased from Sigma-Aldrich, Milwakee, WI. Anhydrous solvents were dried over neutral alumina (MBraun system). All other solvents or reagents were used directly. All reactions were magnetically stirred, monitored by thin layer chromatography (TLC), and performed using glass-backed silica gel plates (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 & ^{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 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 in 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). All ^{2}H (D), ^{13}C, and ^{15}N materials were obtained from Cambridge Isotope Laboratories, Inc. Andover, MA 01810-5413.

Ethyl 2-Chloroacetoacetate (7). Sulfuryl chloride (322μL) was added drop-wise by dropping funnel to ethyl acetoacetate (500 mg) maintained at 0 °C. The mixture was stirred overnight at room temperature. Product 7 was obtained as a slightly yellow liquid by concentrating the reaction *in vacuo*. Both the ^{1}H NMR spectrum and the ^{13}C NMR spectrum showed a keto-enol mixture of ethyl 2-chloroacetoacetate. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.76 (m, 1H), 4.18-4.27 (m, 4H (keto/enol)), 2.38 (s, 3H), 2.19 (s, 3H (enol)), 2.16 (s (br), OH, 1H (enol)), 1.30 (m, 6H (keto/enol)). ^{13}C NMR (75 MHz, $CDCl_3$): δ 196.8, 172.7, 169.3, 164.9, 96.6, 63.1, 61.3, 26.2, 20.1, 14.1.

Chloroacetone (8). A solution of ethyl 2-chloroacetoacetate, **7** (0.62g, 3.74 mmol) dissolved in 3 mL of THF was mixed with 0.27 mL water. Concentrated H_2SO_4 (2 eq.) was added and the reaction mixture was refluxed for 40 h. Subsequently, the mixture was cooled to room temperature followed by the addition of 15 mL of water and 25 mL of diethyl ether. The aqueous layer was extracted with 50 mL (3X) of diethyl ether. The combined organic layers were washed with saturated $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4, filtered and diethylether was then removed via distillation at atmospheric pressure due to volatility of the product. The resulting solution of chloroacetone **8** in THF was used in the following step. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.09 (s, 2H), 2.30 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.5, 48.9, 27.2.

N-acetonyl phthalamide (9). In a dried flask, to a solution of chloroacetone, **8** (1g, 10.8 mmol) in DMF (10 mL) was added potassium phthalimide (2.20 g, 11.8 mmol) with constant stirring (potassium phthalimide was not completely soluble in the DMF). The reaction was stirred at room temperature for 16 h and monitored by TLC. After the reaction was complete, the reaction mixture was poured into water (250 mL). Ivory-white colored solid (**9**) precipitated (2.17 g, 99.3%) which was filtered and dried. ^{1}H NMR ($CDCl_3$, 300 MHz) 2.25(s, 3H), 4.48 (s, 2H), 7.72(dd, 2H, J_1= 2.7 Hz, J_2=5.4 Hz), 7.85(dd, 2H, J_1= 2.7 Hz, J_2=5.4 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 27.1, 47.3, 123.9, 132.2, 134.6, 167.8, 199.9. IR (NaCl, thin film) cm^{-1}: 3055.6, 2925.3, 2860.2, 1770.7, 1729.3, 1616.7, 1418.4, 1190.8. HRMS (ESI+) $C_{11}H_9NO_3$ (M+Li), 210.0742, found, 210.0739.

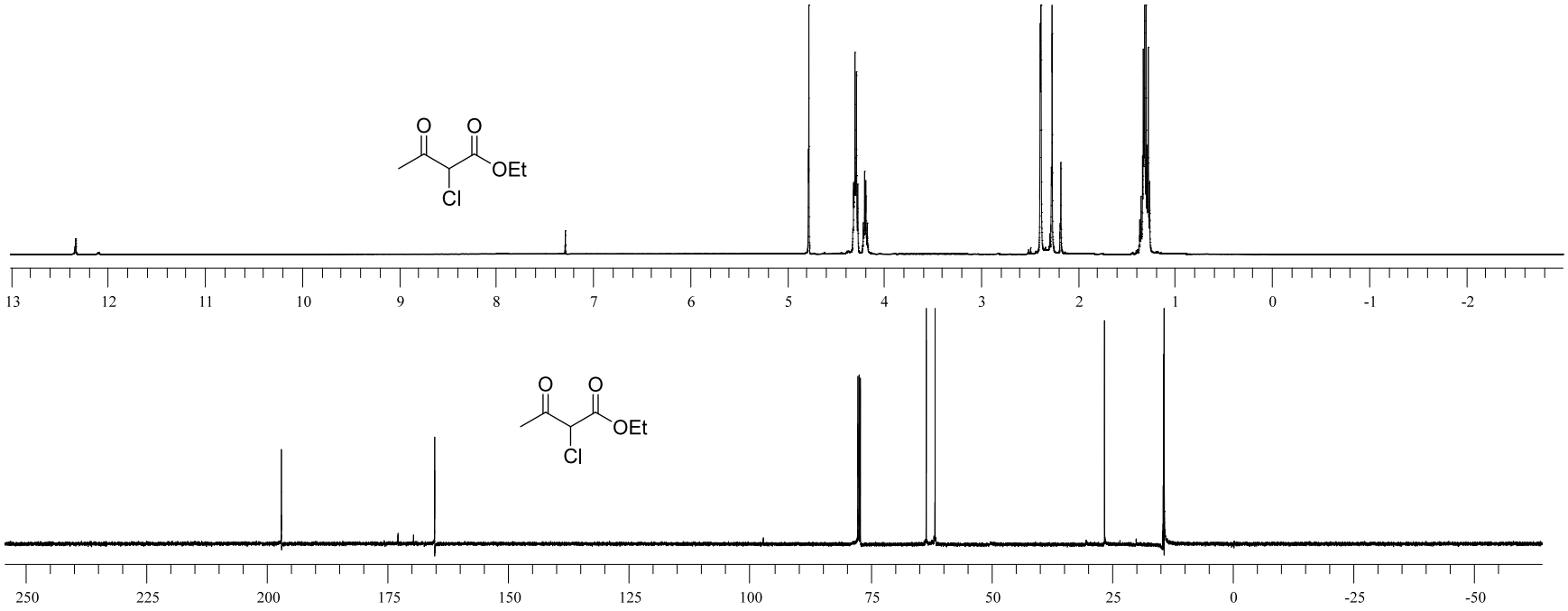
Aminoacetone hydrochloride (5). A suspension of N-acetonylphthalimide, **9** in an aqueous solution (1:1) of conc. HCl was heated to reflux for 7h. The solution was cooled and the precipitated phthalic acid was filtered and washed once with cold water (2 mL). The solvent was evaporated from the filtrate giving the desired product **5** in 77% yield as its hydrochloride salt. ^{1}H NMR (D_2O, 300MHz) 2.10 (s, 3H), 3.92 (s, 2H), 6.70 (br, NH_2, exchangeable). ^{13}C NMR (D_2O, 75 MHz) δ 26.4, 47.4, 203.3. IR (NaCl, thin film) cm^{-1}: 3422.7(br), 2919.4, 2848.3, 1729.3, 1590.1; MS (ESI+) C_3H_7NO (M+H), 74.0606, found, 74.0609. *Note: the product was freeze dried and kept at -20 ^{o}C away from light for prolonged storage.*

b) ^{1}H and ^{13}C Spectra of Compounds 5, 7, 8, 9

Compound 7

^{1}H NMR, 300 MHz, $CDCl_3$

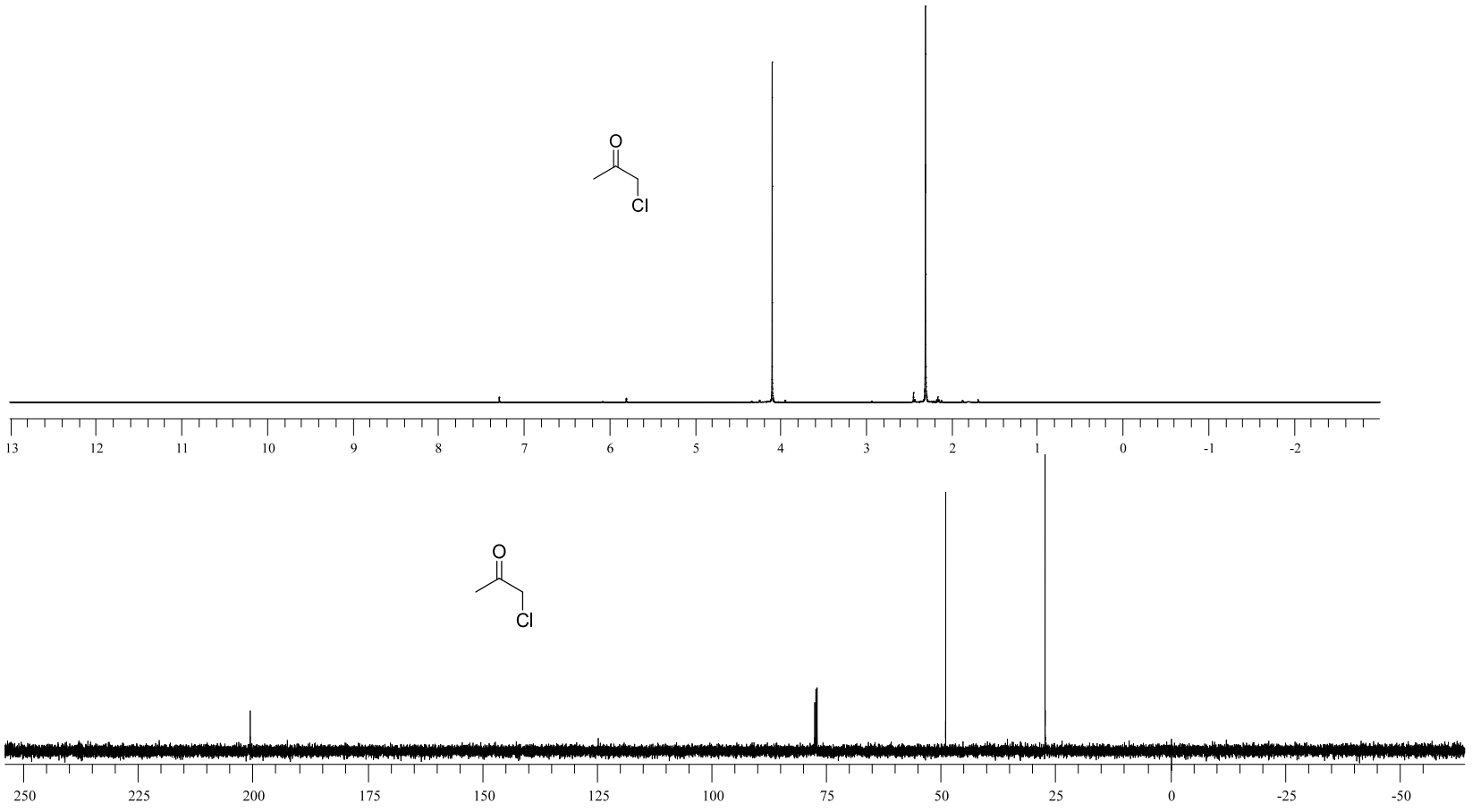
^{13}C NMR, 75 MHz, $CDCl_3$



Compound 8

^{1}H NMR, 500 MHz, $CDCl_3$

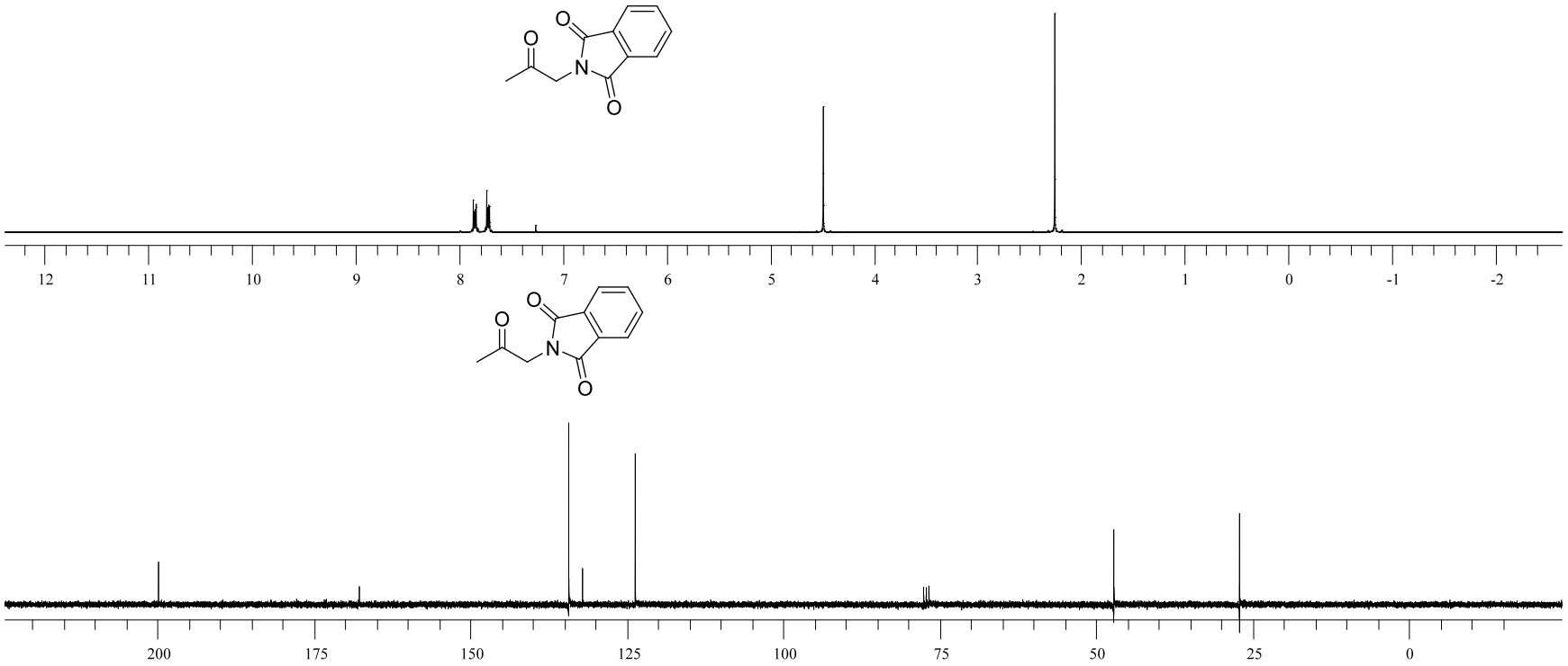
^{13}C NMR, 125 MHz, $CDCl_3$



Compound 9

^{1}H NMR, 300 MHz, $CDCl_3$

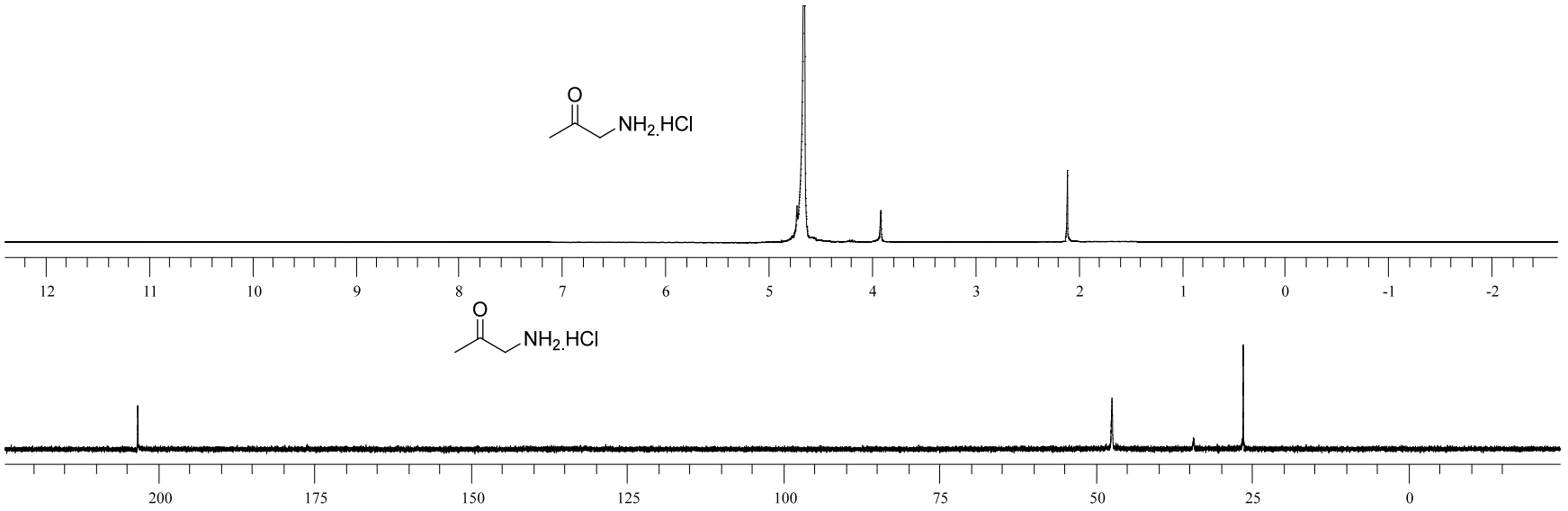
^{13}C NMR, 75 MHz, $CDCl_3$



Compound 5

^{1}H NMR, 300 MHz, D_2O

^{13}C NMR, 75 MHz, D_2O



c) Feeding Studies

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

Culture and feeding conditions. Fermentations were performed on Fermentation Design Inc. Model # MS21 (Allentown, PA, USA). The total capacity of the fermentation system is 15 L. S. *sahachiroi* was cultured as reported previously in Kelly *et al*. 2008. [13] The labeled compounds were fed as per the general feeding regimen detailed in Supporting information 7A.

Isolation and purification of azinomycin A and B. Isolation follows, with a few variations, Nagoaka, *et al*.[1] Following fermentation, the cultures were centrifuged at 7,000 rpm at 4 ^{o}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 1,500 rpm and the supernatant discarded. Diethyl ether (2 mL) was added to the pellet, which was subsequently agitated and centrifuged at 3000 rpm. The diethyl ether soluble fraction was concentrated and chromatographed on silica gel using a solution of dichloromethane – methanol (50:1). A major fraction was concentrated to dryness from which azinomycin A was obtained. The diethyl ether insoluble residue was dissolved in dichloromethane (600 μL) to which hexanes (2 mL) was added. The heterogeneous mixture was centrifuged at 3,000 rpm and the supernatant retained. To the solution was added hexanes (4 mL) and the suspension centrifuged at 3,000 rpm to give azinomycin B as a solid.

If necessary, azinomycin B can be further purified by flash column chromatography (95: 5 CH_2Cl_2: methanol). By TLC azinomycin B 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 ºC under anhydrous diethyl ether. The resulting azinomycins, A and B, matched the NMR spectra provided by Yokoi *et al*.[2]

Complete Characterization of Azinomycin A and B

Azinomycin A. Pale-white amorphous powder (1:9 CH_2Cl_2:hexane); IR (neat) ν_{max} 3450.1, 3323.2, 3025.3, 2972.8, 1735.4(br), 1660.4, 1618.6, 1601.1, 1531.4, 1417.6 1242.3, 1090.7, 1043.6 cm^{-1}; ^1H NMR (300MHz, $CDCl_3$) δ 10.09(1H, dd, *J*=5.1, 5.1), 8.56 (1H, dd, *J*=5.9, 3.3Hz,), 8.54 (1H, s), 7.93 (1H, d, *J*=2.6Hz), 7.48 (1H, d, *J*=2.6Hz), 7.35 (1H, dd, *J*=5.9, 5.9), 7.34 (1H, dd, *J*=5.9, 3.3), 5.53 (1H, d, *J*= 3.8Hz), 5.01 (1H, s), 4.62 (1H, dd, *J*= 5.4, 3.8Hz), 4.28 (1H, dd, *J*= 19.8, 5.1Hz), 3.98 (3H, s), 3.98 (1H, s), 3.23 (1H, m), 3.00 (1H, d, *J*=4.4Hz), 2.84 (1H, d, *J*= 4.4Hz), 2.67 (3H, s), 2.53 (1H, d, *J*= 5.1Hz), 2.21 (1H, d, *J*= 4.5Hz), 2.20 (1H, s), 2.19 (1H, s), 1.52 (1H, s); ^{13}C NMR (75MHz, $CDCl_3$) δ 202.6, 172.7, 165.7, 163.8, 163.2, 156.0, 149.6, 134.4, 133.1, 128.4, 127.7, 126.9, 125.1, 123.9, 121.8, 120.1, 108.6, 84.0, 76.9*, 76.7*, 56.0, 55.6, 53.7, 50.6, 45.4, 35.8, 27.2, 20.7, 20.0, 17.0. (* obscured by $CDCl_3$ solvent peak). TOF-MS (APCI), m/z 596.22 (calcd for $C_{30}H_{33}N_3O_{10}$+ H: 596.22).

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^{-1}; ^1H NMR (300MHz, $CDCl_3$) δ 12.40(1H, br), 12.32(1H, s), 8.54 (1H, dd, *J*=3.6, 7.0Hz,), 8.20 (1H, br), 7.94 (1H, d, *J*=2.9Hz), 7.46 (1H, d, *J*=2.9Hz), 7.32 (1H, s), 7.32 (1H, s), 7.32 (1H, s), 5.50 (1H, d, *J*= 4.0Hz), 5.12 (1H, s), 4.64 (1H, dd, *J*= 4.0, 4.8Hz), 3.96 (3H, s), 3.96 (1H, br), 3.36 (1H, m), 2.98 (1H, d, *J*=4.3Hz), 2.80 (1H, d, *J*= 4.3Hz), 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 (75MHz, $CDCl_3$) 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. (* obscured by $CDCl_3$ solvent peak). TOF-MS (APCI), m/z 624.21 (calcd for $C_{31}H_{33}N_3O_{11}$+ H: 624.21).

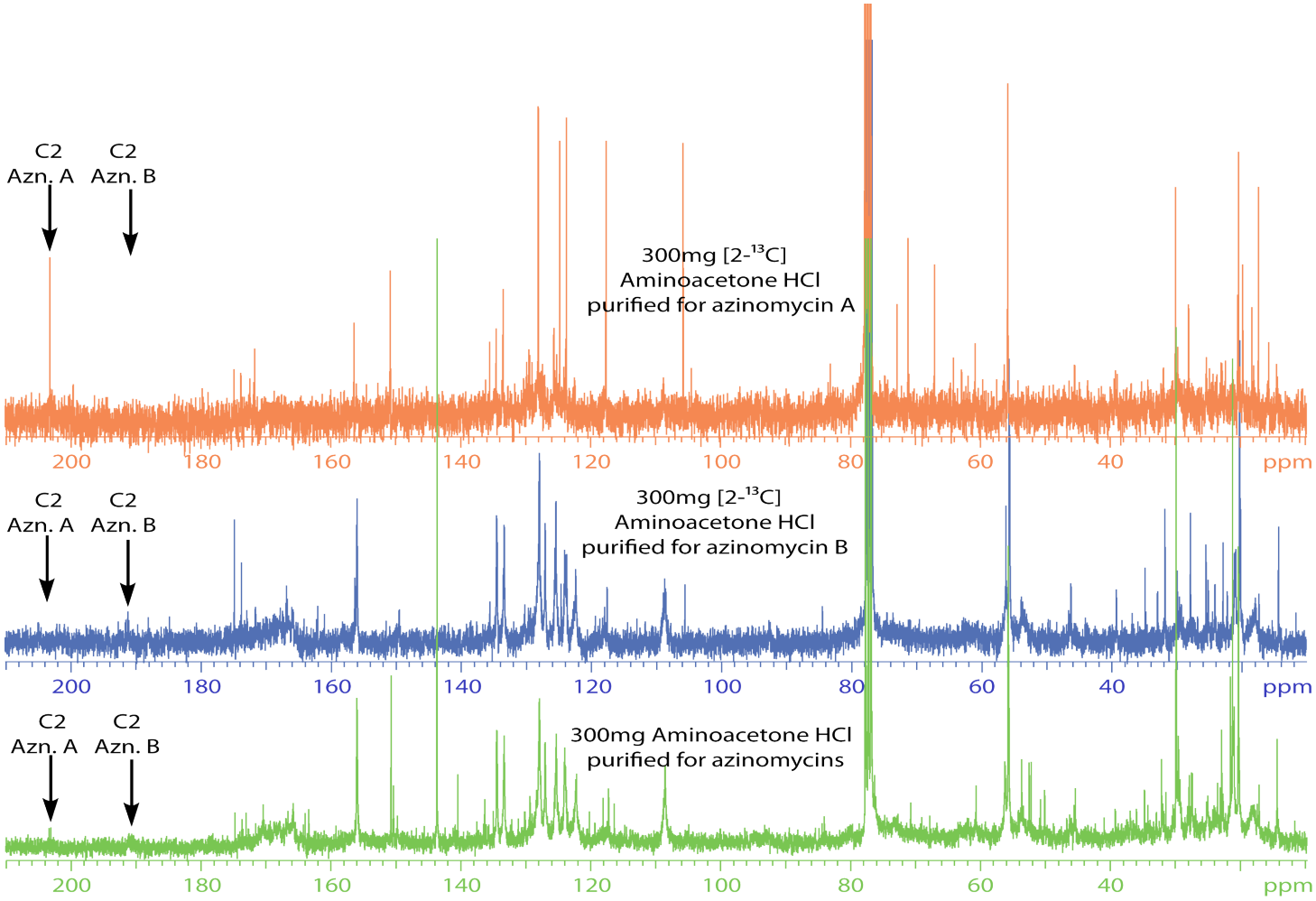


Figure 5. Spectral overlay depicting results arising from feeding 300 mg aminoacetone: [2-^{13}C]-aminoacetone derived azinomycin A, [2-^{13}C]-aminoacetone derived azinomycin B, and unlabeled aminoacetone derived azinomycins. Spectra taken on a Varian Inova 300 instrument at 75 MHz in $CDCl_3$.

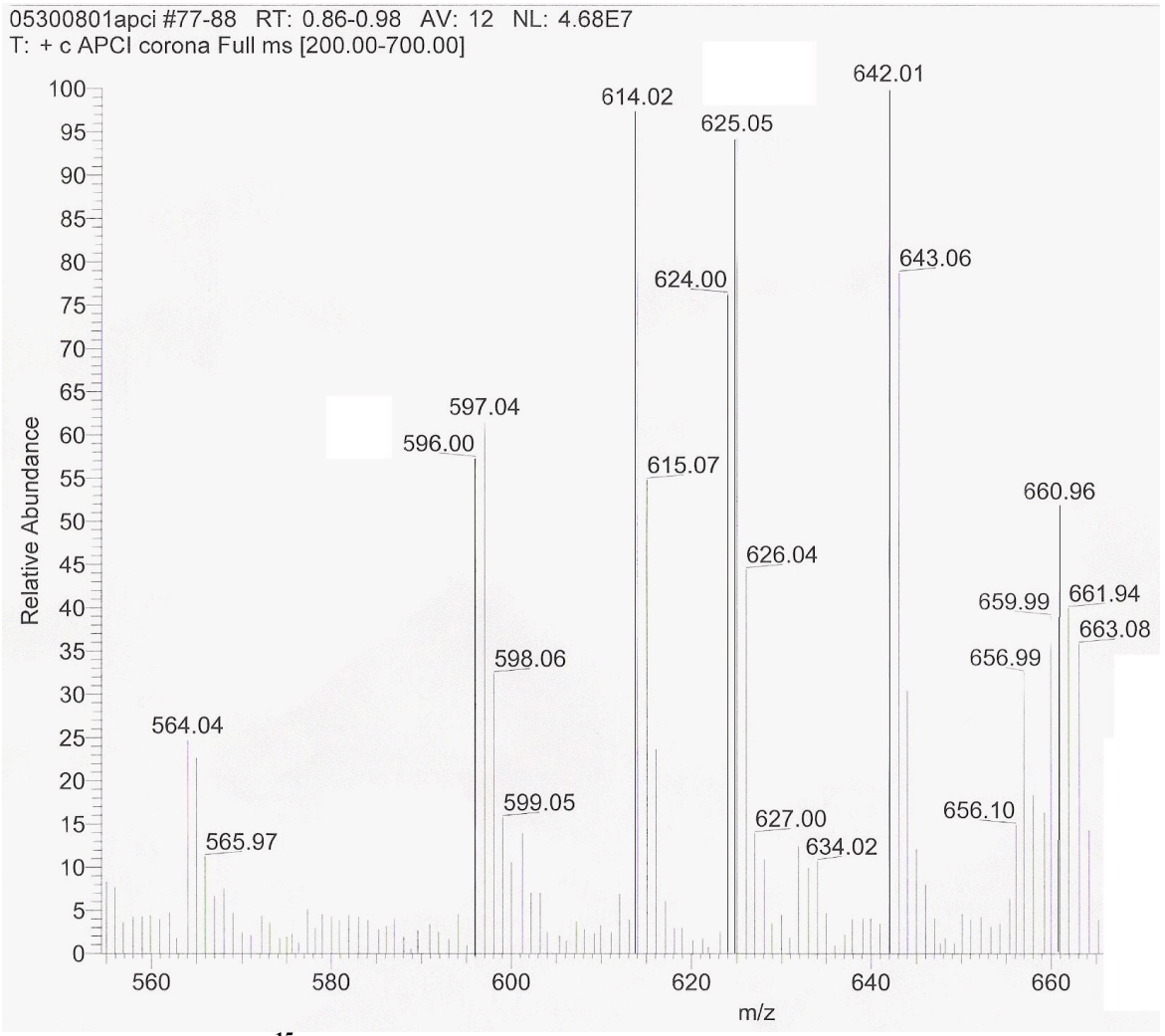


Figure 6. APCI-Mass Spectrometry for [^{15}N]-glycine incorporation into azinomycin A & B
Azinomycin A (M+H = 596, M+H+H_2O = 614) & azinomycin B (M+H = 624, M+H+H_2O = 642, M+H+2H_2O = 660).

Figure 7A. Feeding details and conditions of all compounds fed

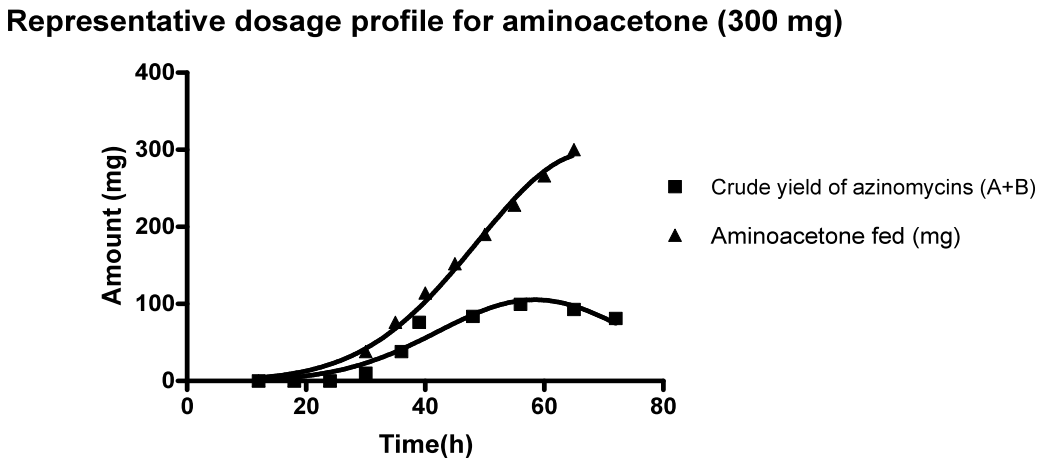
Entry	Compound	Total amount fed
1	[U-^{13}C]-L-threonine	100 mg; 0.8 mmol per 10L culture
2	[^{15}N]-L-threonine	230 mg; 1.9 mmol per 10L culture
3	[U-^{13}C]-2-amino-3-ketobutyrate	114 mg; 0.9 mmol per 10L culture
4	[2-^{13}C]-glycine	1 g; 13.1 mmol per 10L culture
5	[1-^{13}C]-glycine	1 g; 13.1 mmol per 10L culture
6	[1-^{13}C]-glycine	1 g; 13.1 mmol per 10L culture
7	[^{15}N]-glycine	1 g; 13.1 mmol per 10L culture
8	[2,2-D2]-glycine	1 g; 13.1 mmol per 10L culture
9*	[2-^{13}C]-aminoacetone·HCl	0.3 g; 2.7 mmol per 10L culture
10**	aminoacetone·HCl	0.3 g: 2.7 mmol per 10L culture
11**	aminoacetone·HCl	0.7 g; 6.4 mmol per 10L culture
12**	aminoacetone·HCl	1.0 g; 11.4 mmol per 10L culture

Note: Entrys 1-8 were administered as described previously in Ref. 13. Briefly, 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 aliquot 24 h later. The culture was harvested 72 h post-induction (inoculation of the second stage culture into the fermenter).

* Entry 9: Fed in 72 lots of 3 mg/mL each, beginning 24 h after fermenter inoculation (T=0, first feeding). The feeding regimen continued through 72 h post inoculation (i.e. T=48 h, final feeding) (see Figure 7B, S 14). This procedure allows for maximum production of the azinomycins.

**Entry 10-12: The compound was dissolved in autoclaved distilled water and aliquoted in eight equal portions at 3 mg/ml. The first aliquot was administered after 24 h, followed by addition of subsequent aliquots every 5 hours. The culture was harvested 72 h post-induction (inoculation of the second stage culture into the fermenter).

Figure 7B. Dosage profile for aminoacetone within the optimized range for production of azinomycins.



Supporting Information References

(1) Kanehisa, M.; Araki, M.; Goto, S.; Hattori, M.; Hirakawa, M.; Itoh, M.; Katayama, T.; Kawashima, S.; Okuda, S.; Tokimatsu, T.; Yamanishi, Y. *Nucleic Acids Res.* **2008**, *36*, D480-4.
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(3) Kanehisa, M.; Goto, S. *Nucleic Acids Res.* **2000**, *28*, 27-30.
(4) Chenna, R.; Sugawara, H.; Koike, T.; Lopez, R.; Gibson, T. J.; Higgins, D. G.; Thompson, J. D. *Nucleic Acids Res.* **2003**, *31*, 3497-3500.
(5) Kelly, G. T.; Sharma, V.; Watanabe, C. M. H. *Bioorg. Chem.* **2008**, *36*, 4-15.