Macrolide Biosynthesis. 7. Incorporation of Polyketide Chain Elongation Intermediates into Methymycin

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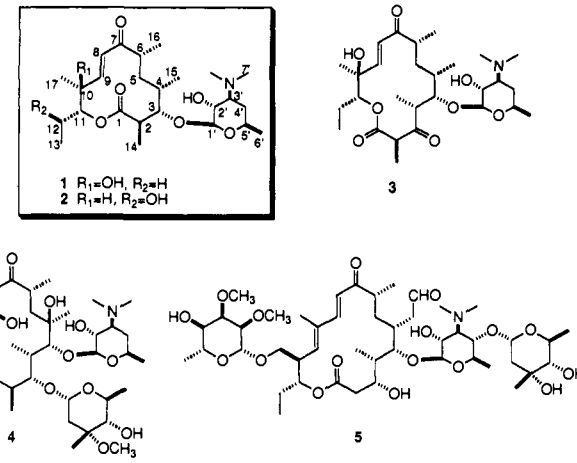
Abstract: Administration of [1-^{13}C]propionate to cultures of *Streptomyces venezuelae* SC 2366 gave methymycin (**1**), which was shown by ^{13}C NMR analysis to be labeled at the predicted sites, C-1, C-3, C-5, C-9, and C-11. Similarly, incorporation of [1,2-$^{13}C_2$]acetate gave methymycin labeled at C-7 and C-8. A series of presumptive intermediates of polyketide chain elongation was also successfully incorporated. Thus, feeding of (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl *N*-acetylcysteamine (NAC) thioester **7a** gave both methymycin (**1**) and neomethymycin (**2**) labeled as expected at C-10 and C-11. In a complementary experiment, (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester **7b** was incorporated into **1** and **2** without loss of deuterium. Finally, incorporation of (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoyl NAC thioester **10a** gave **1** and **2** labeled at C-8 and C-9. These results support a processive model of polyketide chain assembly in which the functionality and oxidation level are adjusted subsequent to each condensation step.

Methymycin (**1**) is a 12-membered macrolide antibiotic first isolated from *Streptomyces venezuelae* in 1954 by researchers at Squibb.[1a] Methymycin was the first macrolide to have its complete structure determined[1b] and the first to succumb to total synthesis.[1c] Methymycin is often accompanied by the cometabolite neomethymycin (**2**).[2] These metabolites show striking structural and stereochemical similarities to the 14-membered macrolides picromycin (**3**)[3] and erythromycin A (**4**) as well as the 16-membered macrolide tylosin (**5**), as first pointed out by Celmer (Chart I).[4] Extensive biosynthetic, enzymological, and molecular genetic investigations have established the polyketide origins of **4** and **5** and elucidated many of the details of the assembly of these functionally and stereochemically complex substances.[5] Significant new insights have recently come from cloning and sequencing and from partial expression of the structural genes encoding the formation of the parent erythromycin aglycone, 6-deoxyerythronolide B.[6] Two groups have independently demonstrated that the *eryA* gene is organized into three large (ca. 10 kb) open reading frames containing a series of domains apparently responsible for each of the microscopic steps of polyketide chain assembly and showing strong homology to analogous regions of fatty acid synthase genes. Remarkably, the organization of these domains appears to be collinear with the presumed order of the biochemical chain assembly steps. In the meantime, our own group and that of Hutchinson has demonstrated that it is possible to incorporate the *N*-acetylcysteamine (NAC) thioesters of putative intermediates of polyketide chain assembly intact into both erythromycin[7] and tylactone,[8] thereby providing strong biosynthetic evidence for the processive nature of reduced polyketide chain elongation. These techniques have subsequently been successfully applied by our own and several other groups in complementary studies of polyketide biosynthesis.[9] We now report the extension of these studies to the investigation of methymycin biosynthesis.

Chart I



Results

The 100.6-MHz ^{13}C NMR spectra of **1** and **2** were assigned using a combination of 1D and 2D NMR experiments including ^{13}C INEPT, ^{1}H COSY, and ^{1}H^{13}C HETCOSY (Table I). The lactonic (C-1, 175.2 ppm) and ketonic (C-7, 204.6 ppm) carbonyl signals for **1** were readily assigned on the basis of standard chemical shift parameters, as was the quaternary, hydroxyl-bearing carbon C-10 (74.1 ppm). The H-17 methyl protons appeared as a singlet at δ 1.35, and the N(CH$_3$)$_2$ signals at 2.27 were easily recognizable. The assignment of the corresponding ^{13}C NMR signals for C-17 (19.2 ppm) and N(CH$_3$)$_2$ (40.2 ppm) followed directly from the HETCOSY spectrum. The olefinic protons H-8

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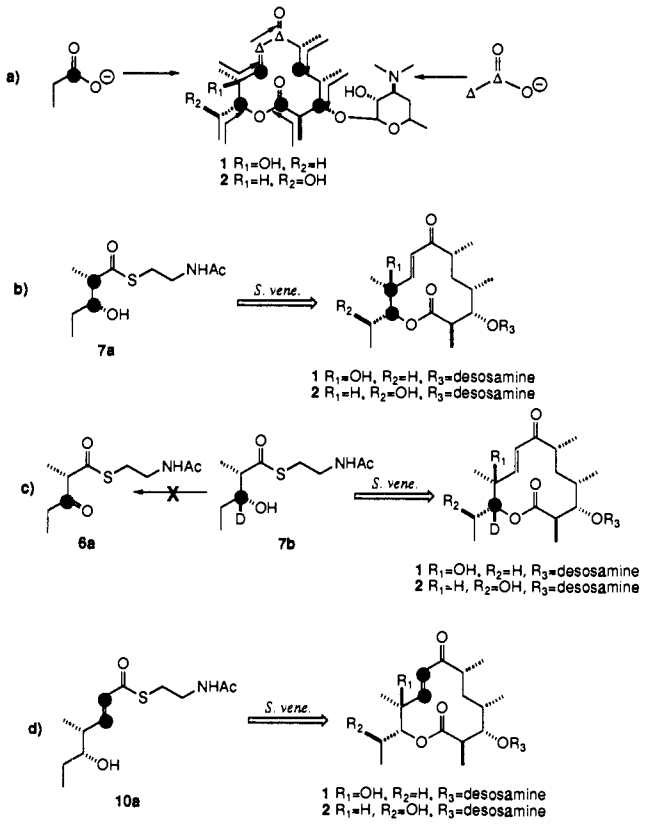
Table I. ^1H (400 MHz) and ^{13}C (100.6 MHz) NMR Spectral ($CDCl_3$) Data of Methymycin (**1**) and Neomethymycin (**2**)

1				**2**			
H	δ (m, *J*(Hz), area)	C	δ (m)	H	δ (m, *J* (Hz), area)	C	δ (m)
2	2.87 (dq, 6.9, 10.5, 1 H)	1	175.2 (s)	2	2.88 (dq, 6.9, 10.5, 1 H)	1	174.8 (s)
3	3.60 (d, 10.5, 1 H)	2	44.2 (d)	3	3.59 (d, 10.4, 1 H)	2	43.9 (d)
4	1.25 (m)	3	85.5 (d)	4	1.25 (m)	3	85.6 (d)
5a	1.75 (m)	4	33.6 (d)	5a	1.68 (m, 1 H)	4	33.4 (d)
5b	1.50 (m)	5	33.9 (t)	5b	1.4 (m, 1 H)	5	34.1 (t)
6	2.5 (m, 1 H)	6	45.1 (d)	6	2.52 (m, 1 H)	6	45.1 (d)
8	6.34 (d, 15.9, 1 H)	7	204.6 (s)	8	6.44 (dd, 1.3, 15.7, 1 H)	7	205.2 (s)
9	6.60 (d, 15.9, 1 H)	8	125.6 (d)	9	6.76 (dd, 5.5, 15.7, 1 H)	8	126.2 (d)
11	4.75 (dd, 10.8, 2.2, 1 H)	9	148.9 (d)	10	3.05 (m, 1 H)	9	147.1 (d)
12a	1.95 (m, 2.2, 7.2, 1 H)	10	74.1 (s)	11	4.79 (dd, 2.3, 9.0, 1 H)	10	35.4 (d)
12b	1.5 (m, 1 H)	11	76.3 (d)	12	3.89 (dq, 6.2, 9.0, 1 H)	11	75.4 (d)
13	0.90 (t, 7.4, 3 H)	12	21.2 (t)	13	1.20 (d, 6.2, 3 H)	12	66.4 (d)
14	1.44 (d, 7.0, 3 H)	13	10.6 (q)	14	1.41 (d, 7.0, 3 H)	13	21.0 (q)
15	1.02 (d, 6.7, 3 H)	14	16.1 (q)	15	1.02 (d, 6.7, 3 H)	14	15.9 (q)
16	1.17 (d, 7.0, 3 H)	15	17.3 (q)	16	1.19 (d, 7.0, 3 H)	15	17.4 (q)
17	1.35 (s, 3 H)	16	17.5 (q)	17	1.16 (d, 6.8, 3 H)	16	17.6 (q)
1′	4.24 (d, 7.3, 1 H)	17	19.2 (q)	1′	4.24 (d, 7.3, 1 H)	17	9.8 (q)
2′	3.22 (dd, 7.3, 10.2, 1 H)	1′	105.0 (d)	2′	3.23 (dd, 7.3, 10.2, 1 H)	1′	105.1 (d)
3′	2.5 (m, 1 H)	2′	70.3 (d)	3′	2.52 (m, 1 H)	2′	70.3 (d)
4a′	1.65 (m, 1 H)	3′	65.8 (d)	4a′	1.68 (m, 1 H)	3′	65.9 (d)
4b′	1.2 (m, 1 H)	4′	28.3 (t)	4b′	1.2 (m, 1 H)	4′	28.3 (t)
5′	3.5 (m, 1 H)	5′	69.4 (d)	5′	3.48 (m, 1 H)	5′	69.5 (d)
6′	1.23 (d, 6.1, 3 H)	6′	21.1 (q)	6′	1.23 (d, 6.1, 3 H)	6′	21.1 (q)
7′	2.27 (s, 6 H)	7′	40.2 (q)	7′	2.28 (s, 6 H)	7′	40.2 (q)

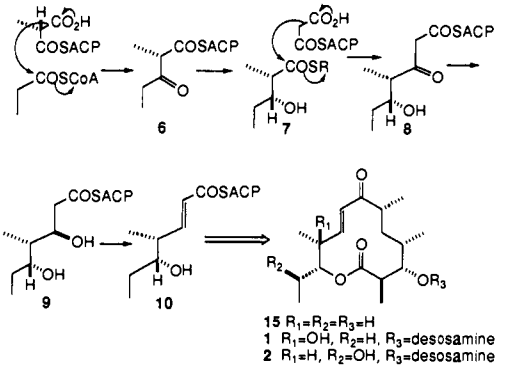
Scheme I



and H-9 appeared as components of an AB quartet at δ 6.34 and 6.60, respectively, correlated with the corresponding ^{13}C NMR signals at 125.6 (C-8) and 148.9 ppm (C-9). The assignments of the remaining macrolide and desosaminyl[10] ^1H and ^{13}C NMR signals were completed in a straightforward manner and are summarized in Table I along with the corresponding assignments for neomethymycin (**2**).

Preliminary incorporation experiments confirmed the expected polyketide origin of methymycin.[11,12] Administration of [1-^{13}C]propionate to cultures of *Streptomyces venezuelae* SC2366 gave methymycin (**1**) which was shown by ^{13}C NMR analysis to be labeled at the predicted sites (C-1, C-3, C-5, C-9, C-11) (20 excess atom % ^{13}C) (Scheme Ia). Similar feeding of sodium [1,2-$^{13}C_2$]acetate gave rise to the expected enhanced and coupled doublets (J_{CC} = 50.3 Hz) corresponding to C-7 and C-8. Thus methymycin was shown to be derived from five propionates and one acetate precursor.

Scheme II



According to the emerging picture of polyketide chain elongation, formation of the macrolide aglycone of methymycin should be initiated by condensation of a propionyl thioester starter unit with an equivalent of (2*R*)-methylmalonyl CoA[13] (as the enzyme-bound ACP thioester) (Scheme II). Reduction of the resulting β-keto ester **6** will give (2*S*,3*R*)-2-methyl-3-hydroxypentanoate as the enzyme-bound thioester **7**. Indeed, this same intermediate has already been implicated in the formation of both erythromycin and nargenicin.[7,9a] Initial attempts at intact in-

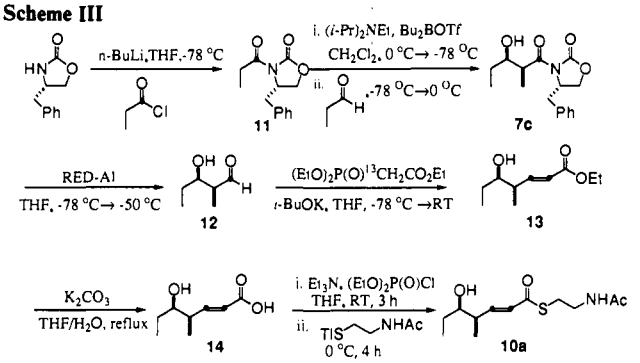
(10) The desosaminyl assignments were in close agreement with those reported for the desosamine moiety of erythromycin A: Everett, J. R.; Tyler, J. W. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2599.

(11) Birch et al. have reported incorporation experiments with ^{14}C-labeled precursors which support the derivation of methymycin from five propionates and one acetate: Birch, A. J.; Djerassi, C.; Dutcher, J. D.; Majer, J.; Perlman, D.; Pride, E.; Rickards, R. W.; Thomson, P. J. *J. Chem. Soc.* **1964**, 5274. Birch, A. J.; Pride, E.; Rickards, R. W.; Thomson, P. J.; Dutcher, J. D.; Perlman, D.; Djerassi, C. *Chem. Ind. (London)* **1960**, 1245.

(12) Biosynthetic studies on the closely related macrolide picromycin (**3**) have been reported: Omura, S.; Takeshima, H.; Nakagawa, A.; Miyazawa, J. *J. Antibiot.* **1976**, *29*, 316.

(13) Cane, D. E.; Liang, T.; Taylor, P. B.; Chang, C.; Yang, C. *J. Am. Chem. Soc.* **1986**, *108*, 4957. Hutchinson, C. R.; Sherman, M. M.; McInnes, A. G.; Walter, J. A.; Vederas, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 5956. Sood, G. R.; Robinson, J. A.; Ajaz, A. A. *J. Chem. Soc., Chem. Commun.* **1984**, 1421.

Scheme III



corporation of the dipropionate intermediate as the corresponding NAC thioester, (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester **7a**, prepared as previously described,[7,9a] proved difficult, resulting largely in degradation of the precursor with no detectable enrichment of the macrolide products. After extensive experimentation, it was eventually found that successful incorporation of **7a** could be achieved by the use of replacement cultures of *S. venezuelae* ATCC 15439 in combination with careful monitoring of the timing of macrolide production, using the UV absorption maximum at λ_{225} (ϵ = 10 500, MeOH) to estimate the macrolide content of $CHCl_3$ extracts of the fermentation broth. It was found to be critical that the cells of *S. venezuelae* be harvested and resuspended in replacement medium immediately following the onset of macrolide production. Both methymycin (**1**) (J_{CC} = 43.0 Hz) and neomethymycin (**2**) (J_{CC} = 36.5 Hz) obtained from the feeding of **7a** (30 mg/100 mL of culture) exhibited the expected pair of enhanced and coupled doublets corresponding to C-10 and C-11 in their respective ^{13}C NMR spectra (0.15 atom excess % ^{13}C) (Scheme Ib).

In order to rule out the possibility that incorporation of **7a** involves initial reoxidation to the corresponding β-keto ester **6a**, we next prepared (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester **7b**[7b] which was fed (120 mg) to a 100-mL replacement culture of *S. venezuelae* supplemented with 4-pentynoic acid[9c] in order to suppress degradation of the substrate by β-oxidation. The resulting samples of methymycin and neomethymycin each carried both ^{13}C and deuterium at C-11 (0.1 atom % excess), as evidenced by the appearance of the characteristic isotope-shifted triplet in the individual ^{13}C NMR spectra (**1**, $\Delta\delta$ = 0.40 ppm, J_{CD} = 17.3 Hz; **2**, $\Delta\delta$ = 0.35 ppm, J_{CD} = 20.9 Hz) (Scheme Ic). In each case, the triplet collapsed to a singlet upon broad-band deuterium decoupling ($^{13}C\{^{2}H,^{1}H\}$ NMR). Again, completely consistent results have previously been reported for the intact incorporation of **7b** into both erythromycin and nargenicin.[7c]

Having established the ability of the dipropionate intermediate to serve as a substrate in whole-cell feeding experiments, we turned our attention to the presumptive triketide intermediate, (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptanoyl NAC thioester **10a**, which was readily prepared in diastereomerically pure form (Scheme III). The synthetic procedures previously developed[7,9a,14] for the preparation of **7a** were used to obtain (4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone (**7c**), which was readily reduced with sodium bis(2-methoxyethoxy)aluminum hydride[15] (RED-Al) in THF to give the corresponding 3-hydroxy aldehyde **12**. Emmons reaction of **12** with triethyl [2-^{13}C]phosphonoacetate in THF using potassium *tert*-butoxide as the base and hydrolysis of the resulting [2,3-$^{13}C_2$]-unsaturated ester **13** with K_2CO_3 in aqueous THF gave the corresponding acid **14**. Finally, **14** was converted to the desired NAC thioester **10a** by treatment with diethyl chlorophosphonate followed by thallous *N*-acetylcysteamine.[16,17] Feeding of **10a** (50 mg) to replacement cultures of *S. venezuelae* gave labeled **1** and **2** (0.5 atom % excess), which exhibited the expected pairs of enhanced and coupled doublets for C-8 and C-9 in the ^{13}C NMR spectrum (**1**, J_{CC} = 70.5 Hz; **2**, J_{CC} = 70.9 Hz) (Scheme Id).

Discussion

The results reported here complement and extend previous reports of the incorporation of intermediates of polyketide chain elongation into the closely related macrolides erythromycin (**4**)[7] and tylosin (**5**),[8] as well as the antibiotic nargenicin.[7b,c,9a] Consistent with the findings of Vederas,[9c] we have observed that precise timing of the administration of labeled precursors and control of unwanted substrate degradation through the use of replacement cultures in combination with appropriate inhibitors of fatty acid β-oxidation can be critical to the successful incorporation of NAC thioesters. In addition, the use of ^{13}C doubly labeled substrates not only allows direct detection of the intact incorporation of precursors but conveniently extends the useful range of reliable detection to 0.1–0.2 atom % excess ^{13}C enrichment.

Taken together, these incorporation studies are fully consistent with a processive model of polyketide chain elongation in which the oxidation level and stereochemistry of the growing polyketide chain are adjusted subsequent to each individual condensation step[6-8] (Scheme II). Interestingly, methymycin (**1**), neomethymycin (**2**), and erythromycin (**4**), as well as nargenicin, all share a common precursor, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl thioester **7**,[7,9a] whereas tylosin is derived from the corresponding (2*R*,3*R*) diastereomer of **7**.[8] The pathways to these macrolides apparently diverge at this point, with **7** undergoing condensation with an enzyme-bound derivative of malonyl CoA (Scheme II) on the way to **1** and **2**, while condensation with methylmalonyl CoA is required for the formation of both erythromycin and nargenicin. The successful incorporation of the triketide substrate, (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoyl NAC thioester **10a**, into both methymycin (**1**) and neomethymycin (**2**) is fully consistent with the proposed intermediacy of **10**, presumably generated from **7** by a sequence of malonyl condensation, keto reduction, and dehydration, as illustrated in Scheme II. In further support of these ideas, we have recently isolated the presumptive product of the polyketide synthase 10-deoxymethynolide (**15**), the parent aglycone of the methymycin family of antibiotics.[18]

The successful incorporation of intermediates of polyketide chain elongation into a variety of macrolides and other polyketide natural products[7-9] assumes particular significance in light of the enormously important molecular genetic discoveries of the groups at Abbott Laboratories[6a] and Cambridge[6b] regarding the organization of the *eryA* gene. Solely on the basis of the inferred organization of the gene product, with each catalytic domain presumably being responsible for individual steps of polyketide chain elongation and functional group modification, one might be tempted to conclude that the structure of the eventually formed 6-deoxyerythronolide B, or by logical extension any macrolide aglycone, would be exclusively the result of the organization of the polyketide synthase. According to this simplified picture, each module of enzymes responsible for a round of condensation and appropriate reductions, dehydrations, and reductions simply accepts as substrate the product of the neighboring module. The finding that it is possible to incorporate intact a number of advanced intermediates of polyketide chain elongation into a variety of macrolides indicates that the actual macrolide biosynthetic mechanism is considerably more sophisticated. The fact that each advanced precursor is properly processed by the polyketide synthase during incorporation experiments with intact cells indicates that there is a significant element of molecular recognition involved in the operation of each biosynthetic module. Which structural features of each intermediate are most critical to proper recognition and whether this molecular recognition involves the acyltransferase, keto synthase,

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(15) Meyers, A. I.; Spohn, R. F.; Linderman, R. J. *J. Org. Chem.* **1985**, *50*, 3633.

(16) Schwab, J. M.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217.

(17) Masamune, S.; Kamata, S.; Diakur, J.; Sugihara, Y.; Bates, G. S. *Can. J. Chem.* **1975**, *53*, 3693.

(18) Lambalot, R. H.; Cane, D. E. *J. Antibiot.*, in press.

ACP domains, or some combination of all three remain to be established. Further investigations of the organization and control of this complex and intriguing biosynthetic process are in progress.

Experimental Section

Materials and Methods. All reactions were run under argon atmosphere using oven-dried syringes and glassware when appropriate. THF and CH_2Cl_2 were distilled from Na/benzophenone. Et_2O was distilled from P_2O_5. ^1H (250 or 400 MHz) and ^{13}C (100.6 MHz) NMR spectra were recorded on Bruker WM-250 and AM-400 NMR spectrometers. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrophotometer. Optical rotation measurements were obtained using a Perkin-Elmer 241 polarimeter. UV/vis spectra were obtained using a Perkin-Elmer 552A spectrophotometer. Mass spectra were obtained by chemical ionization with NH_4^+ or electron ionization using a Kratos MS80RFA mass spectrometer. Reagents and solvents were obtained from Aldrich Chemical Co., Cambridge Isotope Laboratories, and Los Alamos National Laboratories. (2*S*,3*R*)-[2,3-$^{13}C_2$]-2-Methyl-3-hydroxypentanoyl NAC thioester **7a**[7,9a] and (2*S*,3*R*)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester **7b**[7b] were prepared as previously described. *Streptomyces venezuelae* SC2366 was a gift from the Squibb Institute for Medical Research. *Streptomyces venezuelae* ATCC 15439 was received as a freeze-dried pellet. All culture media and glassware were autoclaved prior to use, and all biological manipulations were conducted under a Labconco sterile hood enclosure. Nanopure water obtained from a Barnstead Nanopure II water purification system was used to make all culture media. All *S. venezuelae* cultures were grown at 27 °C and 250 rpm in a New Brunswick Scientific floor model shaker. A Sorvall RC-5 centrifuge equipped with a GSA rotor was used for all harvesting and replacement procedures.

Cultivation of *Streptomyces venezuelae* ATCC 15439 for Advanced Precursor Feeding Experiments. An autoclaved 100-mL seed medium (20 g of glucose, 15 g of soybean flour, 5 g of $CaCO_3$, 1 g of NaCl, and 0.002 g of $COCl_2 \cdot 6H_2O$ per 1 L of nanopure H_2O, pH adjusted to 7.2 with 2 N NaOH) in a 500-mL baffled flask equipped with a sponge stopper was inoculated with a scraping from an agar slant of *S. venezuelae* ATCC 15439. The culture was grown on a shaker at 27 °C and 250 rpm for 48 h. Vegetative cultures (100 mL) were initiated by inoculation of the vegetative medium (20 g of glucose, 30 g of soybean flour, 2.5 g of $CaCO_3$, 1 g of NaCl, and 0.002 g of $COCl_2 \cdot 6H_2O$ per 1 L of nanopure H_2O, pH adjusted to 7.2 with 2N NaOH) with 3 mL of seed culture and grown under the same conditions. At 18 h the vegetative culture was transferred into an autoclaved 250-mL centrifuge bucket and centrifuged at 10000 rpm (Sorvall GSA rotor) for 15 min. The mycelia were resuspended in 100 mL of replacement medium (100 g of glucose, 2.5 g of $CaCO_3$, 1 g of NaCl, and 0.002 g of $COCl_2 \cdot 6H_2O$ per 1 L of nanopure H_2O, pH adjusted to 7.2 with 2 N NaOH) and decanted into an autoclaved 500-mL baffled flask. Isotopically labeled substrates (25–120 mg) were administered in 1 mL of 100% EtOH, and the culture was grown on a shaker at 27 °C and 250 rpm for 72 h.

UV Assay for Monitoring Combined Methymycin and Neomethymycin Production. A 1.5-mL sample was withdrawn from an actively fermenting culture and centrifuged (5 min at 14000 rpm) in a 1.5-mL Eppendorf tube. The supernatant was then decanted into a 13 × 100 mm test tube and basified to pH > 9 (2 drops of 2 N NaOH). The broth was extracted with 2 × 1.5 mL of $CHCl_3$ by gently agitating to avoid an emulsion. Any emulsion formed could be removed by centrifugation for 10 min at 5000 rpm. The organic extract was transferred to a clean dry test tube via a Pasteur pipet and the solvent was removed by rotary evaporation. A gentle stream of nitrogen was then passed through the test tube to remove any traces of $CHCl_3$ from the residue. Spectroscopic grade MeOH (5 mL) was added via volumetric pipet, and the solution was agitated to dissolve the residue. The absorbance of the crude extract was then measured at 225 nm (ϵ = 10500).

Isolation and Purification of Methymycin and Neomethymycin. The vegetative replacement culture was harvested by pouring the 100 mL of fermentation broth into a 250-mL centrifuge bucket and centrifuging for 15 min at 10000 rpm (Sorvall GSA rotor). The supernatant was decanted into a 500-mL flask and the pellet resuspended in 100 mL of acetone. The acetone wash was centrifuged for 15 min at 10000 rpm and the supernatant decanted into a 200-mL round-bottom flask. The solvent was removed by rotary evaporation, and the remaining yellow extract was washed into the first supernatant. The pH of the combined mixtured was adjusted to 9.5 with 2 N NaOH, and the aqueous phase was extracted with 4 × 50 mL of $CHCl_3$. The $CHCl_3$ extract was dried with Na_2SO_4 and the solvent removed by rotary evaporation followed by drying in vacuo to an amber oil (15 mg/100 mL). The crude mixture was purified by flash chromatography on silica gel (10 × 130 mm) with 90% $CHCl_3$/9.9% MeOH/0.1% NH_4OH. Cleaner separations were achieved by slowly ramping the MeOH from 0% to 10%. Fractions were monitored by silica TLC followed by developing with vanillin stain (0.75% vanilla, 1.5% H_2SO_4, MeOH). Methymycin had the higher R_f (0.24) and stained dark brown with vanillin, while neomethymycin had the lower R_f (0.18) and stained orange with vanillin.

(4*S*)-3-[1′-^{13}C]Propionyl-4-benzyl-2-oxazolidinone (11). (4*S*)-3-[1′-^{13}C]Propionyl-4-benzyl-2-oxazolidinone (**11**) was prepared from (4*S*)-4-benzyl-2-oxazolidinone following the previously described procedure:[7,14] mp = 44–45 °C; IR ($CHCl_3$) ν 3025, 1781, 1662, 1387, 1078, 1011 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 7.34–7.19 (m, 5 H, C_6H_5), 4.66 (m, 1 H, NC*H*), 4.17 (m, 2 H, CH_2O), 3.28 (dd, J = 3.2, 13.4 Hz, 1 H, C*H*HC$_6$H$_5$), 3.01–2.87 (m, 2 H, CH_3CH_2), 2.77 (dd, J = 9.6, 13.4 Hz, 1 H, CH*H*C$_6$H$_5$), 1.18 (dt, J = 5.56, 7.32 Hz, 3 H, CH_3CH_2); 100-MHz ^{13}C NMR ($CDCl_3$) δ 173.9 (enriched), 153.4, 135.3, 129.3, 128.8, 127.2, 66.0, 55.0, 37.3, 29.0 (d, J = 50 Hz), 8.1; $[\alpha]_D$ = +59.19° ($CHCl_3$, l = 1.0 dm, 0.016 g/mL); EIMS M^+ 234 m/e.

(4*S*,2′*S*,3′*R*)-3-(2′-Methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone (7c).[7,14] To a stirred solution (0.4 M) of unlabeled **11** (1.0 g, 4.29 mmol) in CH_2Cl_2 at 0 °C (ice bath) in a 100-mL, 2-neck, RB flask under argon atmosphere were added 894 μL of diisopropylethylamine (1.2 equiv) and 4.7 mL of dibutylboron triflate (1.0 M in CH_2Cl_2, 1.1 equiv). The mixture was allowed to stir at 0 °C for 30 min before cooling to −78 °C (dry ice/acetone) and stirring for another 30 min. Following the addition of 340 μL of propionaldehyde (neat, 1.1 equiv) the reaction was stirred for 30 min at −78 °C, after which time the dry ice bath was removed and the reaction allowed to warm to room temperature for 1.5 h. The reaction was cooled to 0 °C and quenched with 20 mL of MeOH and 10 mL of pH 7.4 buffered water, and the borate was oxidized with 10 mL of 30% H_2O_2 while stirring for 1 h. The crude was extracted with 3 × 25 mL of CH_2Cl_2 and purified by flash silica gel chromatography (10% EtOAc/$CHCl_3$) to yield 747 mg (60%) of white crystalline product: R_f = 0.35 (10% EtOAc/$CHCl_3$); mp = 79–81 °C; IR ($CHCl_3$) ν 3530, 3019, 2879, 1781, 1686, 1387 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 7.36–7.20 (m, 5 H, aromatic H's), 4.73–4.69 (m, 1 H, NC*H*), 4.25–4.17 (m, 2 H, CH_2O), 3.87 (m, 1 H, C*H*OH), 3.79 (dq, J = 2.7, 7.04 Hz, 1 H, C*H*CH$_3$), 3.26 (dd, J = 3.35, 13.41 Hz, 1 H, C*H*HPh), 2.87 (b, 1 H, O*H*), 2.79 (dd, J = 9.43, 13.40 Hz, 1 H, CH*H*Ph), 1.58–1.47 (m, 2 H, CH_3CH_2), 1.25 (d, J = 7.02 Hz, 3 H, CHC*H*$_3$), 0.98 (t, J = 7.4 Hz, 3 H, C*H*$_3$CH$_2$); 100-MHz ^{13}C NMR ($CDCl_3$) δ 177.6, 153.0, 135.0, 129.4, 129.0, 127.4, 73.0, 66.2, 55.1, 41.7, 37.8, 26.8, 10.4, 10.3; $[\alpha]_d$ = +51.47° ($CHCl_3$, l = 1.0 dm, 0.0293 g/mL); CIMS (NH_4^+) (M + H)$^+$ 292 m/e.

(4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-Methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone (7c). (4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3 (2′-Methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone ([1′-^{13}C]-**7c**) was prepared from (4*S*)-3-[1′-^{13}C]propionyl-4-benzyl-2-oxazolidinone following the same procedure described for unlabeled **7c**: mp = 81.5–82 °C; IR ($CHCl_3$) ν 3482, 2970, 2878, 1779, 1649, 1455, 1386, 1213, 1112, 969, 757 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 7.34-7.19 (m, 5 H, aromatic H's), 4.70 (m, 1 H, NC*H*), 4.23–4.15 (m, 2 H, CH_2O), 3.86–3.77 (m, 2 H, C*H*OH, C*H*CH$_3$), 3.23 (dd, J = 3.1, 13.4 Hz, 2 H, C*H*HPh, CHO*H*), 2.80 (dd, J = 9.3, 13.4 Hz, 1 H, CH*H*Ph), 1.6–1.4 (m, 2 H, CH_3CH_2), 1.25 (dd, J = 5.39, 6.68 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.35 Hz, 3 H, C*H*$_3$CH$_2$); 100-MHz ^{13}C NMR ($CDCl_3$) δ 177.1 (^{13}C enriched), 152.9 (d, J_{CC} = 4.5 Hz), 134.9, 129.2, 128.7, 127.2, 70.0 (d, J_{CC} = 2.2 Hz), 54.9, 41.6 (d, J_{CC} = 49.3 Hz), 37.5, 26.7 (d, J_{CC} = 3.6 Hz), 10.2; $[\alpha]_D$ = 48.46 ($CHCl_3$, l = 1.0 dm, 0.1881 g/mL); CIMS (NH_4^+) (M + H)$^+$ 293 m/e.

(2*S*,3*R*)-2-Methyl-3-hydroxypentanal (12).[15] A 100-mL, 2-neck, RB flask equipped with an argon inlet, septum, and stir bar was charged with 60 mL of dry THF and 1.30 mL of RED-Al (3.4 M in toluene, 4.4 mmol, 1.1 equiv) under an argon atmosphere. The solution was then cooled to −78 °C, and 1.168 g of unlabeled **7c** (4.0 mmol) in 10 mL of THF was added slowly via cannula. The evolution of gas could be seen as the solution was stirred for 10–15 min at −78 °C. The reaction was then warmed to −50 °C ($CHCl_3/CO_2$) and stirred between −55 and −40 °C for 1 h. The reaction was quenched at −50 °C with 12 mL of EtOAc and 3 mL of MeOH and then poured into a mixture of 25 mL of 1 M HCl (saturated with NaCl) and 45 mL of Et_2O and stirred at −20 °C for 10–15 min. The aqueous layer froze out as a gel. The ethereal layer was decanted and the aqueous layer rinsed quickly with 2 × 30 mL of Et_2O. The combined organic extracts were dried over K_2CO_3 and concentrated to 970 mg of oil. The crude aldehyde was dried in vacuo and taken immediately on to the Wittig reaction.

(2*S*,3*R*)-[1-^{13}C]-2-Methyl-3-hydroxypentanal (12). (2*S*,3*R*)-[1-^{13}C]-2-Methyl-3-hydroxypentanal ([1-^{13}C]-**12**) was prepared from (4*S*,2′*S*,3′*R*)-[1′-^{13}C]-3-(2′-methyl-3′-hydroxypentanoyl)-4-benzyl-2-oxazolidinone following the same procedure as described for unlabeled **12**.

Ethyl (4*R*,5*R*)-4-Methyl-5-hydroxy-2-heptenoate (13).[15] To 50 mL of dry THF in a 100-mL flask under argon was added 912 μL of triethyl phosphonoacetate (4.6 mmol, 1.15 equiv), followed by 493 mg of potassium *tert*-butoxide (4.4 mmol, 1.1 equiv). The mixture was stirred at room temperature for 5 min before cooling to −78 °C (dry ice/acetone).

The crude β-hydroxy aldehyde (**12**) was added in 5 mL of THF and stirred overnight while warming to room temperature. The mixture was poured into 5 mL of brine, extracted with 3 × 30 mL of Et_2O, dried over K_2CO_3, and concentrated in vacuo to 1.12 g of oil (151%). Flash chromatography (60% Et_2O/hexanes) provided 165 mg of pure product as an oil (22%): R_f = 0.31 (60% Et_2O/hexanes); IR (neat) ν 3459, 2969, 1714, 1651, 1462, 1370, 1278, 1184, 1041, 985, 868 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.95 (dd, J = 7.8, 15.8 Hz, 1 H, C*H*=CHCO$_2$), 5.86 (dd, J = 1.25, 15.7 Hz, 1 H, CH=C*H*CO$_2$), 4.19 (q, J = 7.1 Hz, 2 H, OC*H*$_2$CH$_3$), 3.49 (m, 1 H, C*H*OH), 2.43 (m, 1 H, C*H*CH$_3$), 1.92 (b, 1 H, O*H*), 1.5 (m, 1 H, CH$_3$,C*H*H), 1.4 (m, 1 H, CH$_3$CH*H*), 1.29 (t, J = 7.1 Hz, 3 H, OCH$_2$C*H*$_3$), 1.09 (d, J = 6.8 Hz, C*H*$_3$CH), 0.97 (t, J = 7.4 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 166.6, 151.1, 121.5, 75.9, 60.3, 42.2, 27.3, 14.2, 13.9, 10.2; $[\alpha]_D$ = +35.16° ($CHCl_3$, l = 1.0 dm, 0.0062 g/mL); CIMS (NH_4^+) (M + H)$^+$ 187 m/e; exact mass calcd for $C_{10}H_{19}O_3$ 187.1334, found 187.1327.

Ethyl (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoate (13). Ethyl (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoate ([2,3-$^{13}C_2$]-**13**) was prepared from (2*S*,3*R*)-[1-^{13}C]-2-methyl-3-hydroxypentanal and triethyl [2-^{13}C]phosphonoacetate following the same procedure described for unlabeled **13**: IR (neat) ν 3459, 2971, 2878, 1708, 1598, 1459, 1274, 1183, 979, 756 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.95 (dddd, J = 1.93, 7.84, 15.77, 154.28 Hz, 1 H, *H*^{13}C=^{13}CHCO$_2$), 5.86 (ddd, J = 0.77, 15.77, 162.03 Hz, 1 H, H^{13}C=^{13}C*H*CO$_2$), 4.19 (q, J = 7.13 Hz, 2 H, CO$_2$C*H*$_2$CH$_3$), 3.49 (m, 1 H, C*H*OH), 2.44 (m, 1 H, C*H*CH$_3$), 1.9 (b, 1 H, O*H*), 1.60–1.50 (m, 1 H, CH$_3$C*H*H), 1.46–1.36 (m, 1 H, CH$_3$CH*H*), 1.29 (t, J = 7.13 Hz, 3 H, CO$_2$CH$_2$C*H*$_3$), 1.09 (dd, J = 4.90, 6.75 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.40 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 151.2 (enriched, d, J_{CC} = 70.63 Hz), 121.4 (enriched, d, J_{CC} = 70.73 Hz), 75.8 (d, J_{CC} = 3.2 Hz), 60.2 (d, J_{CC} = 1.3 Hz), 42.1 (d, J_{CC} = 41.7 Hz), 27.3, 14.2, 13.9, 10.2; $[\alpha]_D$ = 35.16° ($CHCl_3$, l = 1.0 dm, 0.0442 g/mL); CIMS (NH_4^+) (M + H)$^+$ 189 m/e.

(4*R*,5*R*)-4-Methyl-5-hydroxy-2-heptenoic Acid (14). Unlabeled ester **13** (418 mg, 2.25 mmol), 22 mL, 0.5 M K_2CO_3 (11.24 mmol, 5.0 equiv), and 110 mL of 5:3 THF/H_2O (69 mL THF/41 mL H_2O) were refluxed together for 24 h. After removal of THF, the basic aqueous mixture was extracted with 2 × 50 mL of CH_2Cl_2 and acidified to pH 3.0 with 5% HCl, and the acid was extracted with 3 × 50 mL of CH_2Cl_2 to yield 255 mg of pure acid in 71.7% yield: IR ($CHCl_3$) ν 3407, 3019, 2971, 2937, 2879, 2678, 1698, 1654, 1282 cm^{-1}; 250-MHz ^1H NMR ($CDCl_3$) δ 7.07 (dd, J = 7.8, 16.3 Hz, 1 H, C*H*=CHCO$_2$), 5.87 (dd, J = 1.12, 15.7 Hz, 1 H, CH=C*H*CO$_2$), 3.53 (m, 1 H, C*H*OH), 2.48 (m, 1 H, C*H*CH$_3$), 1.64–1.25 (m, 2 H, CH$_3$C*H*$_2$), 1.10 (d, J = 6.8 Hz, CHC*H*$_3$), 0.92 (t, J = 7.4 Hz, 3 H, C*H*$_3$CH$_2$); 100-MHz ^{13}C NMR ($CDCl_3$) δ 171.3, 153.8, 120.8, 75.9, 42.2, 27.3, 13.7, 10.2; $[\alpha]_D$ = +43.33° ($CHCl_3$, l = 1.0 dm, 0.018 g/mL); CIMS (NH_4^+) (M + H)$^+$ 159 m/e.

(4*R*,5*R*)-[2,3-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoic Acid (14). (4*R*,5*R*)-[1,2-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoic acid ([2,3-$^{13}C_2$]-**14**) was prepared from ethyl (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoate following the same procedure described for unlabeled **14**: 400-MHz ^1H NMR ($CDCl_3$) δ 7.06 (dddd, J = 1.93, 7.78, 15.74, 154.5 Hz, 1 H, *H*^{13}C=^{13}CHCO$_2$), 6.7 (b, 1 H, CO$_2$H), 5.86 (dd, J = 15.6, 162.7 Hz, 1 H, H^{13}C=^{13}C*H*CO$_2$), 3.54 (m, 1 H, C*H*OH), 2.47 (m, 1 H, C*H*CH$_3$), 1.6–1.5 (m, 1 H, CH$_3$C*H*H), 1.45–1.35 (m, 1 H, CH$_3$CH*H*), 1.10 (dd, J = 4.86, 6.78 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.40 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 153.7 (enriched, d, J_{CC} = 70.0 Hz), 120.8 (enriched, d, J_{CC} = 70.1 Hz).

Thallous *N*-Acetylcysteamine.[16] Thallous ethoxide (325 μL, 1.18 g, 459 mmol) was added via syringe to an oven-dried 100-mL round-bottom flask under argon atmosphere containing 20 mL of dry THF. *N*-Acetylcysteamine (574 mg in 20 mL of dry THF) was added dropwise via cannula over 30 min at room temperature. Upon addition of the *N*-acetylcysteamine the solution turned a bright yellow to yield a suspension with a final concentration of 0.12 M.

***N*-Acetylcysteamine Thioester of (4*R*,5*R*)-4-Methyl-5-hydroxy-2-heptenoic Acid (10a).**[16,17] In an oven-dried 10-mL RB flask, 57 mg of unlabeled **14** (0.36 mmol) and 50 μL of Et_3N (0.36 mmol, 1.0 equiv) in 3 mL of dry THF was allowed to stir under an argon atmosphere at room temperature for 10 min. To this mixture was added 52 μL of diethylchlorophosphonate (0.36 mmol, 1.0 equiv) in 1 mL of dry THF dropwise. The reaction was stirred for 3 h after which the fine white Et_3N·HCl salt was filtered out via Schlenk filtration and rinsed with 2 × 10 mL of THF. The solvent was removed and the phosphono anhydride concentrated to an oil. This oil was then dissolved in 12 mL of dry THF in a 50-mL RB flask equipped with a stir bar and septum. The solution was cooled to 0 °C, and 3 mL of thallous *N*-acetylcysteamine as a bright yellow suspension in THF (0.12 M) was added dropwise via cannula. The reaction was allowed to stir at 0 °C for 4 h, after which time the yellow color had dissipated to yield a white suspension. The solvent was removed by rotary evaporation, and the crude was taken up in Et_2O, filtered through Celite, and concentrated to 123 mg of oil. Flash silica gel chromatography (4% MeOH/$CHCl_3$) provided 53 mg of oil (57%): R_f = 0.35 (10% MeOH/0.1% NH_4OH/$CHCl_3$); IR (neat) ν 3303, 3086, 2970, 1660, 1551, 1290, 979 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.95 (dd, J = 7.72, 15.64 Hz, 1 H, C*H*=CHCO$_2$), 6.25 (b, 1 H, N*H*), 6.16 (dd, J = 1.26, 15.66 Hz, 1 H, CH=C*H*CO$_2$), 3.52 (m, 1 H, C*H*OH), 3.45 (m, 2 H, C*H*$_2$NHAc), 3.09 (t, J = 6.4 Hz, 2 H, SC*H*$_2$), 2.45 (m, 1 H, C*H*CH$_3$), 2.22 (b, 1 H, CHO*H*), 1.97 (s, 3 H, COC*H*$_3$), 1.57–1.51 (m, 1 H, CH$_3$C*H*H), 1.44–1.39 (m, 1 H, CH$_3$CH*H*), 1.10 (d, J = 6.83 Hz, 3 H, CHC*H*$_3$), 0.97 (t, J = 7.39 Hz, 3 H, C*H*$_3$CH$_2$); 100-MHz ^{13}C NMR ($CDCl_3$) δ 190.3, 170.4, 148.5, 128.1, 75.7, 42.1, 39.6, 28.3, 27.3, 23.1, 13.7, 10.2; $[\alpha]_D$ = +31.92° ($CHCl_3$, l = 1.0 dm, 0.0026 g/mL); CIMS (NH_4^+) (M + H)$^+$ 260 m/e; exact mass calcd for $C_{12}H_{22}NO_3S$ 260.1320, found 260.1338.

***N*-Acetylcysteamine Thioester of (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-Methyl-5-hydroxy-2-heptenoic Acid (10a).** *N*-Acetylcysteamine thioester of (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoic acid ([2,3-$^{13}C_2$]-**8**) was prepared from (4*R*,5*R*)-[2,3-$^{13}C_2$]-4-methyl-5-hydroxy-2-heptenoic acid following the same procedure as described for unlabeled **10a**: IR (neat) ν 3311, 3085, 2970, 2878, 1658, 1574, 1288, 1218, 758 cm^{-1}; 400-MHz ^1H NMR ($CDCl_3$) δ 6.91 (dddd, J = 1.49, 7.70, 15.6, 153.7 Hz, 1 H, *H*^{13}C=^{13}CHCO$_2$), 6.4 (b, 1 H, N*H*), 6.14 (dd, J = 15.6, 161.5 Hz, 1 H H^{13}C=^{13}C*H*CO$_2$), 3.50 (m, 1 H, C*H*OH), 3.38 (dt, J = 6.20, 6.30 Hz, 2 H, C*H*$_2$NHAc), 3.05 (t, J = 6.56 Hz, 2 H, SC*H*$_2$), 2.55 (b, 1 H, O*H*), 2.40 (m, 1 H, C*H*CH$_3$), 1.55–1.45 (m, 1 H, CH$_3$C*H*H), 1.41–1.30 (m, 1 H, CH$_3$CH*H*), 1.06 (dd, J = 4.9, 6.8 Hz, 3 H, CHC*H*$_3$), 0.93 (t, J = 7.39 Hz, 3 H, C*H*$_3$CHH); 100-MHz ^{13}C NMR ($CDCl_3$) δ 149.0 (enriched, d, J_{CC} = 69.9 Hz), 128.3 (enriched, d, J_{CC} = 69.9 Hz); $[\alpha]_D$ = 27.6° ($CHCl_3$, l = 1.0 dm, 0.0389 g/mL); CIMS (NH_4^+) (M + H)$^+$ 262 m/e.

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