Isolation and Structure Determination of Novamethymycin, a New Bioactive Metabolite of the Methymycin Biosynthetic Pathway in *Streptomyces venezuelae*

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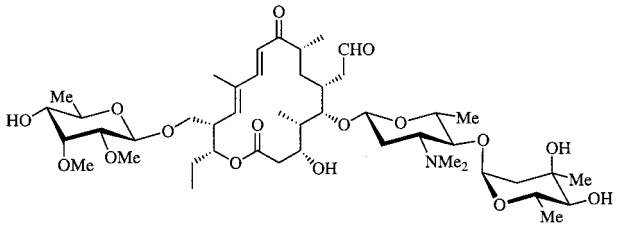
Novamethymycin (**9**), a novel macrolide antibiotic, was isolated from *Streptomyces venezuelae*, the producer of methymycin (**4**) and neomethymycin (**5**). Spectroscopic analysis of **9** indicated that it is highly related to **4** and **5** but contains hydroxy groups at both C-10 and C-12 on the macrolactone ring. Bioconversion studies using the PikC cytochrome P450 hydroxylase demonstrated that **4** is converted to **9**, further broadening the remarkable substrate flexibility of this enzyme.

Natural products of polyketide origin possess the most structural diversity of any other family of compounds.[1] Among them is a category of antibiotics known as macrolides, which are characterized by their large lactone rings. These clinically important antibiotics are usually glycosylated including tylosin (**1**), pikromycin (**2**), erythromycin (**3**), and the unusual 12-membered-ring macrolides



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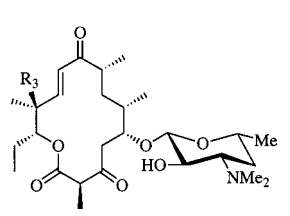
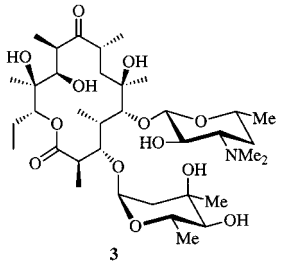


2: R_3 = OH
8: R_3 = H



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methymycin (**4**) and neomethymycin (**5**). The aglycones of these metabolites have been demonstrated to be biosynthetically derived from acetate and propionate through the action of modular polyketide synthases (PKSs).[2−4] *Streptomyces venezuelae* is unique in its ability to produce both 12- and 14-membered-ring macrolide antibiotics, including methymycin/neomethymycin and narbomycin/pikromycin.[5−9] In general, one aglycone requires a unique PKS for its biosynthesis; however, a single PKS from *S. venezuelae* has been shown to generate both the 12-membered-ring macrolactone 10-deoxymethynolide (**6**) and the 14-membered-ring macrolactone narbonolide, the aglycone intermediates for methymycin/neomethymycin and narbomycin/pikromycin, respectively.[10−12] As shown in Scheme 1, **4** and **5** are derived from one acetate and five propionate building blocks followed by glycosylation with desosamine. The biosynthesis of pikromycin involves the condensation of an additional propionate unit to the polyketide chain. The final step in the biosynthesis of methymycin/neomethymycin and pikromycin is the hydroxylation, which has been demonstrated to be catalyzed by a single cytochrome P450 enzyme (PikC).[13−15] Of the macrolide compounds isolated from *S. venezuelae*, they either lack a hydroxyl group (10-deoxymethynolide (**6**), YC-17 (**7**), narbonolide, and narbomycin (**8**)) or are modified by a single hydroxylation step (**4**, **5**, and **2**). Here we report the isolation and structure determination of novamethymycin (**9**), a dihydroxylated macrolide derivative from *S. venezuelae*. The structure of this novel compound strongly suggests that **9** is the final product of the biosynthetic pathway, with **4** and **5** functioning as substrates for the PikC cytochrome P450.

Antibiotic production by *S. venezuelae* has been shown in previous studies to vary according to different culture media. In the nutrient SCM medium, *S. venezuelae* produces both the 12- and the 14-membered-ring macrolide antibiotics.[16] Only the 14-membered-ring macrolide antibiotic **2** was isolated when *S. venezuelae* was cultured in Suzuki glucose-peptone medium.[17] In our current study, antibiotic production was conducted in a vegetative medium that results in accumulation of the 12-membered-ring metabolites.[16] The new metabolite and other related compounds were extracted following similar procedures as previously described.[18] Briefly, the fermentation broth was centrifuged and the pellets were discarded. The supernatant was adjusted to pH 9.5 and extracted with ethyl acetate three times. The organic extract (620 mg) was subjected to silica gel flash chromatography and eluted with a mixture of $CHCl_3$/MeOH (95:5), which was saturated with concentrated ammonium hydroxide. The fraction containing **9** was contaminated initially with **5** and thus was purified again using silica gel flash chromatography. Pure **9** was obtained by preparative high-performance thin-layer chromatography (HPTLC).

The ^{1}H NMR spectrum of novamethymycin (**9**) showed a high degree of similarity to those of **4** and **5**.[18] As in the case of **4**, the olefinic protons (H-8, H-9) appeared to be two doublets, indicating no protons associated with C-10, which is also supported by the fact that the H-17 methyl group (δ 1.52) resonates as a singlet. Similarly, the absence of a triplet methyl signal is characteristic of **5**, which has a hydroxy group substitution at C-12. The absence of a spin coupling between H-3 and H-4 in a COSY spectrum is typical for the **4** and **5** group of compounds. The complete

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Scheme 1

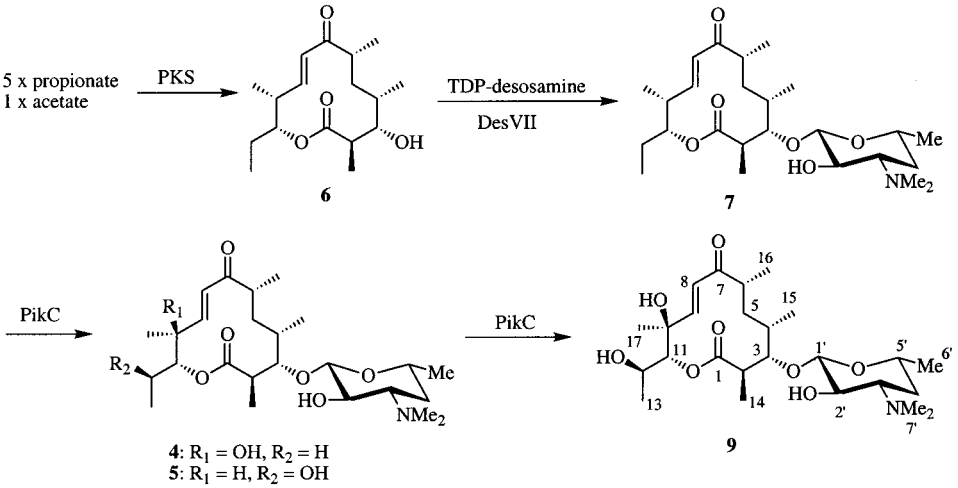

Table 1. NMR Assignment for Novamethymycin (**9**) ($CDCl_3$)

position	^{13}C (δ) (mult)	^{1}H (δ) (mult, *J*)	HMBC
1	174.1 (s)		
2	44.0 (d)	2.86 (dq, 10.5, 7.0)	C1, C14, C3
3	85.4 (d)	3.61 (d, 10)	C14, C2, C4, C15, C1′
4	33.7 (d)	1.19 (overlapped with *Mes*)	
5	33.8 (t)	1.67 (t, 13.5)	C3, C4, C15, C6, C7
		1.44 (ddd, 14.0, 12.0, 4.0)	
6	45.2 (d)	2.57 (m)	C7, C16
7	204.0 (s)		
8	125.5 (d)	6.32 (d, 16)	C7, C9, C10
9	148.1 (d)	6.64 (d, 16)	C7, C17
10	75.5 (s)		
11	74.3 (d)	4.68 (d, 9.5)	C12, C17, C10, C9, C1
12	67.9 (d)	4.14 (dq, 9.5, 6.0)	C11
13	21.11 (q)	1.20 (d, 6.0)	C11,C12
14	15.8 (q)	1.40 (d, 7.0)	C1, C3
15	17.4 (q)	1.01 (d, 7.0)	C3, C4
16	17.6 (q)	1.18 (d, 7.0)	C6, C7
17	20.2 (q)	1.52 (s)	C9, C10
1′	104.8 (d)	4.25 (d, 7.5)	C3, C3′
2′	70.2 (d)	3.28 (dd, 10.5, 7.5)	C1′, C3′
3′	66.1 (d)	2.65 (bt, 12)	
4′	28.9 (t)	1.77 (H_{eq}, bd, 12.5)	
		1.30 (H_{ax}, q, 12)	C3′, C5′
5′	69.3 (d)	3.50 (dqd, 11.0, 6.5, 2.0)	
6′	20.07 (q)	1.24 (d, 5.5)	C4′, C5′
7′	40.3 (q)	2.39 (s)	C3′

assignment of ^{1}H and ^{13}C NMR signals was readily accomplished by analysis of 1D and 2D NMR data (Table 1), and by comparison to those of **4** and **5** (Table 2). Although the HMQC data did not provide information for the assignment of C-4, C-5, and C-3′, their assignments were confirmed by correlations from an HMBC experiment (Table 1). As shown in Table 2, both ^{1}H and ^{13}C signals are in good agreement with those of **4** and **5** except those near the corresponding macrolactone hydroxy groups. Finally, high-resolution FABMS measured on both $[M + H]^+$ and $[M + Na]^+$ ions confirmed the presence of a second hydroxy group and thus concluded the proposed structure for **9**.

The antibiotic activity of novamethymycin (**9**) against *Bacillus subtilis* was evaluated by disk diffusion bioassay on solid medium. At the level of 100 μg/disk, **9** afforded an 18 mm zone of inhibition, while 18 and 20 mm zones of inhibition were observed on the same agar plate for **4** and **5**, respectively. It is noteworthy that YC-17 (16 mm zone of inhibition on the same agar plate) also showed similar activity in comparison to **4** and **5**.[19] These results clearly indicate that the additional hydroxyl group of **9** does not alter significantly the biological activity according to the *B. subtilis* assay system.

To establish the biosynthetic relationship between the new dihydroxylated metabolite and the corresponding monohydroxylated compounds, **4** and **5** were individually incubated with PikC purified from an overproducing recombinant strain, *Escherichia coli* BL-21 (DE3)/pDHS616.[13] After prolonged incubation, a small amount (<10%) of **9** was detected by TLC from the extract of the incubation mixture using **4** as substrate, while **9** could not be detected under the same reaction conditions from the extract of **5**. A control experiment performed under identical conditions showed YC-17 was efficiently converted (>90%) to **4** and **5**. These experimental results clearly demonstrated that **9** could be generated from **4** through the action of the P450 enzyme PikC, albeit with low efficiency. However, we could not eliminate the possibility that **5** is also a substrate for PikC in which product formation was extremely slow or beyond the limit of our detection. Therefore, although these experiments demonstrate that **4** is a substrate for PikC, it is unclear whether **5** can also function as a direct precursor for novamethymycin (**9**) biosynthesis.

Table 2. Comparison of NMR Assignment for Novamethymycin (**9**), Methymycin (**4**), and Neomethymycin (**5**)[a]

	novamethymycin		methymycin		neomethymycin	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	174.1		175.2		174.8	
2	44.0	2.86	44.2	2.87	43.9	2.88
3	85.4	3.61	85.5	3.60	85.6	3.59
4	33.7	1.19	33.6	1.25	33.4	1.25
5	33.8	1.67	33.9	1.75	34.1	1.68
		1.44		1.50		1.4
6	45.2	2.57	45.1	2.5	45.1	2.52
7	204.0		204.6		205.2	
8	125.5	6.32	125.6	6.34	126.2	6.44
9	148.1	6.64	148.9	6.60	147.1	6.76
10	75.5		74.1		35.4	3.05
11	74.3	4.68	76.3	4.75	75.4	4.79
12	67.9	4.14	21.2	1.95	66.4	3.89
				1.5		
13	21.11	1.20	10.6	0.90	21.0	1.20
14	15.8	1.40	16.1	1.44	15.9	1.41
15	17.4	1.01	17.3	1.02	17.4	1.02
16	17.6	1.18	17.5	1.17	17.6	1.19
17	20.2	1.52	19.2	1.35	9.8	1.16
1′	104.8	4.25	105.0	4.24	105.1	4.24
2′	70.2	3.28	70.3	3.22	70.3	3.23
3′	66.1	2.65	65.8	2.5	65.9	2.52
4′	28.9	1.77	28.3	1.65	28.3	1.68
		1.30		1.2		1.2
5′	69.3	3.50	69.4	3.5	69.5	3.48
6′	20.07	1.24	21.1	1.23	21.1	1.23
7′	40.3	2.39	40.2	2.27	40.2	2.28

[a] Data for methymycin and neomethymycin were taken from ref 18.

In summary, novamethymycin (**9**), a novel macrolide antibiotic, was isolated from *S. venezuelae*, and its structure was determined spectroscopically to be the dihydroxylated product of YC-17. Preliminary results indicated that this compound was biosynthetically derived from methymycin (and perhaps neomethymycin), suggesting that novamethymycin is the terminal product of the methymycin biosynthetic pathway. A growing body of evidence has demonstrated that *S. venezuelae* is capable of producing structurally diverse macrolide metabolites not only by construction of alternative aglycones but also by different post-assembly modifications. Although several cytochromes P450 involved in macrolide antibiotic biosynthesis have been studied, only EryF and EryK have been characterized in detail.[20−22] Both enzymes are involved in the biosynthesis of erythromycin A in *Saccharopolyspora erythraea*, and each is responsible for the hydroxylation at a single position on the erythromycin aglycone. In contrast, PikC has exhibited broad substrate flexibility, accepting both 12- and 14-membered-ring macrolides, including those with an altered sugar residue.[23] Our results demonstrated that this enzyme could also accept methymycin (and perhaps neomethymycin) as a substrate. Indeed, an interesting question associated with the isolation of this compound is whether *S. venezuelae* also produces similarly hydroxylated 14-membered-ring macrolide antibiotics (e.g., "neopikromycin" and "novapikromycin").

Experimental Section

General Experimental Procedures. All NMR spectra were recorded on a Varian INOVA 500 spectrometer. Fast-atom bombardment (FAB) mass spectra were measured by the MS facility at the Department of Chemistry of the University of Minnesota. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrophotometer. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 G-254 plates, and the spots were visualized either under UV light or by heating plates previously stained with solutions of vanillin/ethanol/H_2SO_4 (1:98:1). Preparative HPTLC was purchased from Analtech (Newark, Delaware, catalog #59077). Double-deionized water (Milli-Q, Millipore) was used for all fermentations.

Culture Maintenance and Fermentation Conditions. *S. venezuelae* (ATCC 15439) was obtained as a freeze-dried pellet from the American Type Culture Collection (ATCC). Media for vegetative growth and antibiotic production were used as described.[18] Briefly, SGGP liquid medium was used for propagation of *S. venezuelae* mycelia. Sporulation agar was used for production of *S. venezuelae* spores. Methymycin production was conducted in vegetative medium. An autoclaved 50 mL seed medium (20 g of glucose, 15 g of soybean flour, 5 g of $CaCO_3$, 1 g of NaCl, and 2 mg of $CoCl_2 \cdot 6H_2O$ per 1 L of H_2O, pH adjusted to 7.2 with 2 N NaOH) in a 250 mL baffled flask was inoculated with a loop full of spores and was grown on a shaker at 30 °C and 250 rpm for 48 h. An autoclaved 500 mL production culture (20 g of glucose, 30 g of soybean flour, 2.5 g of $CaCO_3$, 1 g of NaCl, and 2 mg of $CoCl_2 \cdot 6H_2O$ per 1 L of H_2O, pH adjusted to 7.2 with 2 N NaOH) in a 2 L baffled flask was initiated by inoculation of the seed medium (30 mL) and grown under the same conditions for 48 h.

Isolation. The fermentation broth (5 L) was centrifuged at 10,000*g* for 15 min. The supernatant was adjusted to pH 9.5 with 2 N NaOH and extracted with EtOAc (3 × 2.5 L). The combined organic extract was dried with Na_2SO_4 and the solvent removed by rotary evaporation under ambient temperature followed by drying in vacuo to yield an amber oil (620 mg). The crude extract was fractionated on a flash silica gel column (2.0 × 25 cm) with 95% $CHCl_3$/4.5% MeOH/0.5% NH_4OH. Methymycin (**4**) was eluted first followed by the majority of neomethymycin (**5**). The next eluted fraction was novamethymycin (**9**), which was contaminated with neomethymycin (**5**) and other minor impurities as judged by ^{1}H NMR spectra. This mixture (45 mg) was further purified by flash chromatography under the same conditions. Pure **9** was obtained by preparative HPTLC (90% $CHCl_3$/9% MeOH/1% NH_4OH). Novamethymycin has a weak UV absorption and its band was visible on the fluorescent HPTLC plates under the short UV_{254} light. The novamethymycin bands were scraped off the TLC plates, and the silica gel was washed with the developing solvent mixture to afford **9** (15 mg).

Novamethymycin (9): white solid; FT-IR (NaCl) ν_{max} 1732, 1690, 1630, 1460, 1377, 1274, 1160, 1111, 1076, 1049 cm^{-1}. For complete ^{1}H and ^{13}C NMR data, see Table 1. Positive ion FABMS *m/z* 486, 352, 329, 176, 154, 77, 51. HRFABMS calcd for $C_{25}H_{44}NO_8$ $[M + H]^+$ 486.3067, found *m/z* 486.3088. HRFABMS calcd for $C_{25}H_{43}NNaO_8$ $[M + Na]^+$ 508.2886, found *m/z* 508.2896.

Bioassay. Sterile Antibiotic Medium 2 (Difco Laboratories, Detroit, Michigan) solution (50 °C) was mixed with 0.2% v/v of a stock mycelia suspension of *B. subtilis* (ATCC 6633), and aliquots (20 mL) were immediately dispensed into Petri dishes and allowed to solidify in a sterile hood. Sterile paper bioassay disks (5 mm in diameter) were treated with methanol solutions (10 μL each) containing an appropriate amount of antibiotics. After the evaporation of methanol, the paper disks were distributed evenly on the agar plates. The diameter of the inhibition zone was measured after incubation at 37 °C for 10 h.

Enzymatic Conversion. To a reaction mixture containing 43 μM PikC, 14 μM spinach ferredoxin (Sigma), 0.2 units ferredoxin-$NADP^+$ reductase (Sigma), and 1 mM NADPH (Sigma) in a total volume of 500 μL of 100 mM potassium phosphate buffer (pH 7.2) was added one of the following compounds: **4**, **5**, and YC-17 (0.5 mM each final concentration) in 12 μL of MeOH. The resulting mixture was incubated at 37 °C for 2 h, and the reaction was terminated by extraction with ethyl acetate (3 × 700 μL). The combined organic extract was evaporated to dryness and the residue dissolved in 30 μL of $CHCl_3$ for TLC analysis. The TLC plate was developed using 90% $CHCl_3$/9% MeOH/1% NH_4OH.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra of novamethymycin (**9**). This information is available free of charge at http://pubs.acs.org.

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