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Neopikromycin and Novapikromycin from the Pikromycin Biosynthetic Pathway of *Streptomyces venezuelae*

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Two new macrolides from the pikromycin biosynthetic pathway of *Streptomyces venezuelae*, neopikromycin (**9**) and novapikromycin (**10**), were identified and structurally characterized through mass spectrometry and NMR spectroscopy. The established structures showed that **9** and **10** have hydroxyl groups at C-14 (**9**) and at both C-12 and C-14 (**10**), on the basis of a comparison with narbomycin (**7**). The purified PikC cytochrome P450 monooxygenase catalyzes the in vitro hydroxylation of **7** and pikromycin (**8**) to yield **9** and **10**, respectively, thus expanding the substrate- and regioflexibility of this enzyme.

Macrolides are a large and structurally diverse class of natural products that possess a wide range of biological activities, which render them useful in human and veterinary medicine, agriculture, and animal nutrition.^{1–3} The biosynthesis of macrolides from actinomycetes involves the formation of the characteristic macrolactone aglycone by modular type I polyketide synthase (PKS) and subsequent post-PKS modification steps such as glycosylation, hydroxylation, methylation, and acylation. The pikromycin biosynthetic pathway in *Streptomyces venezuelae* has a unique feature in that it produces two groups of macrolactones, those based on the 12-membered ring 10-deoxymethynolide (**1**) and the 14-membered ring narbonolide (**6**), by a single PKS.^{4,5} The first post-PKS tailoring step is glycosylation by a *des* gene cluster involved in the biosynthesis and transfer of the desosamine sugar to yield YC-17 (**2**) and narbomycin (**7**).^{6–8} The next step is hydroxylation by PikC cytochrome P450 monooxygenase, which accepts **2** and **7** as substrates to catalyze the hydroxylation at different positions.^{9,10} As shown in Scheme 1, methymycin (**3**), neomethymycin (**4**), and novamethymycin¹¹ (**5**) are the final hydroxylated 12-membered ring products of this biosynthetic pathway. However, in the case of the 14-membered ring, only one hydroxylated macrolide, pikromycin (**8**), has been isolated. In the current study, we confirmed the presence of two novel 14-membered ring macrolides, neopikromycin (**9**) and novapikromycin (**10**), from *S. venezuelae* by mass spectrometry and determined their structures by 1D and 2D NMR spectroscopy. The antibiotic activities of these novel compounds against *Bacillus subtilis* were also evaluated. These results are important not only to realize the diversity of macrolides, produced as secondary metabolites in *S. venezuelae*, but also to offer a valuable insight into the structure–bioactivity relationship of macrolides.

S. venezuelae ATCC 15439 was grown on SPA solid medium for the production and analysis of macrolides. The crude extract containing the new compounds and other products was obtained

by extracting the culture medium with MeOH followed by a purification step using a solid-phase extraction cleanup column.

On conducting an HPLC ESIMS analysis of the crude extracts, the molecular ions $[M + H]^+$ for **9** and **10** were detected at m/z 526 and m/z 542, respectively. Although **9** has the same molecular mass as **8**, the two macrolides were distinguishable from each other on the basis of the difference between the relative retention times by HPLC ESIMS. Pikromycin (**8**), neopikromycin (**9**), and novapikromycin (**10**) were detected in the crude extract from *S. venezuelae* at a ratio of 1500:40:1, indicating the very low production of **9** and **10** as compared to that of **8**. Further analyses of **9** and **10** via MS/MS showed the characteristic fragmentation patterns, including the fragmented desosamine sugar ion at m/z 158.^{12,13} Since HPLC ESIMS and MS/MS spectrometric analyses suggested the presence of the new compounds **9** and **10**, the crude extract was subjected to reversed-phase preparative HPLC to isolate these compounds.

The NMR analysis permitted elucidation of the structures of neopikromycin (**9**) and novapikromycin (**10**). The chemical shifts in the ¹H and ¹³C NMR spectra for the macrolactone moiety in **9** and **10** are very similar to those reported for pikromycin (**8**)¹⁴ (Table 1); the presence of H-2 at 3.81 ppm as a quintet was supported by the fact that both the lactone (C-1, 171.4 ppm) and ketone (C-3, 203.7 ppm) carbonyl signals were recognizable. In the case of the desosamine sugar moiety, the singlet at 2.27 ppm was indicative of the two *N*-methyl group protons in all three compounds, which were verified at 40.1 ppm in the ¹³C NMR spectrum. The major differences among the three compounds were found at positions C-12 and C-14. The presence of H-12 of **9** at 3.21 ppm, which was absent in both **8** and **10**, in addition to the upfield shift of C-12, from 70.8 and 74.2 ppm, respectively, to 30.0 ppm, indicated the removal of the hydroxyl group at the C-12 position in **9**. In contrast, the downfield shifts of C-14 of both **9** and **10** from 19.2 ppm to 64.8 and 67.1 ppm, respectively, in addition to the absence of an AB multiplet at 1.69 and 1.48 ppm, which was found only in **8**, were consistent with the existence of a hydroxyl group at C-14 in both **9** and **10**. In the ¹H NMR spectrum of **8**, there was a triplet at 0.86 ppm for the C-15 methyl protons, whereas **9** and **10** had a doublet instead. This represented the absence of a hydroxyl group at C-14 in **8**, as reported in a previous study.¹⁴ Among the ¹H NMR signals corresponding to the methyl groups attached to the macrolactone in all three compounds, there was another difference in the multiplicity; the C-20 methyl protons adjacent to H-12 in **9**

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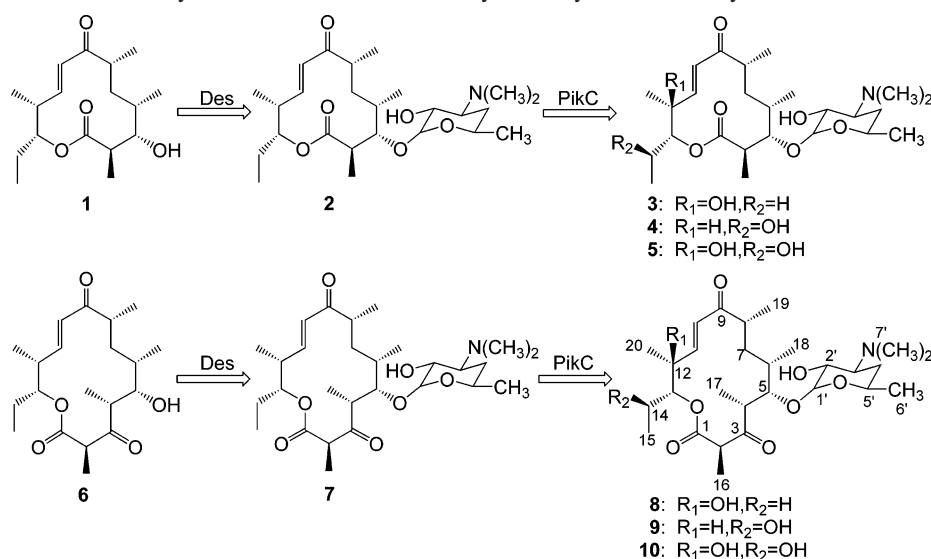
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Scheme 1. Structures of the Secondary Metabolites from the Pikromycin Biosynthetic Pathway of *S. venezuelae***Table 1.** ¹H and ¹³C NMR Data for Neopikromycin (**9**), Novapikromycin (**10**), and Pikromycin (**8**), in CDCl₃

position	neopikromycin (9)		novapikromycin (10)		pikromycin (8)	
	δ _H (m)	δ _C (m)	δ _H (m)	δ _C (m)	δ _H (m)	δ _C (m)
1		171.1 (s)		170.6 (s)		171.4 (s)
2	3.83 (q)	52.2 (d)	3.83 (q)	52.1 (d)	3.81 (q)	52.3 (d)
3		203.9 (s)		203.7 (s)		203.7 (s)
4	2.84 (dq)	45.8 (d)	3.08 (dq)	45.4 (d)	3.18 (dq)	45.5 (d)
5	3.94 (dd)	81.2 (d)	3.95 (dd)	80.8 (d)	3.94 (dd)	80.9 (d)
6	2.15 (m)	32.3 (d)	2.09 (m)	33.1 (d)	2.15 (m)	33.0 (d)
7	1.37 (t)	32.1 (t)	1.38 (t)	31.4 (t)	1.45 (t)	31.6 (t)
	1.17 (ddd)		1.15 (ddd)		1.20 (ddd)	
8	2.64 (m)	42.9 (d)	2.65 (m)	43.0 (d)	2.69 (m)	42.9 (d)
9		197.8 (s)		196.7 (s)		196.4 (s)
10	6.29 (dd)	127.7 (d)	6.27 (d)	127.3 (d)	6.30 (d)	127.5 (d)
11	6.58 (dd)	147.9 (d)	6.56 (d)	148.4 (d)	6.61 (d)	149.0 (d)
12	3.21 (m)	30.0 (d)		70.8 (s)		74.2 (s)
13	5.05 (d)	89.6 (d)	4.68 (d)	88.9 (d)	5.01 (dd)	90.2 (d)
14	3.91 (m)	64.8 (d)	4.12 (m)	67.1 (d)	1.69 (m)	19.2 (t)
			1.43 (d)		1.48 (m)	
15	1.06 (d)	(q)	1.03 (d)	(q)	0.86 (t)	10.4 (q)
16	1.43 (d)	13.5 (q)	1.433 (d)	13.6 (q)	1.43 (d)	13.7 (q)
17	1.31 (d)	12.3 (q)	1.32 (d)	12.5 (q)	1.30 (d)	12.5 (q)
18	1.063 (d)	15.2 (q)	1.06 (d)	15.4 (q)	1.08 (d)	15.1 (q)
19	1.07 (d)	17.9 (q)	1.03 (d)	17.8 (q)	1.10 (d)	17.8 (q)
20	1.26 (d)	(q)	1.42 (s)	(q)	1.31 (s)	21.8 (q)
1'	4.13 (d)	100.5 (d)	4.124 (d)	100.3 (d)	4.25 (d)	100.5 (d)
2'	3.24 (dd)	70.2 (d)	3.26 (dd)	70.3 (d)	3.24 (dd)	70.2 (d)
3'	2.53 (m)	66.0 (d)	2.91 (m)	66.3 (d)	2.51 (m)	65.9 (d)
4'	1.65 (m)	30.8 (t)	1.91 (m)	31.1 (t)	1.62 (m)	30.8 (t)
	1.23 (m)		1.44 (m)		1.23 (m)	
5'	3.49 (m)	69.9 (d)	3.49 (m)	69.9 (d)	3.56 (m)	69.9 (d)
6'	1.24 (d)	22.2 (q)	1.25 (d)	22.1 (q)	1.24 (d)	22.1 (q)
7'	2.27 (s)	40.2 (q)	2.28 (s)	40.2 (q)	2.27 (s)	40.1 (q)

resonated as a doublet, whereas those in **8** and **10** appeared as singlets at 1.31 and 1.42 ppm, respectively. It was also evident that the C-12 hydroxyl group is present in compounds **8** and **10**. The chemical shifts of all the individual protons in **8**, **9**, and **10** were ascertained from a combination of 1D and 2D (¹H–¹H COSY) analyses (Table 1), so that the proposed structures for the new metabolites neopikromycin (**9**) and novapikromycin (**10**) could be confirmed by comparing their ¹H and ¹³C NMR spectra with those of pikromycin (**8**) (Scheme 1).

The antibacterial activity of **9** and **10** was determined against *Bacillus subtilis* using the agar diffusion method as suggested by NCCLS.¹⁵ A 100 μg sample of each of **9** and **10** yielded 8 and 9 mm inhibition zones on agar plates, respectively, while the same amount of pikromycin (**8**) gave a 25 mm inhibition zone. In the case of narbomycin (**7**), an 8 mm inhibition zone was observed.

This result indicated that the absence of the hydroxyl group at C-12 in **9** or the additional presence of the hydroxyl group at C-14 in **10** might be responsible for their reduced in vitro antibacterial activity as compared to that of **8**. Interestingly, the additional hydroxyl group of the 12-membered macrolide novamethymycin (**5**) did not significantly alter the biological activity of methymycin (**3**) or neomethymycin (**4**) when the same *B. subtilis* assay system was used.¹¹

The suggested catalytic effect of PikC on the substrates was investigated by the incubation of **7** and **8** individually with PikC purified from a recombinant strain, *Escherichia coli* BL-21 (DE3)/pYJ106.¹⁶ From the HPLC/MS and MS/MS analyses, it was found that the reaction mixture in which **7** was used as a substrate contained **8** as a major product, as well as a small amount of **9**. Also, **10** was detected from the reaction carried out with **8** as a

substrate. The conversion yields of **9** and **10** were less than 10%. Although **9** is another possible substrate for the production of **10**, the bioconversion of **9** to **10** could not be carried out due to the limited availability of substrate **9**, which is produced in very small amounts from *S. venezuelae*. Although these results demonstrated that PikC catalyzed the hydroxylation of **7** and **8** to yield **9** and **10**, respectively, it is unclear whether **9** can also act as a substrate of PikC for the production of **10**.

In summary, we have isolated neopikromycin (**9**) and novapikromycin (**10**), which are new 14-membered ring macrolides from the pikromycin biosynthetic pathway of *S. venezuelae*, and elucidated their structures. **9** is the C-14 hydroxylation product of narbomycin (**7**), and **10** is a dihydroxylated product of **7**. An in vitro conversion reaction with the purified enzyme suggested that PikC cytochrome P450 monooxygenase catalyzes the hydroxylation of **7** and **8** to yield **9** and **10**, respectively. The results demonstrate that *S. venezuelae* also produces three hydroxylated 14-membered ring macrolides by the action of PikC, as in the case of the 12-membered ring macrolides, thus expanding the substrate- and regioflexibility of PikC hydroxylase. This remarkable flexibility of PikC gives it the potential to be used for the generation of novel hydroxylated macrolides, as shown in previous reports.^{16–18}

Experimental Section

General Experimental Procedures. HPLC ESIMS was conducted on a Thermal Hypersil-Keysone LC and Finnigan LCQ-Advantage equipped with an electrospray source.¹³ ¹H, ¹³C, and 2D ¹H–¹H COSY NMR spectra were acquired on a Varian INOVA 500 spectrometer at 298 K. Chemical shifts were reported in ppm using TMS as an internal reference. The assignment of each compound was carried out by comparison with the previously assigned ¹H NMR spectrum of pikromycin¹⁴ and by a combination of 1D and 2D NMR experiments. All NMR data processing was carried out with MESTREC software.

Culture, Extraction, and Isolation. *S. venezuelae* ATCC 15439 was grown on SPA solid medium (1 g of yeast extract; 1 g of beef extract; 2 g of tryptose; 10 g of glucose; trace amount of ferrous sulfate; and 15 g of agar/L) at 30 °C for 5 days. The grown culture was diced and extracted with 2 volumes of MeOH. The extract was pooled and concentrated under reduced pressure by means of a rotary evaporator. The residues were washed with H₂O and then partitioned with an equal volume of EtOAc. The combined organic phases were concentrated again under vacuum followed by reconstitution into MeOH. Prior to performing the HPLC analysis, a solid-phase extraction (SPE) cleanup column was employed not only to minimize the loss of low-level compounds of interest but also to remove unwanted impurities such as pigments and particles, which were not readily dissolved in MeOH. The crude MeOH extracts were diluted with 9 volumes of H₂O prior to being loaded onto a Waters OASIS HLB extraction cartridge (60 mg), previously conditioned with 5 mL of MeOH and then with 5 mL of H₂O. The column was washed with 5 mL of H₂O and 5 mL of MeOH–H₂O (20:80, v/v); the column was eluted with 1 mL of MeOH. This eluant was separated on a reversed-phase preparative HPLC on a Waters 120 ODS-BP 5 μ m (250 \times 10 mm i.d.) operating at a flow rate of 5 mL/min over a period of 70 min. The analytes were eluted with a gradient of 0.1% aqueous HOAc (A) and MeCN–HOAc (99.9:0.1, v/v) (B) to 15% B from 0 to 15 min, to 60% B at 60 min, then maintained at 60% B for another 10 min. The first eluant was novapikromycin (**10**) followed by neopikromycin (**9**). The next eluant was pikromycin (**8**), and finally narbomycin (**7**) was eluted. The peaks were monitored at 220 nm, and the fractions containing the product of interest were pooled. Finally, they were loaded on an SPE column again and treated as described above, and the eluants were freeze-dried. Each purified compound was subjected to HPLC ESIMS and NMR analyses. NMR samples were prepared by dissolving each compound in 200 μ L of CDCl₃ and placing the solution in a 5 mm Shigemi advanced NMR microtube matched to the solvent.

Neopikromycin (9): white solid; ¹H NMR (500 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃), see Table 1; HRESIMS *m/z* 526.3372 (M + H)⁺ (calcd for C₂₈H₄₈NO₈, 526.3302).

Novapikromycin (10): white solid; ¹H NMR (500 MHz, CDCl₃) and ¹³C NMR (100 MHz, CDCl₃), see Table 1; HRESIMS *m/z* 542.3039 (M + H)⁺ (calcd for C₂₈H₄₈NO₉, 542.3251).

Antibiotic Activity Assay. The antibacterial activity of **9** and **10** was assessed by the National Committee for Clinical Laboratory Standards (NCCLS) method. *B. subtilis* ATCC 23857 was initially grown on Mueller-Hilton broth (Difco), and aliquots of grown culture were dispensed on Mueller-Hilton agar-based medium. An appropriate amount of each compound was reconstituted in a small volume of MeOH and dispensed into paper bioassay disks. An equal volume of MeOH solvent that served as negative control was also dripped onto a disk. The dried disks were placed onto agar plates and incubated at 37 °C for 15 h. The diameter of the inhibition zone showing no growth was indicative of the antibacterial activity.

Purification of PikC and Enzymatic Conversion. The purification of PikC was carried out using the same procedures as those reported previously.¹⁶ The reaction mixture for enzymatic conversion consisted of 2 μ M PikC, 4 mM spinach ferredoxin (Sigma), 0.1 unit of ferredoxin-NADP⁺ reductase (Sigma), 1 mM NADPH (Sigma), and 0.5 mM substrate (**7** and **8**) in a total volume of 1 mL of 100 mM potassium phosphate buffer (pH 7.2). The reaction proceeded at 37 °C for 1 h and was terminated by extraction with EtOAc (\times 2). The partitioned solvent phase was evaporated to dryness, and the residue was dissolved in 100 μ L of MeOH.

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