Nuclear Magnetic Resonance and Biosynthetic Studies of Neoantimycin and Structure Elucidation of Isoneoantimycin, a Minor Metabolite Related to Neoantimycin

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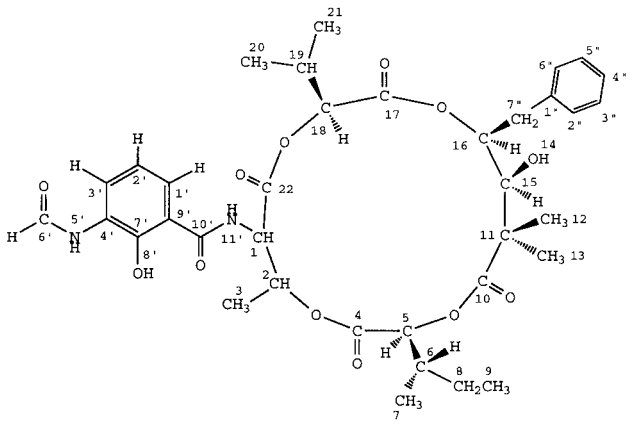
In preparation for biosynthetic studies on the 3,4-dihydroxy-2,6-dimethyl-5-phenylvaleric acid portion of neoantimycin (**1**), the ^{1}H and ^{13}C NMR signals of **1** were assigned unambiguously by means of 2D correlation spectroscopy and NOE experiments. The previously undetermined absolute stereochemistry at C-15 and C-16 was deduced as (*S*) and (*S*). The structure of isoneoantimycin (**2**) was also elucidated. The methyl groups of methionine and propionate were incorporated stereospecifically into C-13 and C-12 of **1**, respectively, and the configuration of the methyl group of methionine is inverted in the process. The results also suggest the intervention of phenylpyruvate as an actual precursor.

Neoantimycin (**1**) is produced by *Streptoverticillium orinoci* (= *Streptomyces fradiae*)[1] and was found to have a large ring ester structure consisting of 3,4-dihydroxy-2,2-dimethyl-5-phenylvaleric acid (DDPVA), (*S*)(+)-α-hydroxy-isovaleric acid, threonine, (2*S*,3*S*)(+)-α-hydroxy-*β*-methyl-valeric acid, and blastmycic acid.[2] Preliminary studies on the biosynthesis of the DDPVA portion of the molecule suggested that this moiety is formed from phenylalanine, propionate, and the methyl group of methionine.[3] In the previous study, however, no consideration was given to the stereochemical aspects of this process. This report deals with biosynthetic studies on the DDPVA portion of neoantimycin (**1**) with special emphasis on stereochemical features. The deduction of the absolute stereochemistry at C-15 and C-16 and the structure elucidation of isoneoantimycin (**2**), which was isolated as a minor metabolite, are also described.



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We employed essentially the same numbering system as reported[4] to avoid confusion.

Results and Discussion

For biosynthetic studies of neoantimycin (**1**) using ^{13}C-labeled precursors, it was necessary to assign the ^{13}C NMR signals of **1**. Prior to the assignments of ^{13}C NMR signals, we reconfirmed the assignments of the ^{1}H NMR signals in $CDCl_3$ solution to obtain better separation of the signals in the upfield region. The ^{1}H assignments were based on the ^{1}H−COSY spectrum, ^{1}H spin−spin decoupling, and comparisons of the data with those reported for **1** in Me_2CO-d_6 solution.[2] The assignments of the signals arising from the blastmycic acid, threonine, (*S*)(+)-α-hydroxy-isovaleric acid, (2*S*,3*S*)(+)-α-hydroxy-*β*-methyl-valeric acid, and phenylpropanol moieties, respectively, were easily performed by interpretation of the ^{1}H−COSY spectrum. The remaining problem was the assignment of the signals (1.31 and 1.41) due to the two geminal methyl groups at C-11. Nuclear Overhauser effects (NOEs)(7.6% and 6.7%) were observed for H-15 (*δ* 3.20) and H-8 (*δ* 1.51) upon irradiation at *δ* 1.31. On the other hand, an NOE (7.7%) was observed for H-15 upon irradiation at *δ* 1.41 (Figure 1). Taking into account that the absolute configurations at both C-5 and C-6 are (*S*), the signals at *δ* 1.31 and 1.41 were thus assigned to the (*pro-S*) (C-13) and (*pro-R*) (C-12) methyl groups, respectively. The assignments of the ^{1}H NMR signals of neoantimycin in $CDCl_3$ solution are summarized in Table 1. Based on the X-ray crystallographic analysis of 3,4-dihydroxy-2,2-dimethyl-5-phenylvaleric acid *γ*-lactone, Benedetti et al. reported that the stereochemistry at C-15 and C-16 are (*R*,*R*) or (*S*,*S*).[4] The above-mentioned results from the NOE experiments demonstrated that the absolute stereochemistry at both C-15 and C-16 is (*S*). The assignments of the ^{13}C NMR signals arising from carbon atoms carrying hydrogen atoms were then determined from the ^{1}H−^{13}C COSY spectrum of **1**. In the aromatic carbon region, there are three quaternary carbon atoms (*δ* 113.0, 127.3, and 150.6), which show cross peaks with H-8′ and are therefore assigned as C-4′, C-7′, or C-9′. The signals at *δ* 127.3 and 150.6 were assigned as C-4′

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Table 1. NMR Data ($CDCl_3$) for Neoantimycin (**1**) and Isoneoantimycin (**2**)

	1			**2**		
position	δ_{13C}	δ_H	multiplicity[a]	δ_{13C}	δ_H	multiplicity[a]
1	55.2	5.14	dd (2.6, 6.5)	55.5	5.14	dd (8.8, 3.1)
2	72.3	5.74	dq (2.6, 6.5)	71.7	5.69	dq (3.1, 6.4)
3	16.3	1.34	d (6.5)	17.0	1.43	d (6.4)
4	168.1			173.8		
5	75.1	4.66	d (8.3)	74.5	4.10	br s
6	36.0	1.96	m	39.1	2.34	m
7	14.2	0.89	d (6.8)	15.2	0.97	d (6.9)
8	24.7	1.21, 1.52	m	24.3	1.27, 1.68	m
9	10.5	0.88	t (7.4)	11.6	0.92	t (7.5)
10	176.9			178.7		
11	45.4			44.6		
12	26.9	1.41	s	22.8	1.33	s
13	21.9	1.31	s	18.4	1.20	s
14		3.57	d (12.4)			
15	79.0	3.20	d (12.4)	79.4	5.32	d (3.6)
16	71.8	5.52	dd (9.6, 5.6)	80.1	4.77	dt (9.5, 3.5)
17	168.3			168.1		
18	76.7	5.45	d (3.5)	77.8	5.03	d (2.9)
19	30.7	1.80	m	29.9	1.80	m
20	18.7	0.81	d (6.9)	16.3	1.13	d (6.9)
21	16.1	0.45	d (6.9)	19.4	1.00	d (6.9)
22	168.3			168.8		
1′	120.5	7.34	dd (8.0, 1.2)	120.1	7.23	dd (8.1, 1.3)
2′	118.9	6.93	t (8.0)	119.1	6.92	t (8.1)
3′	124.8	8.54	dd (8.0, 1.2)	124.9	8.53	dd (8.1, 1.3)
4′	127.3			127.5		
5′		8.01	s		7.94	br s
6′	159.3	8.50	d (1.7)	158.9	8.48	d (1.7)
7′	150.6			150.6		
8′		12.63	br s		12.54	s
9′	113.0			112.9		
10′	170.2			170.1		
11′		7.19−7.29			7.03	d (8.8)
1″	136.8			136.5		
2″, 6″	129.2	7.19−7.29		129.2	7.22−7.34	
3″, 5″	128.6	7.19−7.29		128.7	7.22−7.34	
4″	126.8	7.19−7.29		127.0	7.22−7.34	
7″	40.2	2.93	dd (13.6, 5.6)	35.4	2.83	dd (14.6, 3.5)
		3.16	dd (13.6, 9.6)		3.02	dd (14.6, 9.5)

[a] Coupling constants J (in Hz) are in parentheses.

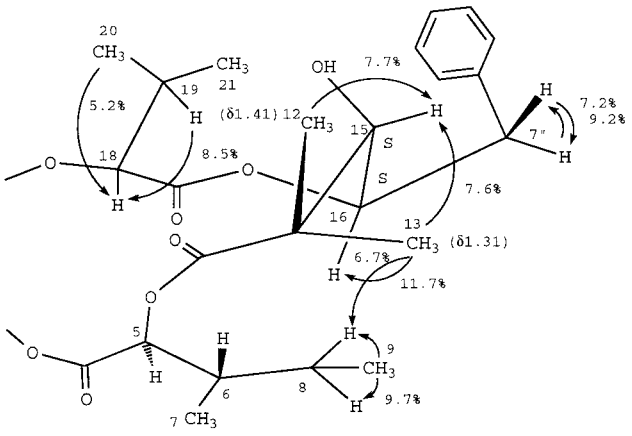

Figure 1. Selected data from NOE experiments on neoantimycin (**1**).

and C-7′, respectively, based on the fact that the former gives cross peaks with H-6′ and the latter with H-1′, H-3′, and H-5′. The signal at δ 113.0 was assigned to C-9′ inasmuch as it shows a cross peak with H-2′.

The signals at δ 45.4 and 176.9 gave cross peaks with H-12 and H-15 and were assigned to C-11 and C-10, respectively, based on consideration of their chemical shifts. On the other hand, the signals at δ 170.2 and 168.1 displayed cross peaks with H-1′ and H-1, and with H-5, respectively, assigning the former to C-10′ and the latter to C-4. The signal at δ 168.3, representing two carbons, was therefore assigned to C-17 and C-22. The assignment of the signals due to the aromatic ring of the benzyl group on C-16 was achieved as follows. There are two signals (δ 136.8 and 129.0) that give cross peaks with H-7″. Based on the DEPT experiment, the former comes from a quaternary carbon atom and the latter from a methine carbon, assigning the former to C-1″ and the latter to C-2″ and C-6″. There remain two signals unassigned (δ 125.8 and 128.6). Because the former corresponds to two carbon atoms and the latter to one carbon, the signals were assigned to C-3″ plus C-5″, and to C-4″, respectively. Thus, all the ^{13}C NMR signals were unambiguously assigned as listed in Table 2.

Table 2. Selected Data[a] of Feeding Experiments with ^{13}C-Labeled Precursors

	natural abundance	L-[^{13}C-Me]methionine	sodium [3-^{13}C]propionate
C-12	0.9	1.3	39.7
C-13	0.7	24.5	0.6
C-15	1.0	1.0	1.0

[a] Signal intensity in relation to that of C-15.

In the course of this work the antibiotic isoneoantimycin (**2**) was isolated from cultures of *Streptoverticillium orinici* IFO 13466 as a minor metabolite. Based

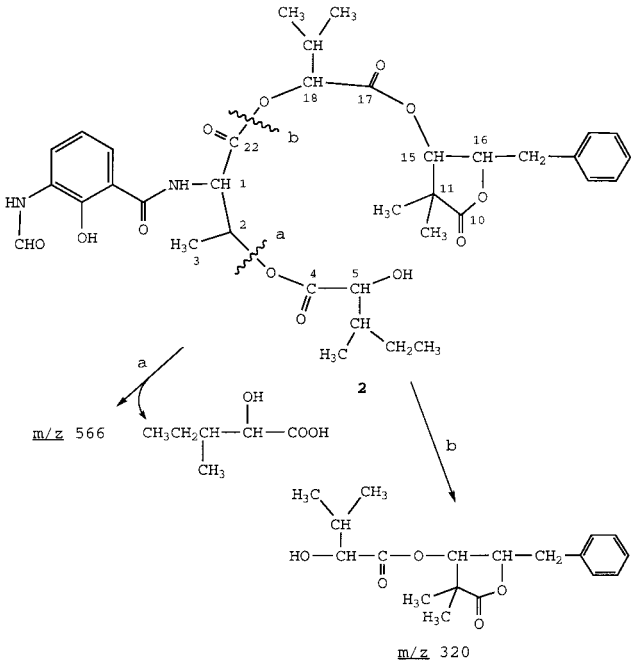

Figure 2. Presumed fragmentation in the EIMS of isoneoantimycin (**2**).

on its FABMS, it has the same molecular weight, 698, as neoantimycin (**1**). It shows UV absorption maxima at 225 (ϵ 31 380) and 320 (ϵ 5663) nm, the latter of which shifted to 340 nm on addition of aqueous NaOH solution. The IR spectrum is very similar to that of **1** except for the appearance of an absorption at 1780 cm^{-1}, which originates from a γ-lactone group. The ^1H and ^{13}C NMR spectra are also very similar to those of neoantimycin (**1**) and show the presence of the same five partial structures as in **1**. Prominent differences in the ^1H and ^{13}C NMR spectra of **1** compared to **2** are as follows: The signals of H-5 and H-16 resonate 0.56 and 0.75 ppm farther upfield and the signals of H-15 and H-21 resonate 2.12 and 0.55 ppm farther downfield, respectively, in the ^1H NMR spectrum. The signals of C-4, C-6, C-16, and C-21 are shifted downfield by 5.7, 3.1, 8.3, and 3.3 ppm, and, on the other hand, the signals of C-12, C-13, and C-20 are shifted upfield by 4.1, 3.5, and 2.4 ppm. These data suggest that isoneoantimycin (**2**) has a structure in which the ester bond between C-5 and C-10 in **1** has been hydrolyzed and a new ester bond formed around C-10−C-17. This presumption is supported by the observation in the EIMS of a fragment ion at *m*/*z* 566, which might be formed through the removal of α-hydroxy-β-methylvaleric acid and one at *m*/*z* 320, which might be formed by the fission of the bond between C-22 and an oxygen atom (Figure 2). The structure of the lactone ring was established as shown based on the results obtained in the $^1H-^{13}C$ long-range COSY spectrum (J = 10 Hz) summarized in Figure 3. Thus, the planar structure of isoneoantimycin was elucidated as **2**. The stereochemistry is assumed to be the same as that of neoantimycin, although a definitive conclusion will require further data.

The biosynthesis of **1** was investigated by the use of stable isotope and chiral metionine precursor. Based on the results of preliminary studies, including the time course of **1** production, precursors were added to the cultures 24 h after inoculation. Feeding experiments

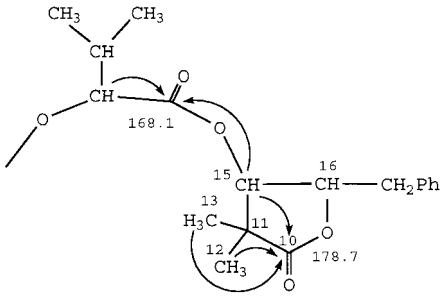

Figure 3. Selected correlations from the $^1H-^{13}C$ long-range COSY (J = 10 Hz) of isoneoantimycin (**2**).

with [^{13}C-Me]-L-methionine, sodium [3-^{13}C]propionate, and sodium [2-^{13}C]acetate revealed unambiguously that the C-10−C-13 portion of neoantimycin (**1**) is biosynthesized from propionate and the methyl group of methionine. The methyl group of methionine and carbon 3 of propionate were incorporated specifically into C-13 (δ 21.9) and C-12 (δ 26.9), respectively, as judged from the ^{13}C NMR spectra as shown in Table 2. The ^{13}C NMR spectrum of neoantimycin (**1**) derived from [2-^{13}C]acetate did not show any specific enrichments except for a slight one [threefold enrichment at C-11 (δ 45.4) compared with natural abundance]. This could be attributed to the possibility that a small amount of acetate is used for the synthesis of propionate by the microorganism. We then examined the stereochemistry of the transfer of a methyl group in chiral fashion from methionine to C-13. Neoantimycin (**1**) obtained from a culture fed with [(*S*)-^{1}H,^{2}H,^{3}H-Me]-methionine[5] was subjected to Kuhn−Roth oxidation to give tritiated HOAc from C-11 and C-13. The configurational analysis[6] revealed that the resulting acetate was 60% ee *R* on the basis of its *F* value (67.4). Thus, the stereochemistry of the chiral methyl group is inverted during its transfer to C-11, a finding consistent with the stereochemistry of most methyltransfers studied.[7] Phenylalanine has also been reported as a precursor in the biosynthesis of **1**. We presumed that the actual intermediate may be phenylpyruvate. Thus, we fed L-[U-^{14}C, 2,3-$^3H_2$]phenylalanine ($^3H/^{14}C$ = 10) to the culture. The neoantimycin isolated showed a $^3H/^{14}C$ < 3. Taking into consideration the fact that the ratio of tritium at C-2 and C-3 of the precursor is 51:49, the expected value is at most 5 if phenylpyruvate is an intermediate in the biosynthesis. The observed figure is well explained by loss of tritium from C-3 due to enolization at the stage of phenylpyruvate, suggesting that phenylpyruvate indeed serves as an actual intermediate in the biosynthetic process.

Experimental Section

General Experimental Procedures. UV spectra were recorded with a Shimadzu UV-160 spectrometer and IR spectra with a Hitachi 215 spectrometer. Optical rotations were measured with a Union Giken PM 106 digital polarimeter. NMR spectra were measured with a JEOL JNM GSX-400 or a Bruker AM-400 spectrometer. Chemical shifts are expressed in parts per million (ppm) relative to tetramethylsilane as internal standard. MS were determined with a JEOL JMS D-300 spectrometer. Si gel 60 (Merck, 70−230 mesh) was used for column chromatography. Precoated Si gel plates F_{254} (0.25 mm and 0.5 mm in thickness) were used for

Table 3. Summary of Feeding Experiments

	precursor			Neoantimycin (**1**)	
	specific radioactivity (μCi/mmole)	amount added (mg/60 mL)	total volume of culture ($\times$ 60 mL)	amount isolated (mg)	specific radioactivity (μCi/mmole)
sodium [3-^{13}C]propionate		10	6	9	
L-[^{13}C-Me]methionine		10	6	10	
sodium [2-^{13}C]acetate		10	6	9	
L-[U-^{14}C, 2, 3-$^{3}H_2$]phenylalanine	165 (^{3}H) 16.5 (^{14}C)	10	8	7	2.05 (^{3}H) 0.70 (^{14}C)
L-[(*S*)-^{1}H, ^{2}H, ^{3}H-Me]methionine	27.1 (^{3}H)	4	6	12	2.0 (^{3}H)

thin-layer chromatography and preparative layer chromatography, respectively. *Streptomyces fradiae* IFO 12773 (ISP 5063)(*Streptoverticillium orinoci* IFO 13466) was purchased from the Institute of Fermentation, Osaka. L-[methyl-^{13}C]Methionine, sodium [3-^{13}C]propionate, and sodium [2-^{13}C]acetate (99% enrichments) were obtained from Cambridge Isotope Laboratories. L-[U-^{14}C]Phenylalanine and L-[2,3-$^{3}H_2$]phenylalanine were obtained from Daiichi Chemical Co. Ltd., Japan, and diluted to 1.6 μCi/mmol(^{14}C) and 16.5 μCi/mmol-(^{3}H), respectively. L-(*methyl-S*)-[methyl-$^{2}H_1$, ^{3}H]Methionine (106.9 μCi/mmol) was synthesized by a literature procedure from sodium (*R*)-[$^{2}H_1$, ^{3}H]acetate.[5]

Isolation of Neoantimycin (1) and Isoneoantimycin (2). Nutrient broth (600 mL), consisting of 3% dextrin, 0.5% casein, 0.3% corn steep liquor, 0.1% $(NH_4)_2SO_4$, and 0.01% K_2HPO_4 at pH 7.0, in a 3-L Erlenmeyer flask was inoculated with a seed culture, maintained on a potato−dextrose agar slant, and shaken (150 rpm) for 4 days at 28 °C. A total of 8.4 L of culture was separated into culture broth and mycelia by filtration through a layer of Celite. The mycelia were extracted with MeOH repeatedly, and the combined extract was concentrated in vacuo. The culture filtrate was passed through a column of the synthetic high porous polymer Dianion HP-20 (1 L). After washing with H_2O (2 bed volumes), the column was eluted with MeOH (3 bed volumes) and Me_2CO (3 bed volumes). The MeOH and Me_2CO eluates were combined and evaporated in vacuo. The residues from the MeOH extract of the mycelia and from the MeOH and Me_2CO eluate of the HP-20 column were combined and partitioned between EtOAc (200 mL) and H_2O (200 mL). After washing with H_2O, the EtOAc extract was dried and evaporated in vacuo to give a residue (1.2 g) that was chromatographed on Si gel (100 g) with $CHCl_3$ containing increasing amounts of Me_2CO. The eluate with $CHCl_3$ (408 mg) was purified by repeated Si gel chromatography (solvent: $CHCl_3$) and preparative layer chromatography (solvent: $CHCl_3$−Me_2CO 8:2) to give neoantimycin (**1**) (83 mg) as an amorphous powder. The eluate (69.0 mg) with $CHCl_3$−Me_2CO (19:1) was purified by repeated HPLC [YMC-packed column 1 × 15 cm, solvent: hexane−EtOAc 1:1, 1 mL/min; detection 319 nm] and preparative TLC [solvent: $CHCl_3$−Me_2CO 17:3 and then 9:1] to give isoneoantimycin (**2**) (8 mg) as an amorphous powder.

Neoantimycin was identified by direct comparison with an authentic sample.[2] The physicochemical data of isoneoantimycin (**2**) are as follows.

Isoneoantimycin (2): amorphous powder, $[\alpha]^{20}_D$ +53.8(*c* 0.26, EtOH); UV λ_{max} (EtOH) 225 (31 380) and 320 nm (5663); IR ν_{max} ($CHCl_3$) 3680, 3550, 3405, 1780, 1760, 1750, 1700, 1645, 1615, 1595, 1430, 1360, 1260−1180, 1130, 1070, 1005, 950, 870, and 840 cm^{-1}; ^{1}H NMR (see Table 1); ^{13}C NMR (see Table 1); FABMS *m/z* 699 $[M + H]^+$, 721 [M + Na], and 737 $[M + K]^+$ (+KI).

Feeding Experiments. Production cultures were grown in 500-mL Erlenmyer flasks containing 60 mL of medium consisting of the same components as described above. The cultures were incubated at 28 °C with shaking at a speed of 150 rpm for 72 h. Labeled precursors were added to the cultures as Millipore-sterilized solutions 24 h after inoculation. The cultures were worked up as described above to give pure neoantimycin (**1**). The results are summarized in Table 3.

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