BIOSYNTHETIC STUDIES ON VALIDAMYCINS

I. ^{1}H AND ^{13}C NMR ASSIGNMENTS OF VALIDAMYCIN A†

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The ^{1}H and ^{13}C NMR spectra of validamycin A have been assigned by use of 2D homonuclear correlation spectroscopy for protons, and off-resonance decoupling, single frequency off-resonance decoupling, and comparison studies with model compounds for carbons. Effects of pH on carbon chemical shifts have also been studied.

Validamycin A is the major and most active component of the validamycin complex isolated in 1971 by IWASA *et al.*[1] from *Streptomyces hygroscopicus* var. *limoneus*. The antibiotic is widely used in the Orient for the treatment of sheath blight disease of rice plants.

The structure of validamycin A, first proposed by HORII and KAMEDA,[2] was recently revised by SUAMI *et al.*,[3,4] on the basis of synthetic studies, to the 4-*O*-glucosyl structure shown in Scheme 1. Validamycin A (**1**) is characterized by the novel structure of its aglycone, validoxylamine A (**2**), which consists of two aliphatic C_7 units, validamine and valienamine (**3** and **4**, respectively). These units share a single nitrogen atom and differ from one another only in their degrees of unsaturation. Both of them constitute potential "*m*-C_7N" units[5,6] and are, thus, particularly intriguing in connection with our continuing biosynthetic studies on *m*-C_7N antibiotics.[7]

Since we have used ^{13}C NMR spectroscopy for multi-labeled *m*-C_7N antibiotics in studies on the conversion of ^{13}C-enriched precursors[8] and expected to do so for validamycin, complete assignment of the ^{13}C resonances in spectra of **1** and **2** was a first priority. In the present paper the ^{13}C NMR assignments of **1** and **2**, as well as their ^{1}H NMR assignments, are reported.

Results and Discussion

Assignment of ^{1}H NMR Spectra

For a single frequency off-resonance decoupling (SFORD) experiment, it is necessary to assign the individual signals in the ^{1}H NMR spectrum. In aminocyclitol antibiotics, however, complete assignment of ring protons, other than anomeric protons, is always formidable††† due to the severe overlap of spin-coupled multiplets of these protons.

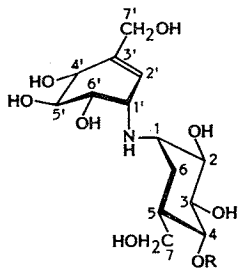
The spectra for **1** and **2** were measured at 500 MHz, to separate nearly all the resonances (Table 1). More than half of the peaks in the spectrum of **2** could be assigned readily from chemical shift and coupling constant considerations; this was true of protons 2′ (olefinic), 5, 6_{ax}, 6_{eq}, 7a and 7b

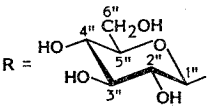
† Dedicated to Professor TETSUO SUAMI, our long-term collaborator and friend and an inspiring teacher at Keio University, on the occasion of his retirement from Keio University.

†† On leave of absence, 1981~1983, from the Institute of Antibiotics, Chinese Academy of Medical Sciences, Beijing.

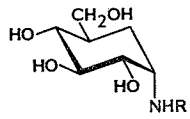
††† Recently, the ^{1}H NMR spectrum of neomycin B was assigned completely by use of 2D *J*-resolved spectroscopy at 400 MHz.[9]

Scheme 1. Structures of validamycin A and related compounds.



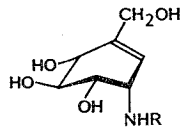
Validamycin A (**1**) R = 

Validoxylamine A (**2**) R = H



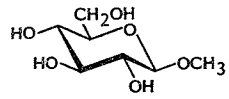
Validamine (**3**) R = H

N-Methylvalidamine (**5**) R = CH_3

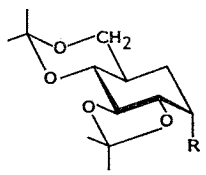


Valienamine (**4**) R = H

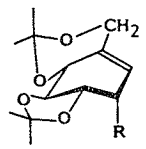
N-Methylvalienamine (**6**) R = CH_3



Methyl β-D-glucopyranoside (**7**)



8 R = NH_2
10 R = NHCHO
11 R = $NHCH_3$



9 R = N_3
12 R = NH_2
13 R = NHCHO
14 R = $NHCH_3$

(aliphatic), 4′ (allylic), and 7′a and 7′b (split AB quartet). Moreover, H-4 is assigned by its coupling constant to H-5 (11.0 Hz), H-1 by its coupling constants to H-6_{ax} and H-6_{eq} (3.0 and 4.0 Hz), and H-1′ by its coupling constant to H-2′ (5.0 Hz). There was some overlap of multiplets in the region near 3.7 ppm (Fig. 1), where the assignments were greatly facilitated by employing 2D homonuclear correlation spectroscopy (COSY).[10,11] Contour plots of the COSY maps for **2** (and **1**) are shown in Fig. 1. Four hydroxymethine protons (H-2, H-3, H-5′ and H-6′) for **2** appear in the range δ 3.6~3.7 ppm and make up complex multiplets. The coupling constants for two protons, each a doublet of doublets, at δ 3.62 ppm (J=9.5 and 4.0 Hz) and 3.68 ppm (J=10.0 and 5.0 Hz), suggest that each of these signals is due to an axial proton split by vicinal axial and equatorial protons.[12] In addition, the contour map for **2** clearly identifies the coupling between H-1 and H-2 and between H-1′ and H-6′. Therefore, these two peaks can be assigned to H-2 (δ 3.62 ppm) and H-6′ (δ 3.68 ppm). However, resonances for H-3 and H-5′ appearing around δ 3.65 ppm are not completely dispersed, which leaves their assignments ambiguous.

For **1**, some of the assignments can be made from chemical shift and coupling constant considerations—H-5, H-6_{ax}, H-6_{eq}, H-7a, H-7b, H-2′, H-4′, H-7′a and H-7′b. In addition, starting with the anomeric proton H-1″ at δ 4.59 ppm, all the resonances due to the glucose protons are assigned completely (Fig. 1). The observed coupling constants agree with the assignment. Similarly,

Table 1. ^{1}H NMR data for validamycin A (**1**) and validoxylamine A (**2**).

Proton	δ, m, (J)[a]					
	1 (free base, pH 7.6)			**2** (free base, pH 8.0)		
H-1	3.49	br s		3.33	td	(4.0, 3.0)
H-2	3.76	dd	(9.5, 3.5)	3.62	dd	(9.5, 4.0)
H-3	3.81	dd	(10.0, 9.5)	~3.65	[c]	
H-4	3.60	dd	(10.0, 8.0)	3.32	dd	(11.0, 8.5)
H-5	2.16	m		1.94	dddt	(13.0, 11.0, 6.0, 4.0)
H-6_{ax}	1.54	br t	(~14.0)	1.36	ddd	(14.5, 13.0, 3.0)
H-6_{eq}	2.10	m		2.01	dt	(14.5, 4.0)
H-7a	3.84	dd	(11.0, 4.0)	3.71	dd	(11.0, 6.0)
H-7b	3.86	dd	(11.0, 4.5)	3.79	dd	(11.0, 4.0)
H-1′	3.66	br s		3.43	t	(5.0)
H-2′	6.07	dq	(5.0, 1.5)	6.08	dq	(5.0, 1.5)
H-4′	4.16	br ddd	(7.0, 1.5, 1.0)	4.14	br d	(~7.0)
H-5′	3.76	dd	(10.0, 7.0)	~3.65	[c]	
H-6′	~3.80	[b]		3.68	dd	(10.0, 5.0)
H-7′a	4.22	br d	(13.5)	4.18	br d	(13.5)
H-7′b	4.31	ddd	(13.5, 2.5, 1.5)	4.29	ddd	(13.5, 2.5, 1.5)
H-1″	4.59	d	(8.0)			
H-2″	3.40	dd	(9.5, 8.0)			
H-3″	3.58	t	(9.5)			
H-4″	3.49	dd	(10.0, 9.5)			
H-5″	3.55	ddd	(10.0, 5.5, 2.5)			
H-6″a	3.80	dd	(12.5, 5.5)			
H-6″b	3.97	dd	(12.5, 2.5)			

[a] δ in ppm downfield from 3-(trimethylsilyl)propionate (TSP); multiplicity; (J in Hz).
[b] The peak was overlapping those of H-3 and H-6′a so that unambiguous assignment could not be made.
[c] The peaks were overlapping and the assignments thus remained obscure.

based on the signals of H-2′ at δ 6.07 ppm and H-5 at δ 2.16 ppm, the spin coupling network of the protons for the valienamine and validamine units are identified. However, unequivocal assignment of H-6′ was not possible due to overlap with the H-3 and H-6″a signals.

The coupling constants indicate that the validamine and valienamine units adopt energetically favorable chair and half-chair conformations,[13] respectively, in both **1** and **2**.

It is obvious from Table 1 that the introduction of glucose causes downfield shifts (0.07~0.28 ppm) for the protons in the validamine unit. Surprisingly, H-1′, in the valienamine unit of **1** and thus rather remote from the glucose-bearing carbon (C-4), is also shifted downfield by 0.23 ppm relative to its signal in **2**.

Assignment of ^{13}C NMR Spectra

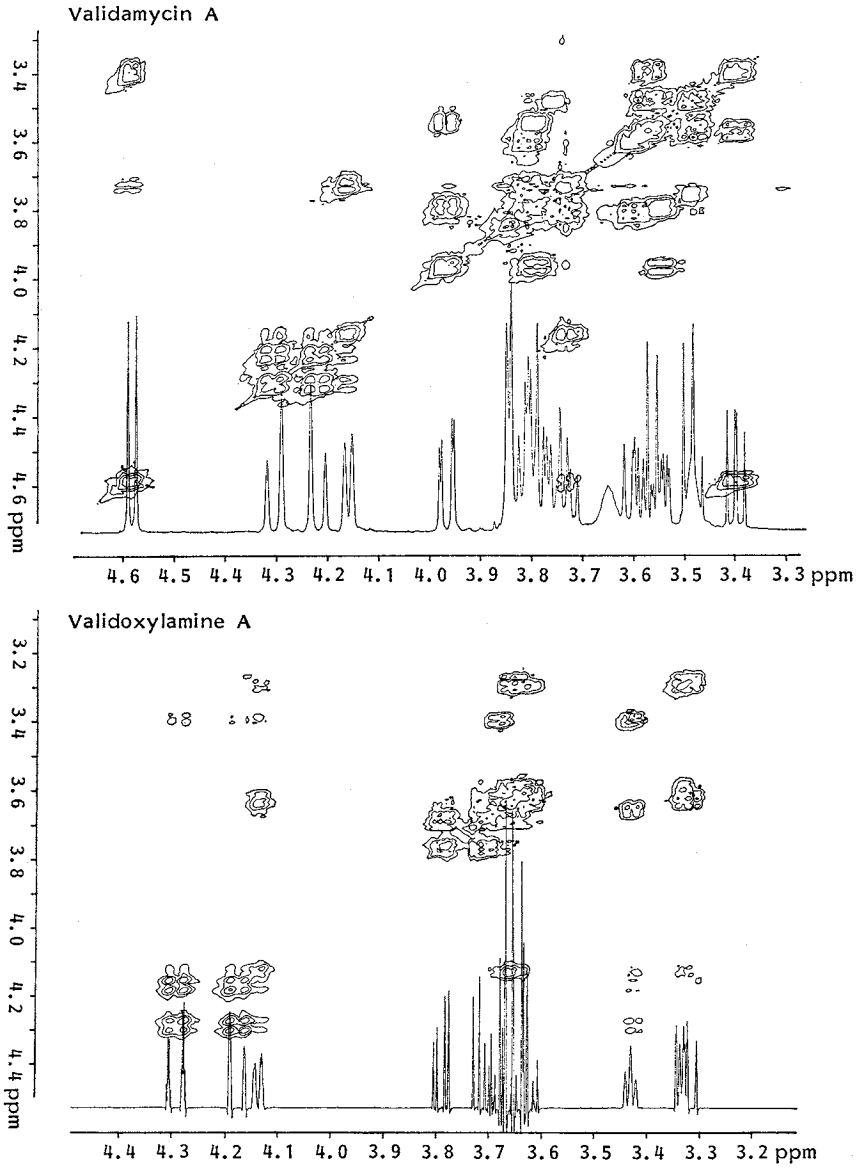
Since the effects of pH on the carbon shifts in aminocyclitol antibiotics are well known,[14] the spectra were recorded together with pH measurements; the results are found in Table 2.

Validamine (**3**), Valienamine (**4**) and Their *N*-Methyl Derivatives (**5** and **6**)

First, ^{13}C NMR spectra of **3** and **4**, which are components of the antibiotic, were assigned. Carbons bearing nitrogen atoms (C-1 and C-1′), methylene and methine carbons (C-6 and C-5) and olefinic carbons (C-2′ and C-3′) were easily assigned on the basis of chemical shifts.

Hydroxymethine carbons (C-2, C-3 and C-4) in **3** were tentatively distinguished on the basis of substituent effects. Thus, it can be argued that the carbon β to an alkyl group (C-4) should appear

Fig. 1. COSY maps for validamycin A and validoxylamine A (D_2O).



at the highest field, and that the carbon β to nitrogen (C-2) should be at higher field than that β to oxygen (C-3).†

Applying the same argument to **4**, C-6′ should appear at higher field than C-5′. Since $\Delta\delta$ (pH shift) for the carbon β to nitrogen should be the largest ($\sim$3 ppm)[15] among those for the saturated β-, γ- and δ-carbons, the signal at δ 67.9 ppm (pH 3.5) can be assigned to C-6′, β to nitrogen, which is found at 70.2 ppm for the free base. However, unambiguous assignments for C-4′ and C-5′ can not be made because of the similarity in their chemical shifts.

As model compounds for **1** and **2**, mono-*N*-methyl derivatives of validamine and valienamine (**5**

† Comparison of the reported spectra of *N*-methylcyclohexylamine[15] and cyclohexanol[16] supports the latter argument.

Table 2. ^{13}C NMR data for validamycin A and related compounds.

Carbon	δ (ppm from TSP)													
	3		5		4		6		2			7	1	
	pH 8.5[a]	pH 4.0	pH 8.5[a]	pH 3.0	pH 8.5[a]	pH 3.5	pH 8.5[a]	pH 3.5	pH 8.0[a]	(m)[b]	Change(s) on irradiating H-X		pH 7.6[a]	(m)[b]
C-1	50.4	52.1	59.5	60.4					56.4	(d)	H-1[d] → s		56.2	(d)
C-2	74.6[c]	70.9	74.3	71.1					76.0	(d)			75.6	(d)
C-3	74.8[c]	74.6[c]	75.4[c]	74.9					76.8	(d)			75.2	(d)
C-4	74.2[c]	72.9[c]	74.3[c]	72.8					75.9	(d)	H-1[d] → signal shape changed		86.8	(d)
C-5	38.7	38.8	38.8	38.8					40.5	(d)			39.9	(d)
C-6	30.3	26.8	26.3	23.8					29.4	(t)			29.4	(t)
C-7	63.3	62.4	63.4	62.4					65.0	(t)			64.3	(t)
C-1′					49.9	50.5	57.5	57.7	54.8	(d)	H-1′ → s		54.9	(d)
C-2′					123.1	116.7	123.3	114.2	125.5	(d)			125.5	(d)
C-3′					141.8	147.0	140.7	147.9	141.6	(s)			141.7	(s)
C-4′					72.5[c]	71.9[c]	72.4[c]	71.5[c]	73.9	(d)	H-4′ → s		74.0	(d)
C-5′					72.9[c]	72.9[c]	73.5[c]	72.7[c]	75.9	(d)			76.2	(d)
C-6′					70.2	67.9	70.3	67.4	71.8	(d)			72.0	(d)
C-7′					62.2	62.2	62.5	62.0	64.0	(t)	A center of H-7′a, b → s		64.1	(t)
C-1″												105.8	105.3	(d)
C-2″												75.7	75.9	(d)
C-3″												78.3[c]	78.1	(d)
C-4″												72.2	72.0	(d)
C-5″												78.5[c]	78.5	(d)
C-6″												63.3	63.0	(t)
NCH_3			34.1	32.6			34.0	31.8						
OCH_3												59.8		

[a] Compounds in aqueous solution were passed through a short column of Amberlite IRA 400 (OH^-) resin and dried *in vacuo* overnight with sodium hydroxide pellets in order to obtain their free bases.

[b] Multiplicity in off-resonance decoupled spectra.

[c] Signals may be interchanged in the column where they appear.

[d] The signal is partially overlapping with H-4 in the ^1H NMR spectrum.

and **6**, respectively) were prepared (see Experimental section) and their ^{13}C NMR spectra were measured. Again, the assignments for hydroxymethine carbons were made tentatively, using the same criteria as above.

Upon *N*-methylation, α-carbons (C-1 and C-1′) show the expected downfield shifts, by 9.1 and 7.6 ppm, respectively, while the remaining carbons show less effect, except C-6, which shifts upfield by 4.0 ppm.

Interestingly, protonation causes an unusually large downfield shift in valienamine and its *N*-methyl derivative (5.2 ppm in **4** and 7.2 ppm in **6**) for C-3′, γ to nitrogen. This seems to be explicable in terms of an allylic effect, which suggests that a positive charge introduced by protonation of an allylic nitrogen atom could polarize a π-bond, resulting in decreased electron density around C-3′.†

It is also noteworthy that the α-carbons (C-1 and C-1′) shift slightly downfield upon protonation, while the *N*-methyl carbons, also α to nitrogen, shift upfield (*ca.* 1.5 ppm).

Comparing the chemical shifts of **3** and **4** and of **5** and **6**, it can be deduced that the carbons in the valienamine unit appear at 0.5~4.0 ppm higher field than corresponding carbons in the validamine unit.[18,19]

Validoxylamine A (**2**)

Assignments were made by use of off-resonance decoupling and SFORD experiments and by comparison of the ^{13}C NMR spectrum with those of **5** and **6**.

The olefinic carbons (C-2′ and C-3′) and the methylene (C-6) and methine (C-5) carbons bearing no oxygen are also easily recognized and distinguished by their off-resonance patterns.

The hydroxymethyl carbons (C-7 and C-7′) were identified by the off-resonance decoupled spectrum and by the triplet → singlet transformation undergone by the higher field signal (C-7′) when the center (at *ca.* 4.2 ppm) of the AB quartet due to the two H-7′ protons was irradiated.

Differentiation of C-1 and C-1′, the only nitrogen-bearing carbons, is also based on SFORD studies. Irradiation of H-1′ (near 3.4 ppm) caused a doublet → singlet conversion for the higher field signal (54.8 ppm). Similarly, irradiation of H-1 (near 3.3 ppm), partially overlapping H-4 in the ^1H NMR spectrum, converted the lower field doublet (56.4 ppm) to a singlet, and there was also a change in the shape and an enhancement in the intensity of the 75.9-ppm resonance,†† which could then be attributed to C-4. SFORD at H-4′ (near 4.15 ppm) afforded a doublet → singlet conversion of the 73.9-ppm resonance, assigning the C-4′ carbon.

It was difficult to apply SFORD for the assignment of the remaining hydroxymethine carbons (C-2, C-3, C-5′ and C-6′) because the chemical shifts of their attached protons (H-2, H-3, H-5′ and H-6′) were nearly identical. The above assignments for C-1, C-1′; C-4, C-4′; and C-7, C-7′ indicate that the carbons of the valienamine unit appear upfield relative to the corresponding carbons in the validamine unit, just as they do in the monomers **3**~**6**. If this correlation is considered, the resonance at δ 71.8 ppm should belong to a valienamine carbon and that at δ 76.8 ppm to a validamine carbon. Moreover, consideration of the effect of the adjacent substituents allows the assignment of the 71.8-ppm signal to C-6′, β to nitrogen, and of the 76.8-ppm resonance to C-3, β to oxygen. By comparing

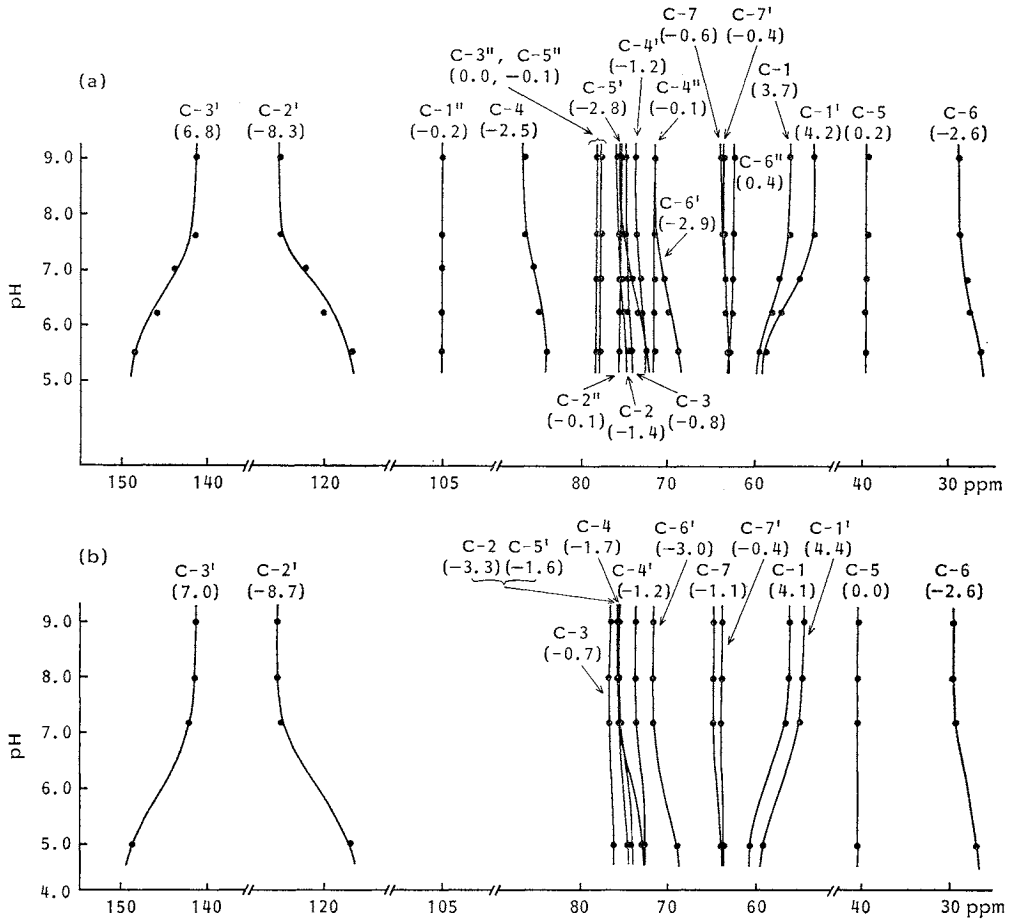
† DAVIES *et al.*[17] listed ^{13}C chemical shifts for sisomicin as free base and at pH 1.5. A similar allylic effect upon protonation is seen in their data (5.2 ppm for C-4′ in their table).

†† A singlet at δ 75.9 ppm in the noise-decoupled spectrum becomes two closely overlapping doublets in the off-resonance decoupled spectrum. This indicates that the 75.9-ppm singlet consists of two methine carbon signals.

Fig. 2. Protonation effects for (a) validamycin A and (b) validoxylamine A.

Parenthesis indicates protonation shifts, $\Delta=\delta_{\text{pH 5.5}}-\delta_{\text{free base}}$ for validamycin A and $\Delta=\delta_{\text{pH 5.0}}-\delta_{\text{free base}}$ for validoxylamine A.

Chemical shift: δ, ppm from TSP.



the chemical shifts of C-2 and C-5′ in **3** and **5** as well as in **4** and **6**, the higher field signal (δ 75.9 ppm) can be tentatively assigned to C-5′ and the signal at δ 76.0 ppm to C-2. The protonation shift shown in Fig. 2 confirms the above assignments, showing that protonation causes upfield shifts of about 3 ppm for C-2 and C-6′, β to nitrogen, and of about 1.2 ppm for C-3 and C-5′, γ to nitrogen.

Like *N*-methylation of **3** and **4**, formation of an imino linkage between **3** and **4** produced significant downfield shifts for C-1 and C-1′ (6.0 and 4.9 ppm, respectively), α to nitrogen, with smaller effects (0.9 to 3.0 ppm) on other carbons.

As expected from the shifts in **3**, **4**, **5** and **6**, protonation of **2** resulted in sizable downfield shifts for the α-carbons (4.1 ppm for C-1 and 4.4 ppm for C-1′) and the γ-olefinic carbon (7.0 ppm for C-3′).

Validamycin A (**1**)

The results obtained for validoxylamine A facilitated the assignment of the ^{13}C NMR spectrum of **1**. Assignment of the glucose carbons was based on the chemical shifts for methyl β-D-glucopyranoside (**7**).† Comparison of validoxylamine A and validamycin A spectra identifies the C-4

† The assignment of methyl β-D-glucopyranoside was made by reference to the review article.[20]

carbon as that bearing the glucose unit, in agreement with the revised structure.[3]

The assignments shown in Table 2 are supported by considering glycosidation effects. Thus, glucoside formation produces a 10.9-ppm downfield shift for the α-carbon (C-4) and 1.6-ppm and 0.6-ppm upfield shifts for the β-carbons (C-3 and C-5, respectively). The γ-carbons (C-2 and C-6) shift less than 0.4 ppm. These shifts are in good agreement with the results previously reported for bluensomycin.[21]

Although the protonation effect observed was similar to that in validoxylamine A, it must be mentioned that the carbons C-1, C-1′, C-2, C-2′, C-3, C-3′, C-6 and C-6′ exhibited significant line-broadening at pH 6.8 and 6.2, probably due to a rapid equilibration between protonated and non-protonated forms.

Experimental

General

One-dimensional and COSY ^{1}H NMR spectra of validamycin A and validoxylamine A were recorded by S. A. MIZSAK at The Upjohn Company, Kalamazoo, MI, on a Bruker WM-500 spectrometer in deuterium oxide (D_2O) containing sodium 3-(trimethylsilyl)propionate (TSP) as an internal standard. ^{1}H NMR spectra of other compounds and ^{13}C NMR spectra were measured on a Nicolet NT-360 spectrometer in deuteriochloroform ($CDCl_3$) or D_2O. Chemical shift standards were TMS ($CDCl_3$) and TSP (D_2O). The pH was adjusted with either 1 M hydrochloric acid or 0.5 M sodium hydroxide and measured with pHydrion papers (3.0 to 5.5, 6.0 to 8.0, and 8.0 to 9.5). Fast atom bombardment mass spectra (FAB-MS) were obtained using a VG Analytical 7070E spectrometer. TLC was performed on precoated Silica gel $60F_{254}$ plates (Merck, Darmstadt; 0.25-mm thickness). Silica gel used for column chromatography was purchased from Brinkmann (particle mesh size 0.05 to 0.2 mm). Solutions were evaporated at 50°C under reduced pressure.

Production of Validamycin A (1)

Streptomyces hygroscopicus var. *limoneus* was grown in a seed medium (Tryptone 0.5%, yeast extract 0.3%, D-glucose 0.3% and tap water 100 ml) in a 500-ml Erlenmeyer flask at 28°C on a rotary shaker at 20 rpm for 2 days. The seed (5 ml) was then used to inoculate 100-ml portions of production medium (D-glucose 1.0%, soluble starch 5.0%, peptone 1.5%, corn gluten meal 3.0%, NaCl 0.5%, $CaCO_3$ 1.0% and distilled water) in a 500-ml flask. The pH of the production medium was adjusted to 9.3~9.5 with NaOH before sterilization. Cultures were incubated at 28°C on a rotary shaker for 7 days. The antibiotic was isolated according to the reported procedure[22] and purified further by chromatography over Dowex 1-X2 (OH^-) with water, which gave a colorless powder after solidification from ethanol: NMR, see Tables 1 and 2, Figs. 1 and 2; FAB-MS *m/z* 498 (M+H).

Validoxylamine A (2)

Validamycin A (**1**) was hydrolyzed according to the reported procedure[23] to give **2** in 80% yield: NMR, see Tables 1 and 2, Figs. 1 and 2; FAB-MS *m/z* 336 (M+H).

Racemic Validamine (3)

Racemic 2,3: 4,7-di-*O*-isopropylidene-(1,2,4/3,5)-2,3,4-trihydroxy-5-(hydroxymethyl)cyclohexylamine (di-*O*-isopropylidenevalidamine, **8**,[24] 25 mg) was treated with 1 M hydrochloric acid (1 ml) at room temperature overnight. The mixture was neutralized with Amberlite IRA-400 (OH^-) resin. After the resin had been removed by filtration, the filtrate was evaporated to give a pale yellow syrup (18 mg, 98%), which was purified by passage through a short column of Dowex 1-X2 (OH^-, 100~200 mesh) resin with water. The ninhydrin-positive fractions were combined and evaporated to give **3** (15 mg, 86%) as a colorless syrup. FAB-MS gave *m/z* 178 (M+H).

Racemic Valienamine (4)

According to the previously reported procedure,[25] racemic 1,2: 3,7-di-*O*-isopropylidene-(1,3,6/2)-

6-azido-4-hydroxymethyl-4-cyclohexene-1,2,3-triol (**9**, 20 mg) was reduced with hydrogen sulfide to give a yellow solid, which was chromatographed over silica gel with toluene - ethanol (1 : 1). The ninhydrin-positive fractions were combined and evaporated to give racemic 4,7: 5,6-di-*O*-isopropylidene-(1,4,6/5)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexenylamine (di-*O*-isopropylidenevalienamine, **12**, 17 mg, 90%) as a colorless foam, which was then treated with 1 M hydrochloric acid in the same manner as described for the preparation of **3**. Chromatography over Dowex 1-X2 (OH^-, 100~200 mesh) with water yielded **4** (10 mg, 82% from **9**) as a colorless syrup. FAB-MS gave *m/z* 176 (M+H).

Racemic Mono-*N*-methylvalidamine (**5**)

A solution of **8** (30 mg) in 97% ethyl formate (4 ml) was heated at reflux for 2 hours. The reaction mixture was evaporated to give racemic *N*-formyl-di-*O*-isopropylidenevalidamine (**10**, 32 mg, 96%) as a colorless foam, which on TLC gave a single spot at Rf 0.4 (toluene - ethanol, 4 : 1). Without further purification, the product was dissolved in dry THF (8 ml) and the mixture was heated at reflux with lithium aluminum hydride (50 mg) for 7 hours. The excess hydride was destroyed by addition of water and the mixture was evaporated to dryness. The product was chromatographed on a silica gel column eluted with toluene - ethanol (4 : 1). The fractions containing a single spot at Rf 0.2 (toluene - ethanol, 4 : 1) were combined and evaporated to give racemic mono-*N*-methyl-di-*O*-isopropylidenevalidamine (**11**, 26 mg, 82% from **8**) as a colorless syrup: ^{1}H NMR ($CDCl_3$) δ 0.96 (1H, br t, J=13.3 Hz, H-6_{ax}), 1.37 (3H), 1.38 (6H) and 1.44 (3H) (isopropylidene CH_3), 1.66 (1H, br d, J=*ca.* 13 Hz, H-6_{eq}), 1.95~2.07 (1H, m, H-5), 2.36 (3H, s, NCH_3), 3.14 (1H, d, J=2.8 Hz, H-1), 3.46 (1H, dd, J=7.7 and 2.8 Hz, H-2), 3.58~3.74 (3H, m, H-3, H-7a and H-7b), 3.98 (1H, t, J=9.5 Hz, H-4).

Anal Calcd for $C_{14}H_{26}NO_4$: M_r 272.1862. Found: M_r 272.1854 (high resolution FAB-MS (HRFAB-MS)).

Compound **11** (22 mg) was treated with 1 M hydrochloric acid (2 ml) at 70°C for 1 hour. After neutralization with Amberlite IRA 400 (OH^-) resin, the filtrate was concentrated to give a pale yellow syrup which was purified by column chromatography on Dowex 1-X2 (OH^-, 100~200 mesh) with water to give **5** (15 mg, 97% from **11**) as a colorless syrup: ^{1}H NMR (D_2O) δ 1.11 (1H, td, J=14.8 and 3.3 Hz, H-6_{ax}), 1.58 (1H, m, H-5), 1.83 (1H, dt, J=14.8 and 3.0 Hz, H-6_{eq}), 2.16 (3H, s, NCH_3), 2.81 (1H, br s, H-1), 3.08 (1H, dd, J=10.7 and 8.7 Hz, H-4), 3.35 (1H, t, J=8.7 Hz, H-3), *ca.* 3.45 (2H, m, H-2 and H-7a), 3.56 (1H, dd, J=11.2 and 3.5 Hz, H-7b).

Anal Calcd for $C_8H_{18}NO_4$: M_r 192.1236. Found: M_r 192.1232 (HRFAB-MS).

Racemic Mono-*N*-methylvalienamine (**6**)

Compound **12** (20 mg) was *N*-formylated with 97% ethyl formate (4 ml) as described for the preparation of **10**. After evaporation, the residual syrup of racemic *N*-formyl-di-*O*-isopropylidenevalienamine (**13**, 22 mg, 99%), giving a single spot at Rf 0.5 on TLC (toluene - ethanol, 4 : 1), was dissolved in dry THF (8 ml) upon heating. To the solution was added lithium aluminum hydride (40 mg) and the mixture was heated at reflux for 7 hours. The reaction was quenched by addition of water and the mixture was evaporated to dryness. Silica gel column chromatography of the residue with toluene - ethanol (4 : 1) yielded racemic mono-*N*-methyl-di-*O*-isopropylidenevalienamine (**14**, 17 mg, 81% from **12**) as a colorless syrup: ^{1}H NMR ($CDCl_3$) δ 1.42 (3H, s), 1.48 (6H, s) and 1.56 (3H, s) (isopropylidene CH_3), 2.55 (3H, s, NCH_3), 3.47 (1H, br t, J=*ca.* 4.5 Hz, H-1), 3.65 (1H, dd, J=10 and 4.8 Hz, H-2), 4.07 (1H, dd, J=10 and 8.0 Hz, H-3), 4.20 (1H, d) and 4.47 (1H, br d) (J=14 Hz, H-7a and H-7b), 4.54 (1H, d, J=8.0 Hz, H-4), 5.64 (1H, d, J=4.5 Hz, H-6).

Anal Calcd for $C_{14}H_{24}NO_4$: M_r 270.1705. Found: M_r 270.1697 (HRFAB-MS).

Compound **14** (15 mg) was hydrolyzed and the product was purified in the same manner described for the preparation of **5** to give **6** (9.5 mg, 96% from **14**) as a colorless syrup: ^{1}H NMR (D_2O) δ 2.24 (3H, s, NCH_3), 3.18 (1H, t, J=4.5 Hz, H-1), 3.61 (1H, dd, J=12.0 and 6.0 Hz, H-5), 3.65 (1H, dd, J=12.0 and 5.2 Hz, H-6), 3.92 (1H, d, J=6.0 Hz, H-4), 3.97 (1H, d) and 4.07 (1H, d) (J=13.7 Hz, H-7a and H-7b), 5.73 (1H, dd, J=4.5 and 1.3 Hz, H-2).

Anal Calcd for $C_8H_{16}NO_4$: M_r 190.1079. Found: M_r 190.1080 (HRFAB-MS).

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