Pyralomicins, Novel Antibiotics from *Microtetraspora spiralis*

II. Structure Determination

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Novel antibiotics, pyralomicins 1a~1d, 2a~2c were isolated from the culture broth of *Microtetraspora spiralis* MI178-34F18. The structures of pyralomicins were determined by various NMR spectral analyses including ^{1}H-^{15}N HMBC and $^{13}C\{^{1}H\}$ NOE difference experiments.

In the course of our screening program for novel antibiotics, we have found pyralomicins 1a~1d, 2a~2c (**1**~**7**), from a culture broth of *Microtetraspora spiralis* MI178-34F18. In the preceding paper[1], production, fermentation, isolation, physico-chemical and biological properties of **1**~**7** were reported. In this paper, we describe the structure determination of **1**~**7** (Fig. 1).

Results and Discussion

The structural studies were carried out first for pyralomicin 1a (**1**), the major component of the antibiotics. The structures of the other components were subsequently determined by comparing their spectral data with those of **1**.

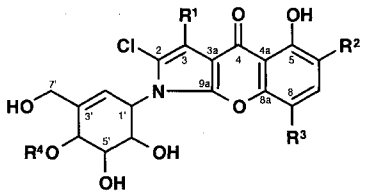
In this work, ^{1}H-^{15}N HMBC and $^{13}C\{^{1}H\}$ NOE difference experiments were employed, as well as ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC and HMQC. These two methods were necessary to determine the structure of the chromophore part of **1** which have only a small number of protons.

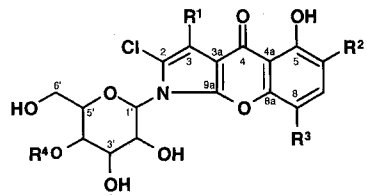
Structure of Pyralomicin 1a (**1**)

The molecular formula of **1** was elucidated as $C_{20}H_{19}NO_7Cl_2$ (MW455) from the HRFAB-MS, which was supported by the ^{1}H and ^{13}C NMR spectra of **1** (Tables 1 and 2). The ^{13}C NMR, DEPT and HMQC spectra of **1** revealed the presence of seven sp^3 carbons, consisting of a methyl (δ 14.8), a methoxy (δ 59.4), four methine (δ 60.7, 73.0, 76.8 and 82.8) and a methylene (δ 61.8) carbons. In addition, **1** contains thirteen sp^2 carbons, consisting of three methine (δ 99.7, 119.4 and 135.6) and ten quaternary (δ 105.2, 110.1, 114.2, 117.8, 119.8, 143.5, 150.2, 151.4, 155.5 and 177.5) carbons. The ^{1}H NMR spectrum indicated the presence of four deuterium exchangeable protons (δ 5.01, 5.40, 5.72 and 13.81) other than the protons which were attributed to the carbons described above. The proton at δ 13.81 was deduced to be a chelated phenolic proton because of its chemical shift and the bathochromic shift in alkaline solution in the UV spectra of **1**[1].

The two partial structures (Fig. 2 **a** and **b**) were established by the ^{1}H-^{1}H COSY, HMQC and HMBC

Fig. 1. Structures of pyralomicins 1a, 1b, 1c, 1d, 2a, 2b and 2c.





	R1	R2	R3	R4
Pyralomicin 1a (**1**)	H	Cl	CH_3	CH_3
Pyralomicin 1b (**2**)	H	CH_3	Cl	CH_3
Pyralomicin 1c (**3**)	H	Cl	CH_3	H
Pyralomicin 1d (**4**)	Cl	Cl	CH_3	H

	R1	R2	R3	R4
Pyralomicin 2a (**5**)	H	Cl	CH_3	CH_3
Pyralomicin 2b (**6**)	H	CH_3	Cl	CH_3
Pyralomicin 2c (**7**)	H	Cl	CH_3	H

Table 1. ^{1}H NMR assignments of pyralomicins 1a, 1b, 1c, 1d, 2a, 2b and 2c in DMF-d_7.

Position	1a (1)	1b (2)	1c (3)	1d (4)	2a (5)	2b (6)	2c (7)
3	6.74 s	6.74 s	6.74 s	–	6.80 s	6.80 s	6.79 s
5-OH	13.81 s	13.47 s	13.82 s	13.60 s	13.68 s	13.40 s	13.69 br s
6-CH_3	–	2.25 s	–	–	–	2.27 s	–
7	7.71 s	7.72 s	7.71 s	7.73 s	7.76 s	7.77 s	7.75 s
8-CH_3	2.38 s	–	2.36 s	2.34 s	2.48 s	–	2.46 s
1'	5.15 br d	5.15 br d	5.17 br d	5.17 br d	5.53 d, (9.3)	5.49 d, (9.3)	5.52 d, (9.3)
2'	5.82 m	5.74 br s	5.74 br s	5.80 br s	4.39 dt (5.4, 9.3)	4.55 dt (5.4, 9.3)	4.37 br t
2'-OH	–	–	–	–	5.91 d (5.4)	5.90 d (5.4)	5.88 br s
3'	–	–	–	–	3.77 dt (4.9, 9.3)	3.77 dt (4.4, 9.3)	3.63 m
3'-OH	–	–	–	–	5.62 d (4.9)	5.64 d (4.4)	5.33* br s
4'	4.13 br d	4.16 br d	4.37 m	4.35 br d	3.44 t (9.3)	3.35 t (9.3)	3.63 m
4'-OH	–	–	5.35 d, (5.4)	5.37 br s	–	–	5.50* br s
4'-OCH_3	3.60 s	3.59 s	–	–	3.63 s	3.61 s	–
5'	3.89 ddd (4.4, 6.8, 9.8)	3.88 m	3.72 ddd (3.4, 7.8, 10.3)	3.72 m	3.65 m	3.65 m	3.63 m
5'-OH	5.40 d, (4.4)	5.43 br d	5.31 d, (3.4)	5.33 br s	–	–	–
6'	4.28 m	4.38 m	4.24 m	4.20 m	3.73 m, 3.83 m	3.69 m, 3.84 m	3.74 m, 3.90 m
6'-OH	5.72 d, (5.0)	5.72 br d	5.61 br d	5.68 br s	4.93 br t	4.80 br t	4.81 br t
7'	4.22 m	4.22 m	4.29 m	4.29 m	–	–	–
7'-OH	5.01 br t	4.87 br t	4.93 br t	4.95 br s	–	–	–

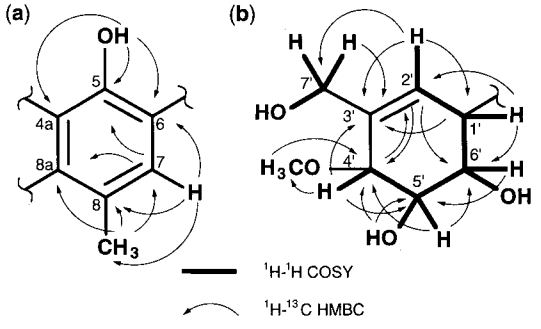
Chemical shifts in ppm from TMS as an internal standard.
J values (Hz) are in parentheses.
*These assignments are exchangeable.

Table 2. ^{13}C NMR assignments of pyralomicins 1a, 1b, 1c, 1d, 2a, 2b and 2c in DMF-d_7.

Position	1a (1)	1b (2)	1c (3)	1d (4)	2a (5)	2b (6)	2c (7)
2	119.8	119.5	119.8	117.0*	119.1	119.0	119.1
3	99.7	99.9	99.7	102.6*	100.6	100.8	100.5
3a	105.2	105.3	105.2	103.6*	105.5	105.6	105.5
4	177.5	177.6	177.5	177.2	177.7	177.8	177.7
4a	110.1	109.8	110.1	110.1	110.1	109.9	110.1
5	155.5	158.9	155.5	155.5	155.4	158.8	155.4
6	114.2	122.1	114.3	114.5	114.3	122.2	114.3
6-CH_3	–	14.4	–	–	–	14.4	–
7	135.6	135.8	135.9	136.1	135.9	136.0	135.9
8	117.8	109.8	117.9	117.9	118.0	109.9	117.9
8-CH_3	14.8	–	14.7	14.6	14.9	–	14.8
8a	151.4	148.1	151.4	151.2	151.5	148.3	151.5
9a	150.2	149.8	150.2	148.8	150.4	149.9	150.4
1'	60.7	61.0	61.2	62.3	86.0	86.0	86.3
2'	119.4	118.3	117.3	117.0	71.9	71.5	71.8
3'	143.5	144.0	145.1	145.2	77.9	78.0	78.0
4'	82.8	82.9	73.7	73.7	79.8	80.2	70.9
4'-OCH_3	59.4	59.4	–	–	60.5	60.5	–
5'	76.8	76.7	78.0	77.8	79.6	79.9	81.0
6'	73.0	72.8	72.9	73.1	61.4	62.0	62.0
7'	61.8	61.7	61.9	61.9	–	–	–

Chemical shifts in ppm from TMS as an internal standard.
*These assignments are exchangeable.

Fig. 2. Partial structures of pyralomicin 1a (**1**).

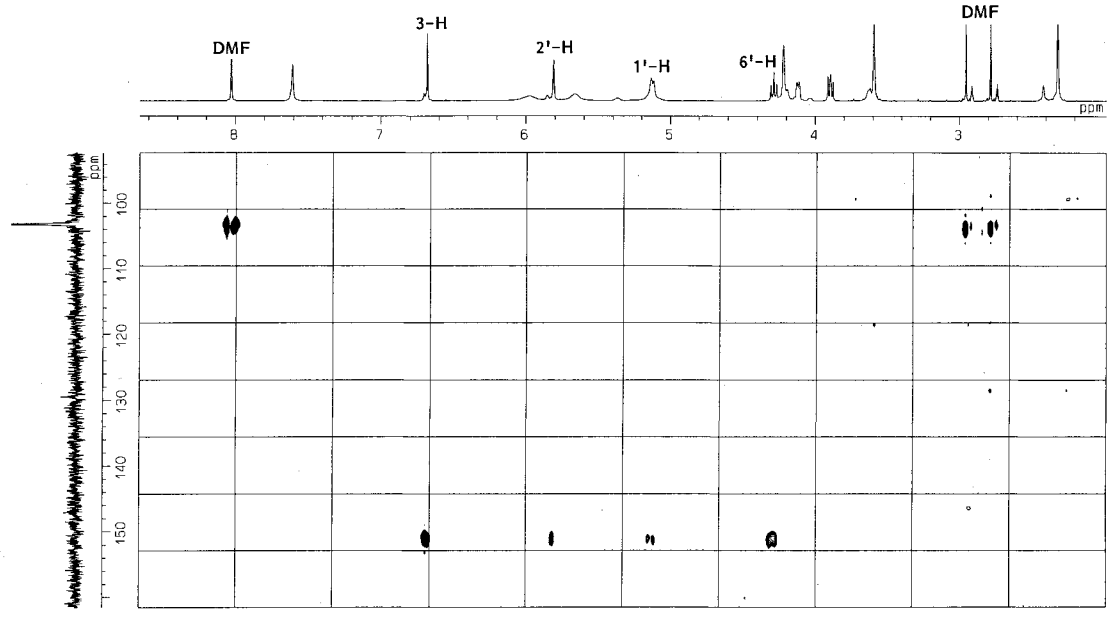


spectra. The structure of the remaining part was determined as follows.

The carbonyl carbon at δ 177.5 (C-4) did not show any correlation with proton signals in the HMBC spectrum. However, the carbon was deduced to connect to C-4a or C-6 of the partial structure **a**, because the carbonyl group should chelate with the phenolic hydroxyl group at δ 13.81 (5-OH).

In the HMQC spectrum of **1**, an aromatic proton at δ 6.74 (3-H) was correlated to an aromatic carbon at δ 99.7 (C-3), and in the HMBC spectrum this proton showed cross peaks with three aromatic carbons at δ 105.2, 119.8 and 150.2 (C-3a, C-2 and C-9a). C-2 and C-9a were deduced to be adjacent to a nitrogen atom, since these two carbons showed cross peaks with 1'-H of the partial structure **b** in the HMBC spectrum, and the chemical shift of 1'-H (δ 5.15) and C-1' (δ 60.7) suggested that C-1' bonded to the nitrogen atom. These data suggested that C-2, C-3, C-3a, C-9a and the nitrogen atom formed a pyrrole ring, and the nitrogen atom of

Fig. 3. ^{1}H-^{15}N HMBC spectrum of pyralomicin 1a (**1**) in DMF-d_7.



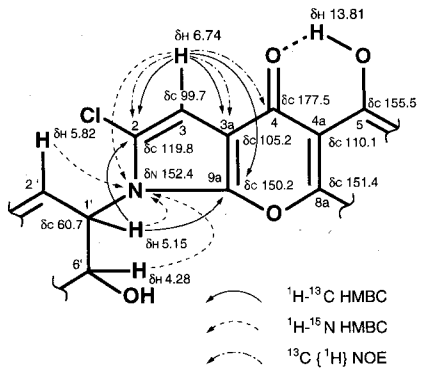
the pyrrole ring connected to the partial structure **b**.

For the confirmation of the pyrrole ring structure and of the connectivity between the partial structure **b** and the pyrrole ring, the ^{1}H-^{15}N HMBC experiment[2,3] was employed. In this case, the application of a decoupled-HMBC (D-HMBC) technique[4] was effective to observe the long-lange ^{1}H-^{15}N cross peaks. Fig. 3 shows the ^{1}H-^{15}N HMBC spectrum of **1**. The ^{1}H-^{15}N cross peaks showed the correlations from 1'-H, 2'-H, 6'-H and 2-H to the nitrogen atom at δ 151.2* (Fig. 4). This chemical shift was appropriate for a pyrrole nitrogen[6].

In the $^{13}C\{^{1}H\}$ NOE difference experiment[7] at 35°C, the irradiation of 3-H gave signal enhancements for three carbons, C-2, C-3a and C-4. Hence, the pyrrole carbons C-2 and C-3a should be adjacent to C-3, and the carbonyl carbon C-4 would bind to C-2 or C-3a (Fig. 4).

Considering the molecular formula and the chemical shifts of C-8a (δ 151.4) in the partial structure **a** and C-9a (δ 150.2) in the pyrrole ring, they were determined to be connected through the remaining oxygen atom. Therefore, the carbonyl carbon C-4 was deduced to connect to C-4a in the partial structure **a** and C-3a in the pyrrole ring to form a pyrone ring (Fig. 4). The other three combinations in connectivity of C-4, to C-2 and C-4a, to C-2 and C-6, and to C-3a and C-6 were distinctly deniable, from the inspection of the molecular model.

Fig. 4. Connectivities between partial structures **a** and **b** of pyralomicin 1a (**1**).



The remaining two chlorine atoms were properly attributed to the carbons C-2 (δ 119.8) and C-6 (δ 114.2). Consequently, the planar structure of **1** was determined as shown in Fig. 1. **1** has a benzopyranopyrrole[8] as a chromophore, the nitrogen atom of which is alkylated by a cyclitol.

Structure of Pyralomicin 1b (**2**)

The molecular formula of pyralomicin 1b (**2**) was elucidated as $C_{20}H_{19}NO_7Cl_2$ (MW455) from the HRFAB-MS, which was the same as that of **1**. The UV and IR spectra, and the ^{1}H and ^{13}C NMR spectra of **2**

* The chemical shift was determined on the ^{1}H-^{15}N HMBC spectral chart using DMF-d_7 as a reference (δ 103.2). It contains the deviation derived from the digital resolution. In the biosynthetic studies of pyralomicin 1a[5], the chemical shift of the enriched ^{15}N signal was observed at δ 152.4.

were closely similar to those of **1** (Tables 1 and 2). However in the HMBC spectrum of **2**, methyl protons at δ 2.25 (6-CH_3) showed a cross peak to a carbon at δ 158.9 (C-5) bearing a chelated hydroxyl group at δ 13.47 (5-OH), while in the HMBC spectrum of **1**, the methyl protons assigned to 8-CH_3 did not show a cross peak to C-5 which was bearing 5-OH. The NMR data suggested that **2** possessed a methyl group at C-6 instead of C-8 in **1** (Fig. 5). Consequently, the remaining chlorine atom was deduced to be at C-8, and the structure of **2** was determined as shown in Fig. 1.

Structure of Pyralomicin 1c (3)

The molecular formula of pyralomicin 1c (**3**) was elucidated as $C_{19}H_{17}NO_7Cl_2$ (MW441) from the HRFAB-MS. This molecular formula has one less carbon atom and two less protons than that of **1**. The methoxy proton and carbon signals corresponding to 4′-OCH_3 (δ 3.60, δ 59.4) of **1** were not observed in the ^1H NMR spectrum of **3** (Table 1). Instead of these signals, an additional hydroxyl proton signal appeared at δ 5.35 (4′-OH). Accordingly, the carbon signal corresponding to C-4′ (δ 73.7) of **3** resonated at higher field than that of **1** (δ 82.8) (Table2). Therefore, the structure of **3** was determined as a 4′-*O*-demethyl analogue of **1** (Fig. 1).

Structure of Pyralomicin 1d (4)

The molecular formula of pyralomicin 1d (**4**) was elucidated as $C_{19}H_{16}NO_7Cl_3$ (MW475) from the HRFAB-MS, which was one more chlorine atom and one less proton than those of **3**. The ^1H and ^{13}C NMR spectra of **4** were closely similar to those of **3** (Tables 1 and 2). However, the corresponding signal to H-3 (δ 6.74) of **3** was not observed in the ^1H NMR spectrum of **4**. Therefore, the structure of **4** was determined as a 3-chloro analogue of **3** (Fig. 1).

Structure of Pyralomicin 2a (5)

The molecular formula of pyralomicin 2a (**5**) was elucidated as $C_{19}H_{19}NO_8Cl_2$ (MW459) from the HRFAB-MS, which was one more oxygen atom and one less carbon atom than that of **1**. The UV and IR spectra of **5** were closely similar to those of **1**. The ^1H and ^{13}C NMR spectra of **5** were almost coincident with those of **1** (Tables 1 and 2), except for the cyclitol portion. The two partial structures of **5** was established by the ^1H-^1H COSY, HMQC and HMBC spectra of **5**. One was the same as the partial structure **a** of **1**, and the other, the structure from C-1′ to C-6′, was suggested to be a sugar as shown in Fig. 6. The cyclitol of **1** was replaced by a sugar in **5**. Consequently, the structure of **5** was determined as shown in Fig. 1.

Structure of Pyralomicin 2b (6)

The molecular formula of pyralomicin 2b (**6**) was elucidated as $C_{19}H_{19}NO_8Cl_2$ (MW459) from the HRFAB-MS, which was the same as that of **5**. The UV and IR spectra, and the ^1H and ^{13}C NMR spectra of **6** were closely similar to those of **5** (Tables 1 and 2). However in the HMBC spectrum of **6**, methyl protons at δ 2.27 (6-CH_3) showed a cross peak to a carbon at δ 158.8 (C-5) bearing a chelated hydroxyl group at δ 13.40 (5-OH). The NMR data of **6** suggested that **6** possessed a methyl group at C-6 instead of C-8 in **5**. Consequently, the remaining chlorine atom was deduced to be at C-8, and the structure of **6** was determined as shown in Fig. 1.

Structure of Pyralomicin 2c (7)

The molecular formula of pyralomicin 2c (**7**) was elucidated as $C_{18}H_{17}NO_8Cl_2$ (MW445) from the HRFAB-MS. This molecular formula has one less carbon atom and two less protons than that of **5**. In comparison of the ^1H and ^{13}C NMR spectra of both **7** and **5** (Tables 1 and 2), the methoxy group, 4′-OCH_3 (δ 3.63, δ 60.5) of **5** was replaced by a hydroxyl group (δ 5.50 or 5.33) of **7**, and accordingly the carbon signal corresponding to C-4′ (δ 70.9) of **7** was shifted upfield from that of **5** (δ 79.8). Therefore, the structure of **7** was determined as a 4′-*O*-demethyl analogue of **5** (Fig. 1).

Fig. 5. Partial structure of pyralomicin 1b (**2**).

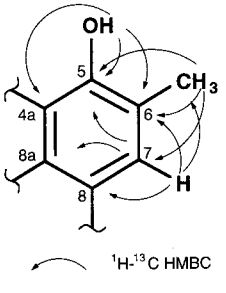


Fig. 6. Partial structure of pyralomicin 2a (**5**).

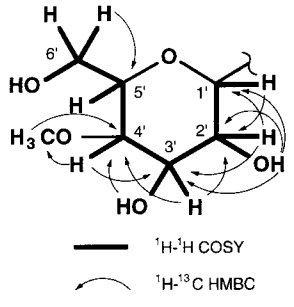


Fig. 7. Relative stereochemistry of pyralomicin 1a (**1**).

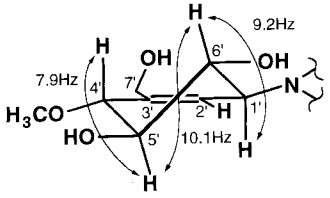
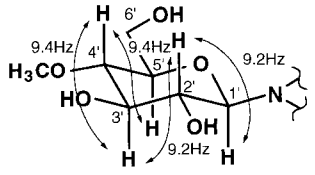


Fig. 8. Relative stereochemistry of pyralomicin 2a (**5**).



Relative Stereochemistry of **1**, **2**, **3** and **4**

The relative stereochemistry of **1** was determined by the analysis of spin-spin coupling constants. Spin decoupling experiments were performed under the following conditions to elucidate the intricate ^{1}H NMR signals; sample (6.2 mg) was dissolved in *N*,*N*-dimethylformamide(DMF)-d_7 - methanol-d_4 (20 : 1), the spectra were measured at −20°C to avoid the line broadening of the ^{1}H signals, irradiations were performed at δ 3.90 (5′-H), 4.22 (7′-H_2), 5.17 (1′-H) and 5.84 (2′-H). As shown in Fig. 7, the cyclitol was in the half-chair form and 1′-H, 4′-H, 5′-H and 6′-H were in pseudo-axial, because the coupling constants between vicinal protons in the cyclitol were large (7.9 ~ 10.1 Hz) except for the coupling constant between 1′-H and 2′-H.

This result was confirmed by the NOE difference experiment of 5,5′,6′,7′-tetra-*O*-acetyl-pyralomicin 1a (**8**). NOEs were observed between 1′-H and 2′-H, 1′-H and 5′-H, and 4′-H and 6′-H, respectively.

The relative stereochemistry of **2**, **3** and **4** were determined by comparing their ^{1}H NMR spectral data with those of **1**. Their cyclitols were deduced to be in the half-chair form as same as **1**.

Relative Stereochemistry of **5**, **6** and **7**

The relative stereochemistry of **5** was determined by the analysis of spin-spin coupling constants. Spin decoupling experiments were performed under the following conditions to elucidate the intricate ^{1}H NMR signals; sample (5.3 mg) was dissolved in DMF-d_7, signals were measured at −30°C, irradiations were performed at δ 3.48 (4′-H) and 4.39 (2′-H). The sugar moiety of **5** was found to be β-glucopyranoside shown in Fig. 8, because the four vicinal coupling constants in the sugar were all large (9.2 ~ 9.4 Hz). This result was confirmed by the NOE difference experiment of 5,2′,3′,6′-tetra-*O*-acetyl-pyralomicin 2a (**9**). NOEs were observed between 1′-H and 3′-H, 1′-H and 5′-H, 3′-H and 5′-H, and 2′-H and 4′-H, respectively.

The relative stereochemistry of **6** and **7** were determined by comparing their ^{1}H NMR spectral data with those of **5**. Their sugars were deduced to be β-glucopyranoside as same as **5**.

Experimental

General

MPs were determined on a Yanagimoto micro melting point apparatus. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. IR spectra were recorded with a Hitachi I-5020 spectrometer. Mass spectra were measured with a JEOL JMS-SX102 spectrometer.

NMR Spectrometry

NMR spectra were recorded on JEOL JNM-A500 and JNM-GX400 NMR spectrometers at room temperature except for ^{13}C{^{1}H} NOE difference experiment at 35°C.

The ^{1}H-^{15}N HMBC spectrum of **1** was measured by a JEOL JNM-A500 spectrometer using a 5 mm inverse probe. Other conditions were as follows: sample 66 mg, solvent 0.7 ml of DMF-d_7, $f_1 \times f_2 = 6{,}974 \times 5{,}000$ Hz, $t_1 \times t_2 = 256 \times 512$ points, transients = 256, repetition time = 1.10 seconds, $\Delta = 180$ milli-seconds.

The ^{13}C{^{1}H} NOE difference spectrum of **1** was measured by a JEOL JNM-A500 spectrometer using a 5 mm tunable probe at 35°C. Other conditions were as follows: sample 66 mg, solvent 0.7 ml of DMF-d_7, irradiation of 3-H at δ 6.64, pulse flip angle = 45°, data points = 32,768, spectral width = 34 kHz, repitition time = 5.97 seconds, scan times = 20,000.

Preparation of 5,5′,6′,7′-Tetra-*O*-acetylpyralomicin 1a (**8**)

To a solution of pyralomicin 1a (**1**, 30 mg) in pyridine (5 ml), acetic anhydride (100 μl) was added and stirred at room temperature for 3 days. After the removal of the solvent by evaporation, the residue was purified with silica gel column chromatography (Wakogel C-300, 5 g; *n*-hexane - EtOAc, 2 : 1) to give **8** (27 mg) as white powder.

FAB-MS *m/z* 624 $(M+H)^+$; MP 107 ~ 109°C; IR (KBr) cm^{-1} 1754, 1656, 1523, 1506, 1367, 1228, 1189, 1043; UV λ_{max}^{MeOH} nm (log ε) 212 (4.44), 240 (4.51), 294 (3.71), 330 (3.98); $[\alpha]_D^{24}$ −154.5° (*c* 0.2, MeOH); Rf 0.47 (silica gel TLC Merck, Kiesel gel 60F_{254}, EtOAc); ^{1}H NMR ($CDCl_3$) δ 7.56 (1H, s), 6.53 (1H, s), 5.83 (1H, br s), 5.72 (1H, dd, J = 9.1, 10.7 Hz), 5.57 (1H, dd, J = 7.8, 10.7 Hz), 5.32 (1H, d, J = 9.1 Hz), 4.80 (1H, d,

$J=13.9$ Hz), 4.65 (1H, d, $J=13.9$ Hz), 4.36 (1H, d, $J=7.8$ Hz), 3.49 (3H, s), 2.49 (3H, s), 2.46 (3H, s), 2.11 (3H, s), 2.10 (3H, s), 1.89 (3H, s).

Preparation of 5,2′,3′,6′-Tetra-*O*-acetylpyralomicin 2a (**9**)

To a solution of pyralomicin 2a (**5**, 30 mg) in pyridine (5 ml), acetic anhydride (100 μl) was added and stirred at room temperature for 3 days. After the removal of the solvent by evaporation, the residue was purified with silica gel column chromatography (Wakogel C-300, 5 g; *n*-hexane - EtOAc, 2 : 1) to give **9** (33 mg) as white powder.

FAB-MS *m/z* 628 $(M+H)^+$; MP 111~113°C; IR (KBr) cm^{-1} 1754, 1658, 1506, 1369, 1228, 1103, 1052; UV λ_{max}^{MeOH} nm (log ε) 213 (4.36), 240 (4.54), 293 (3.63), 328 (3.96); $[\alpha]_D^{24}$ −51.5° (*c* 0.2, MeOH); Rf 0.47 (silica gel TLC Merck, Kiesel gel $60F_{254}$, EtOAc); ^1H NMR ($CDCl_3$) δ 7.59 (1H, s), 6.54 (1H, s), 5.81 (1H, t, $J=9.3$ Hz), 5.53 (1H, br d), 5.37 (1H, t, $J=9.3$ Hz), 4.34 (2H, m), 3.80 (1H, m), 3.54 (1H, t, $J=9.3$ Hz), 3.50 (3H, s), 2.60 (3H, s), 2.48 (3H, s), 2.11 (3H, s), 2.08 (3H, s), 1.85 (3H, s).

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