THE STRUCTURE OF TAUTOMYCETIN, A DIALKYLMALEIC ANHYDRIDE ANTIBIOTIC

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The chemical structure of tautomycetin ($C_{33}H_{50}O_{10}$) was determined by chemical degradation and spectroscopic evidence. Tautomycetin exists in methanol-buffer solution (1% diethylamine-formic acid, pH 7.3) as an equilibrium mixture of a 2,3-dialkylmaleic anhydride and its dicarboxylic acid. The structure of tautomycetin is similar to tautomycin in many respects.

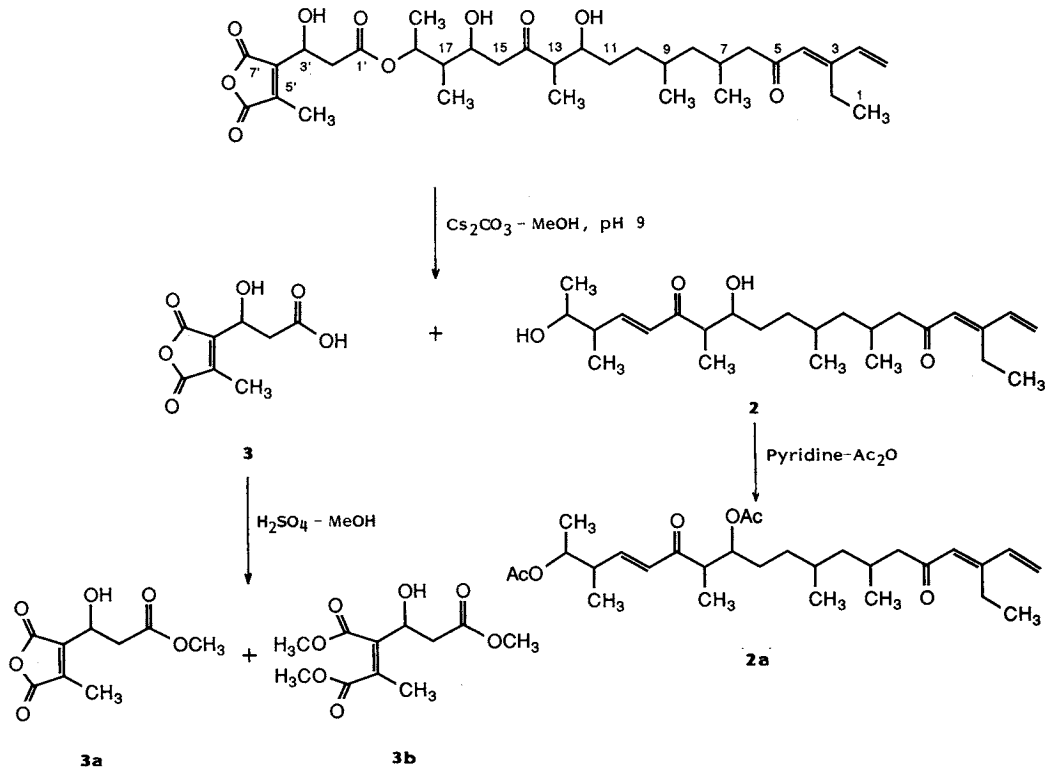
In the course of screening of soil microorganisms for new antibiotics for agricultural use, a strain of *Streptomyces griseochromogenes* was found to produce a new antibiotic, tautomycetin (**1**), which has high activity against *Sclerotinia sclerotiorum*. Besides antifungal activity, the antibiotic was found to induce the morphological change (bleb formation) of human leukemia cells K562. The biological activity of tautomycetin was previously described[1]. This paper presents experimental results and discussion for the structure elucidation of tautomycetin.

Purified tautomycetin **1** gave two peaks on the HPLC column[1], each peak was collected and analyzed by HPLC: ODS-H, MeOH - H_2O - buffer (1% diethylamine - formic acid, pH 7.3) (75 : 15 : 10). Immediately after separation, each fraction showed virtually single peaks. However, a second peak appeared and gradually increased. Equilibrium was reached after standing at 0°C for over 12 hours. The ratio of the mixture was approximately 4 : 6. This behavior could be also observed in the case of tautomycin which was described previously[2,3]. This suggested that the same dialkylmaleic anhydride moiety[4,5] exists in the structure of tautomycetin as in the case of tautomycin.

SI-MS (*m/z* 607 $(M+H)^+$) and the total number of carbons detected by ^{13}C NMR spectrum established the molecular formula of $C_{33}H_{50}O_{10}$. If measured in MeOH solution, SI-MS gave the molecular ion: *m/z* 661 $(M+CH_3OH+Na)^+$ and *m/z* 677 $(M+CH_3OH+K)^+$. IR absorption bonds at 1825 and 1760 cm^{-1} suggested the presence of 2,3-dialkylmaleic anhydride moiety, 1730 and 1700 cm^{-1} suggested the presence of ester and ketone. UV maximum at 268 nm (MeOH) suggested the presence of dienone moiety[6]. Fully decoupling (COM), INEPT and ^1H-^{13}C correlation spectrum of **1** indicated the presence of $7\times CH_3$, $7\times CH_2$, $8\times CH$, $1\times CH_2{=}$, $2\times CH{=}$, $3\times C{=}$ and $5\times C{=}O$. ^1H NMR spectrum of **1** in DMSO-d_6 showed three deuterium exchangeable protons (5.84, 4.66 and 4.46 ppm) which could be also observed in the case of tautomycin. The above data suggested that a portion of the structure of tautomycetin is similar to tautomycin.

Treatment of **1** at pH 9 (20% Cs_2CO_3, MeOH) resulted in hydrolysis of the ester bond and dehydration between C-15 and C-16, giving anhydrodeacyltautomycetin **2** and an acid **3** as shown in Fig. 1. ^1H and ^{13}C NMR (INEPT) of **2** indicated the presence of $6\times CH_3$, $5\times CH_2$, $6\times CH$, $1\times CH_2{=}$, $4\times CH{=}$, $1\times C{=}$ and $2\times C{=}O$. SI-MS (*m/z* 407 $(M+H)^+$) and the NMR data suggested the molecular formula of $C_{25}H_{42}O_4$ accounting for five unsaturations. The coupling constant ($J=15.2$ Hz) between 15-H at 6.19 ppm

Fig. 1. Degradation of tautomycetin.



and 16-H at 6.88 ppm suggested the *E*-configuration of the double bond. Acetylation of **2** (Ac_2O, pyridine) gave diacetate **2a** (Fig. 1). SI-MS (*m/z* 491 $(M+H)^+$) and the number of carbons indicated the molecular formula of $C_{29}H_{46}O_6$ which is consistent with the molecular formula of **2**. Homonuclear proton spin decoupling experiment revealed four structural fragments A, B, C and D possessing the connections shown in Fig. 2. These four fragments are separated by two carbonyls (201.0 and 200.4 ppm) and one quaternary carbon (156.2 ppm). The latter must be connected with one methine CH= (6.06 ppm, s). HMBC spectrum (Fig. 3) provided the total connectivity of carbons in **2a** and hence established the structure (Fig. 4). A carbon at 200.4 ppm gave a cross peak with the proton (6.88 ppm) on C-16, the proton (3.14 ppm) on C-13 and the protons (1.07 ppm) on 13-CH_3. The carbon at 201.0 ppm was coupled to the proton (2.38 and 2.88 ppm) on C-6 and the proton (6.07 ppm) on C-4. The quaternary carbon (156.2 ppm) was coupled with the protons (5.45 and 5.70 ppm) on the vinyl carbon, the protons (1.08 ppm) on C-1 and the protons (2.74 ppm) on C-2. The mutual coupling constant for 15-H and 16-H ($J=15.2$ Hz) indicated the typical *E*-configuration of the olefin. Difference NOE study indicated the *E*-configuration for C-3 to C-4 double bond. Irradiation at 4-H (6.07 ppm) resulted in 18% enhancement in the signal intensity at vinyl-H (6.28 ppm). Table 1 summarizes NMR data of **2** and **2a**. The assignments were based on the data from homonuclear proton spin decoupling, HMBC and carbon-proton correlation spectra.

Treatment of the acid **3** with methanol - H_2SO_4 gave monomethyl ester **3a** (EI-MS *m/z* 215 $(M+H)^+$) and trimethyl ester **3b** (EI-MS *m/z* 261 $(M+H)^+$) (Fig. 2). Table 2 presents a list of NMR data of **3a** and **3b** which are the same as the monomethyl ester and trimethyl ester of the acid moiety of tautomycin[3]. The above data confirmed the 2,3-dialkylmaleic anhydride structure of **3**.

Fig. 2. Partial structure of tautomycetin.

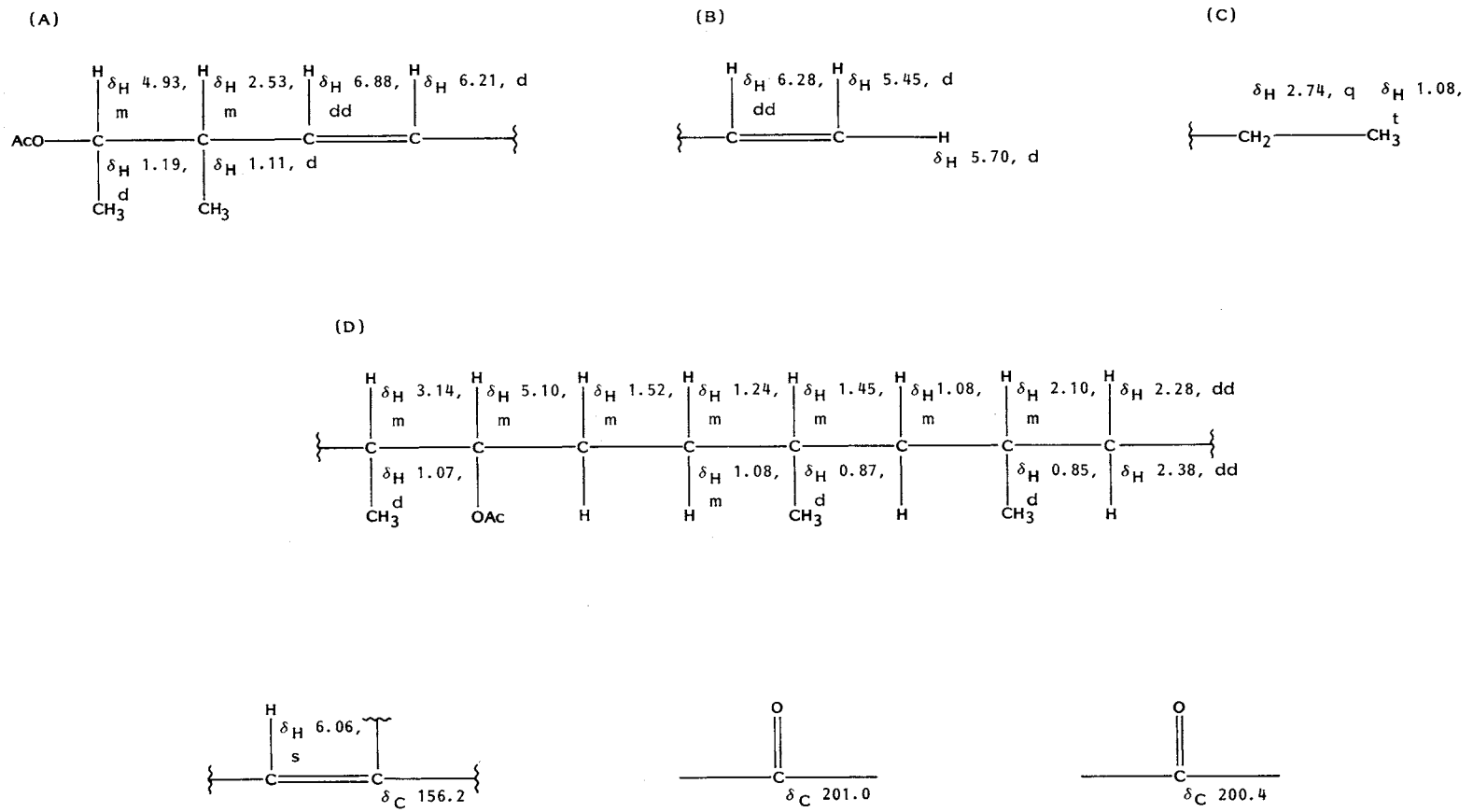


Fig. 3. HMBC spectrum of **2a**.

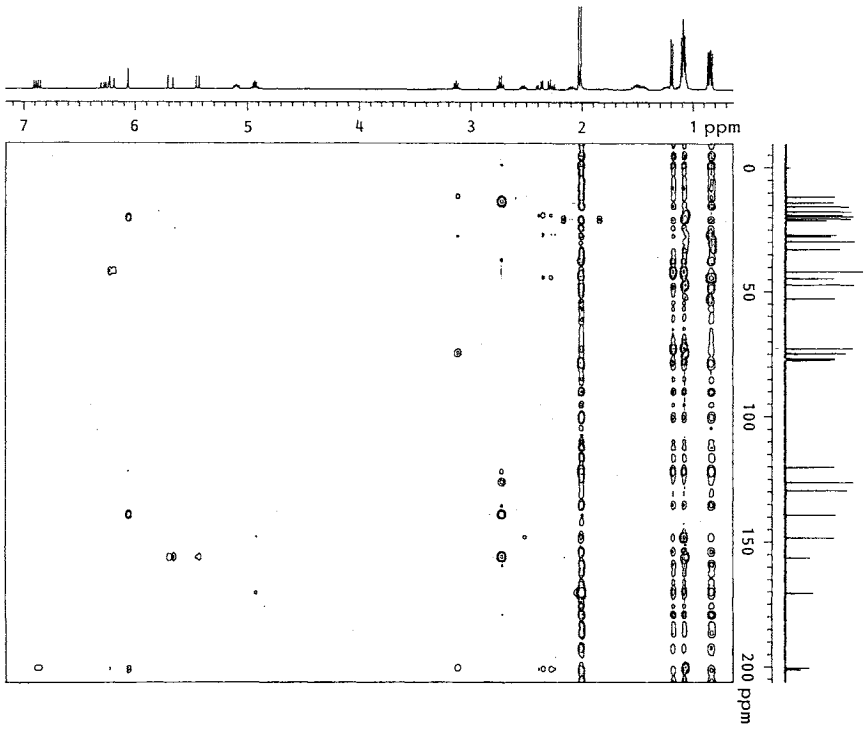
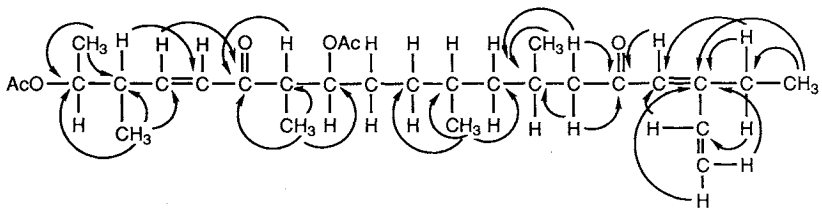


Fig. 4. ^{1}H-^{13}C coupling pattern of **2a**.



The equilibrium of **1** in HPLC comes from ring opening and closure of the dialkylmaleic anhydride moiety as in the case of tautomycin reported in the preceding paper[3].

The data reported above establishes the structure of tautomycetin. Table 3 presents the total ^{1}H and ^{13}C NMR assignment of **1**. The structure of tautomycetin and tautomycin are very similar (Fig. 5) in left half of the molecule from C-9 in tautomycetin and C-15 in tautomycin.

Experimental

General Methods

The ^{1}H NMR spectra and 2D NMR spectra were recorded on a JMN GX-400 FT-NMR spectrometer, and ^{13}C NMR spectra were recorded on a Jeol FT 100 FT-NMR spectrometer. UV spectra were recorded on a Hitachi 220 A spectrophotometer, IR spectra were recorded on a Shimadzu IR spectrophotometer IR-27 G. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. EI-MS, SI-MS and HREI-MS were recorded on Hitachi M-80 spectrometer. Positive and negative FAB-MS were performed on JMS DSX-300 and SX-102 mass instruments.

Table 1. ^{13}C and ^{1}H NMR chemical shifts of **2** and **2a** in $CDCl_3$.

	2		**2a**	
	^{13}C (100 MHz) ppm	^{1}H (400 MHz) ppm (J, Hz)	^{13}C (100 MHz) ppm	^{1}H (400 MHz) ppm (J, Hz)
C-1	13.9 q	1.08 t, J=7.2	13.9 q	1.08 t, J=7.0
C-2	20.6 t	2.73 q, J=7.2	20.5 t	2.74 q, J=7.0
C-3	156.3 s	—	156.2 s	—
3-vinyl-CH	139.2 d	6.25 dd, J=10.7, 17.6	139.2 d	6.28 dd, J=10.7, 17.6
3-vinyl-CH_2	120.0 t	5.44, 5.68 dd, J=10.7, 17.6	120.1 t	5.45, 5.70 dd, J=10.7, 17.6
C-4	126.3 d	6.07 s	126.3 d	6.07 s
C-5	201.3 s	—	201.0 s	—
C-6	52.9 t	2.28, 2.38 dd, dd, J=8.0, 15.5; 6.0, 15.5	52.8 t	2.28, 2.38 dd, dd, J=8.0, 15.5; 6.0, 15.5
C-7	27.3 d	2.10 m	27.1 d	2.10 m
7-CH_3	19.7 q	0.86 d, J=7.2	19.6 q	0.86 d, J=7.0
C-8	44.8 t	1.09 m	44.6 t	1.08 m
C-9	30.0 d	1.46 m	29.8 d	1.45 m
9-CH_3	19.2 q	0.86 d, J=7.2	19.0 q	0.84 d, J=7.0
C-10	33.4 t	1.32 m	32.9 t	1.24 m
C-11	32.2 t	1.46 m	27.7 t	1.52 m
C-12	73.9 d	3.70 m	74.7 d	5.10 m
$COCH_3$	—	—	170.4 s	—
	—	—	21.2 s	2.03 s
C-13	48.6 d	2.90 m	47.2 d	3.14 m
13-CH_3	14.7 q	1.16 d, J=7.2	13.9 q	1.07 d, J=7.0
C-14	204.9 s	—	200.4 s	—
C-15	130.4 d	6.19 d, J=15.2	129.5 d	6.21 d, J=15.2
C-16	149.7 d	6.90 dd, J=7.9, 15.2	148.3 d	6.88 dd, J=7.2, 15.2
C-17	44.3 d	2.36 m	41.8 d	2.53 m
17-CH_3	15.7 q	1.09 d, J=7.2	15.4 q	1.11 d, J=7.0
C-18	70.8 d	3.75 m	72.7 d	4.93 m
18-CH_3	20.9 d	1.19 d, J=7.2	17.5 q	1.19 d, J=7.0
$COCH_3$	—	—	170.4 s	—
	—	—	21.1 q	s

Table 2. ^{13}C and ^{1}H NMR chemical shifts of **3a** and **3b** in $CDCl_3$.

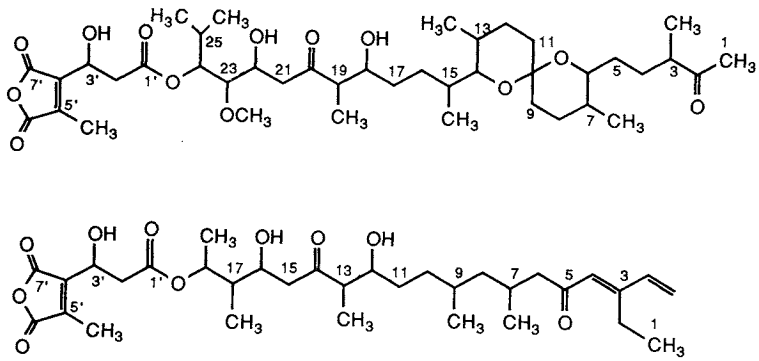
	3a	**3b**	
	^{1}H (400 MHz) ppm (J, Hz)	^{13}C (100 MHz) ppm	^{1}H (400 MHz) ppm (J, Hz)
C-1	—	171.1 s	—
OCH_3	3.78 s	52.3 q	3.72 s
C-2	2.88 m	39.8 t	2.58 dd, J=4.0, 17.0, 2.95 dd, J=1.0, 17.0
C-3	5.13 dd, J=4.0, 8.5	66.1 d	5.10 dd, J=4.0, 10.0
C-4	—	132.0 s	—
C-5	—	140.0 s	—
C-6	—	167.6 s	—
$COCH_3$	—	51.9 q	3.72 s
C-7	—	167.9 s	—
OCH_3	—	52.0 q	3.80 s
C-8	2.28 s	14.8 q	2.00 s

All reactions were monitored by TLC carried out on 0.25 mm E. Merck Silica gel plates ($60F_{254}$). Preparative TLC was performed on 0.5 mm 20 × 20 cm E. Merck Silica gel plates ($60F_{254}$). E. Merck Silica gel (60 particle size 63~200 μm) was used for column chromatography.

Table 3. ^{13}C and ^{1}H NMR chemical shifts of tautomycetin in $CDCl_3$.

	^{13}C (100 MHz) ppm	^{1}H (400 MHz) ppm (J, Hz)		^{13}C (100 MHz) ppm	^{1}H (400 MHz) ppm (J, Hz)
C-1	13.9 q	1.06 t, J=7.0	13-CH_3	13.5 q	1.05 d, J=7.0
C-2	20.5 t	2.70 q, J=7.0	C-14	215.0 s	—
C-3	156.0 s	—	C-15	46.6 t	2.43, 2.73 dd, dd overlaping
3-vinyl-CH	139.0 d	6.25 dd, J=10.7, 17.6			
3-vinyl-CH_2	120.0 t	5.40, 5.70 d, d, J=10.7, 17.6	C-16	66.0 d	4.30 m
			C-17	42.8 d	1.68 m
C-4	126.0 d	6.05 s	17-CH_3	10.2 q	1.05 d, J=7.0
C-5	201.0 s	—	C-18	73.4 d	4.98 m
C-6	52.8 t	2.35, 2.24 dd, dd, J=7.2, 15.5	18-CH_3	18.3 q	1.24 d, J=7.0
			C-1′	170.0 s	—
C-7	27.1 d	2.10 m	C-2′	40.6 t	2.80 dd, dd, J=9.4, 16.0; 9.4, 3.0
7-CH_3	19.9 q	0.85 d, J=7.0			
C-8	44.9 t	1.05, 1.20 m	C-3′	63.0 d	5.15 dd, J=9.4, 3.0
C-9	29.8 d	1.46 m	C-4′	142.2 s	—
9-CH_3	19.2 q	0.87 d, J=7.0	C-5′	142.9 s	—
C-10	32.5 t	1.32 m	C-6′	165.7 s	—
C-11	31.7 t	1.50 m	C-7′	164.8 s	—
C-12	73.6 d	3.70 m	5′-CH_3	10.2 q	2.25 s
C-13	52.5 d	2.62 m			

Fig. 5. Structures of tautomycetin (lower) and tautomycin (upper).



Preparation and Purification of Tautomycetin

Fermentation was carried out at 28°C for 72 hours in a jar fermenter containing 18 liters of a medium which is composed of glucose 1%, soluble starch 0.55%, meat extract 0.05%, dry yeast 0.2%, soybean flour 1.25%, NaCl 0.1% and K_2HPO_4 0.0025%. The filtered broth (36 liters) was extracted with EtOAc at pH 4. The mycelial cake was extracted with acetone (15 liters). The extract was evaporated *in vacuo* to give an aqueous solution which was extracted with 5 liters portions of EtOAc. The combined organic layer was washed with H_2O, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. A portion of oily residue (10.1 g) was applied onto a silica gel column (size 7.5 × 30 cm) developed successively with the solvent, $CHCl_3$ - MeOH, 10 : 1 (2 liters), 4 : 1 (2 liters), 2 : 1 (1 liter) and 1 : 1 (1 liter) (in 1 liter portions). Active portions were collected and concentrated *in vacuo* to give a yellowish oily material (1.73 g). Further purification of the oily material carried out by HPLC (Senshu-Pak-ODS-H, 250 × 20 mm, i.d., MeOH - H_2O, 7.5 : 2.5 plus 1% diethylamine - formic acid, pH 4.0) gave pure sample (100 mg). **1**: Yellowish gum; $[\alpha]_D^{25}$ +19.4° (c 0.83, $CHCl_3$); UV λ_{max}^{MeOH} nm (ε) 268 (298); IR ν_{max} (film) cm^{-1} 3400, 1825, 1760, 1730, 1700; SI-MS (m/z 607 $(M+H)^+$).

Anal Calcd for $C_{33}H_{50}O_{10}$: C 62.56, H 8.12.
Found: C 62.32, H 8.12.

Alkaline Hydrolysis of **1**

To a stirred solution of **1** (49 mg, 0.08 mmol) in MeOH (5 ml) was added dropwise 20% Cs_2CO_3 solution until the mixture solution reached pH 9, and the solution was allowed to stirr at room temperature for 1.5 hours. The resulting solution was adjusted to pH 4 with 0.1 N HCl and concentrated to remove MeOH. The aqueous solution was extracted with EtOAc. The extract was evaporated to dryness *in vacuo*. The residue was subjected to preparative TLC (silica gel, MeOH - $CHCl_3$, 1 : 50, Rf 0.15) to give compound **2**, (8.1 mg, 18% yield). Further purification was achieved by HPLC (Senshu-Pak-ODS-H, MeOH - H_2O, 80 : 20). **2**: Colorless oil, Rf 0.15 (silica gel TLC; MeOH - $CHCl_3$, 1 : 50); $[\alpha]_D^{25}$ +4.9° (*c* 1.0, $CHCl_3$); UV λ_{max}^{MeOH} nm (ε) 217 (39), 275 (26); IR ν_{max} (film) cm^{-1} 3400, 1660, 1570; SI-MS (*m/z* 407 $(M+H)^+$).

Anal Calcd for $C_{25}H_{42}O_4$: C 73.89, H 10.35.
Found: C 73.54, H 10.39.

Acetylation of **2**

2 (20 mg, 0.05 mmol) was dissolved in dry pyridine 0.5 ml and acetic anhydride 0.5 ml. The reaction mixture was stirred at room temperature for 12 hours. After quenching with ice, the mixture was extracted with EtOAc (30 ml). The organic solution was washed successively with 1 N HCl, saturated aqueous $NaHCO_3$ and H_2O, and then dried over Na_2SO_4. After filtration, the solution was concentrated *in vacuo* to dryness. The residue was subjected to preparative TLC (silica gel, EtOAc - benzene, 1 : 4), giving compound **2a** (5.6 mg, 23% yield). Further purification was achieved by HPLC (Senshu-Pak-ODS-H, MeOH - H_2O, 86 : 14). **2a**: Colorless oil; Rf 0.5 (silica gel TLC; EtOAc - benzene, 1 : 4); $[\alpha]_D^{25}$ +46.2° (*c* 2.0, $CHCl_3$); UV λ_{max}^{MeOH} nm (ε) 226 (55), 264 (62); IR ν_{max} (film) cm^{-1} 1730, 1660, 1580, 1230; SI-MS (*m/z* 491, $(M+H)^+$).

Anal Calcd for $C_{29}H_{46}O_6$: C 70.02, H 9.39.
Found: C 70.41, H 9.35.

Preparation of Trimethyl Ester of **3**

A water layer of the hydrolysate of **1** (tautomycetin 250 mg, 80% purity, 0.33 mmol) was acidified to pH 4 and lyophilized. The dry powder was suspended in MeOH (30 ml). The stirred suspension was cooled to 0°C and treated dropwise with conc H_2SO_4 (36 N, 3 ml). After 48 hours of stirring at room temperature, water (20 ml) was added. The solution was extracted with EtOAc. The EtOAc extract was washed with water and dried over Na_2SO_4. After filtration, the solution was concentrated *in vacuo*. The residue was subjected to preparative TLC (silica gel, MeOH - $CHCl_3$, 1 : 50) to give **3b** (11 mg, 12% yield). **3b**: Colorless oil, Rf 0.30 (silica gel TLC; MeOH - $CHCl_3$, 1 : 50); $[\alpha]_D^{25}$ −15.9° (*c* 0.93, $CHCl_3$); UV $\lambda_{max}^{CH_3CN}$ nm (ε) 225 (43); IR ν_{max} (film) cm^{-1} 1720; EI-MS (*m/z* 261 $(M+H)^+$).

Anal Calcd for $C_{11}H_{16}O_7$: C 50.77, H 6.15.
Found: C 51.08, H 6.32.

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References

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