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Structure of TMC-69, a new antitumor antibiotic from *Chrysosporium* sp. TC 1068

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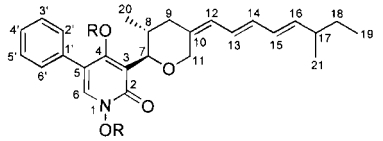
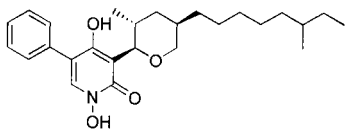
Abstract—A new antitumor antibiotic, TMC-69, possessing inhibitory activity against cdc25A phosphatase, has been isolated from the fermentation broth of a mitosporic fungus, *Chrysosporium* sp. TC 1068. This antibiotic was labile in solid state as well as in solution, and thus was converted to the more stable derivatives, diacetyl TMC-69 and hexahydro TMC-69, to elucidate the structure and to evaluate the biological activities. The planar structure of TMC-69 was determined, through the analysis of various NMR experiments of diacetyl TMC-69 and ESI-MS data of TMC-69, to be [5*Z*(*E*,*E*)]-1,4-dihydroxy-5-phenyl-3-[tetrahydro-3-methyl-5-(6-methyl-2,4-octadienylidene)-2*H*-pyran-2-yl]-2(1*H*)-pyridinone. Furthermore, the absolute configuration of tetrahydropyranyl moiety was determined by degradation studies of TMC-69, followed by the application of modified Mosher's method.

In the course of our screening program for new antitumor substances, a new antitumor antibiotic, TMC-69 (**1**) was isolated from the fermentation broth of a mitosporic fungus, *Chrysosporium* sp. TC 1068. TMC-69 (**1**) was a labile compound, and the more stable derivatives, diacetyl TMC-69 (**2**) and hexahydro TMC-69 (**3**) were prepared to elucidate the structures and to evaluate the biological activities. TMC-69 (**1**) and the derivatives **2** and **3** showed cytotoxic activities in vitro against various tumor cell lines, and **3** induced significant prolongation of survival time of mice transplanted with B16 melanoma as well as P388 leukemia. Moreover, hexahydro TMC-69 (**3**) was found to have inhibitory activity against cdc25A phosphatase. In this paper, we report the isolation, structure elucidation and cytotoxicity in vitro of **1**–**3**. The taxonomy, fermentation, and detailed evaluation of biological activities will be reported elsewhere.[1]



TMC-69 (**1**) R = H

Diacetyl TMC-69 (**2**) R = Ac



Hexahydro TMC-69 (**3**)

Keywords: TMC-69; antitumor antibiotic; *Chrysosporium*; 2(1*H*)-pyridinone.

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1. Results

1.1. Isolation

The fermentation broth (6.3 L) of *Chrysosporium* sp. TC 1068 was extracted with 1-butanol. The extract was purified by Sephadex LH-20 column chromatography and preparative HPLC to afford 43.3 mg of TMC-69 (**1**).

1.2. Structure determination

TMC-69 (**1**) was obtained as colorless powder, which was completely decomposed after two weeks at 0°C under air. In the organic solvent, e.g. DMSO, methanol, acetone, ethyl acetate, chloroform and benzene, up to 50% of **1** was decomposed within two days. TMC-69 (**1**) showed characteristic UV absorption maxima at 262, 275 and 287 nm, suggesting the presence of a triene group. The IR spectrum of **1** indicated the presence of a hydroxyl (3230 cm^{-1}) and an amide (1640 and 1550 cm^{-1}) group.

Diacetyl TMC-69 (**2**), obtained by acetylation of **1** with acetic anhydride and pyridine, was stable in methanol at room temperature for three days, even though about 50% of **2** was decomposed in chloroform or DMSO.

Hexahydro TMC-69 (**3**), obtained by catalytic hydrogenation of **1** with palladium charcoal, was stable in the organic solvents described above at room temperature for more than two weeks. The UV spectrum of **3** lacked the characteristic absorption, attributed to triene group, observed in **1**.

The ^{1}H and ^{13}C NMR spectra in $CDCl_3$ of **1** are shown in

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Table 1. ^{13}C and ^{1}H NMR data for **1**–**3**

Position	**1**[a]		**2**[b]		**3**[a]	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	157.0 s[c]		157.3 s		157.0 s	
3	108.6 s		124.4 s		109.3 s	
4	160.8 s		157.1 s		160.9 s	
5	113.6 s		117.6 s		113.5 s	
6	129.9 d	7.69 (1H, s)	134.9 d	7.87 (1H, s)	129.9 d	7.67 (1H, s)
7	80.8 d	4.88 (1H, d, 9.8)[d]	79.4 d	4.62 (1H, d, 9.9)	81.7 d	4.68 (1H, d, 10.5)
8	38.3 d	2.08 (1H, m)	37.1 d	2.40 (1H, m)	31.0 d	2.08 (1H, m)
9	41.7 t	2.41 (1H, brd, 12)[e]	43.0 t	2.45 (1H, m)[e]	36.1 t	1.77 (1H, brd, 12)[e]
		2.23 (1H, t, 12)[f]		2.15 (1H, m)[f]		1.53 (1H, m)[f]
10	133.4 s[g]		137.1 s		34.0 d	1.63 (1H, m)
11	67.3 t	4.88 (1H, d, 13.0)[e]	67.3 t	4.77 (1H, d, 12.8)[e]	72.5 t	3.96 (1H, brd, 11.6)[e]
		4.01 (1H, d, 13.0)[f]		3.83 (1H, d, 12.8)[f]		3.72 (1H, dd, 11.6, 2.5)[f]
12	125.6 d[g]	5.91 (1H, d, 9.8)	125.1 d	5.94 (1H, brd, 11.3)	30.9 t	1.52 (2H, m)
13	124.3 d[g]	6.21–6.23 (1H, m)	126.3 d	6.46 (1H, dd, 11.3, 14.2)	30.0 t	
14	134.2 d	6.21–6.23 (1H, m)	134.0 d	6.22 (1H, dd, 10.6, 14.2)	27.1 t	
15	128.6 d[g]	6.05 (1H, dd, 8.9, 15.0)	130.1 d	6.13 (1H, ddd, 0.9, 10.6, 14.8)	27.8 t	1.25–1.35 (9H, m)
16	141.9 d	5.62 (1H, dd, 7.8, 15.0)	141.2 d	5.60 (1H, dd, 7.9, 14.8)	36.9 t	
17	38.6 d	2.11 (1H, m)	39.4 d	2.10 (1H, m)	34.4 d	
18	29.7 t	1.33 (2H, m)	30.4 t	1.33 (2H, m)	29.5 t	1.11 (2H, m)
19	11.7 q	0.85 (3H, t, 7.4)	12.0 q	0.85 (3H, t, 7.4)	11.4 q	0.85 (3H, t, 7.3)
20	17.6 q	0.88 (3H, d, 6.4)	17.8 q	0.80 (3H, d, 6.5)	17.9 q	0.80 (3H, d, 6.6)
21	20.0 q	1.00 (3H, d, 6.7)	20.4 q	0.98 (3H, d, 6.9)	19.2 q	0.83 (3H, d, 6.3)
1′	133.0 s[g]		134.2 s		133.2 s	
2′,6′	122.9 d	7.43 (2H, m)	129.7 d		129.2 d	7.46 (2H, m)
3′,5′	128.4 d	7.39 (2H, m)	129.3 d	7.33–7.42 (5H, m)	128.4 d	7.41 (2H, m)
4′	127.7 d[g]	7.33 (1H, m)	128.7 d		127.7 d	7.35 (1H, m)
CH$_3$CO			17.8 q	2.35 (3H, s)		
CH$_3$CO			20.5 q	1.90 (3H, s)		
CH$_3$*CO*			167.4 s			
CH$_3$*CO*			167.3 s			
OH		9.22 (1H, brs)				9.51 (1H, brs)
OH		N.d.				N.d.

[a] Measured in $CDCl_3$.
[b] Measured in acetone d_6.
[c] Multiplicity.
[d] Proton number, multiplicity and coupling constants in Hz.
[e] Equatorial protons.
[f] Axial protons.
[g] May be exchangeable.

Table 1. Owing to unstability of **1**, poorly resolved 2D-NMR spectra of **1** were obtained in DMSO-d_6, acetone-d_6 and benzene-d_6 as well as in $CDCl_3$. The more stable derivative **2**, however, showed well-resolved 2D-NMR spectra in acetone-d_6. The structure of **1** was thus derived from the analysis of various NMR experiments of **2**.

1.3. Diacetyl TMC-69 (2) and TMC-69 (1)

The molecular formulae of **1** and **2** were elucidated to be $C_{26}H_{31}NO_4$ and $C_{30}H_{35}NO_6$ from their HRFAB-MS [*m/z* **1**: found 444.2173 $(M+Na)^+$, calcd 444.2151; **2**: found 528.2373 $(M+Na)^+$, calcd 528.2362] and ^{1}H and ^{13}C NMR data. The molecular formula of **2** differed from that of **1** by $C_4H_4O_2$ unit. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, except for the additional signals of two methyls (^{1}H: δ 2.35 and 1.90; ^{13}C: δ 17.8 and 20.5) and two carbonyls (^{13}C: δ 167.4 and 167.3). These results indicated that the structure of **2** was diacetyl derivative of **1**. The ^{13}C NMR spectrum of **2** displayed 30 signals composed of 3 methyls, 3 methylenes, 3 methines, 17 sp^2 carbons as well as two acetyl groups. In the DQF-COSY experiment, the cross peaks, observed unambiguously, indicated the presence of a 6-methyl-2,4-octadienylidene moiety (C-12–C-19 and C-21) as shown in Fig. 1.

The structure of a tetrahydro-3-methyl-2*H*-pyranyl moiety was deduced from a proton network of –H7–H8(H20)– H9–,

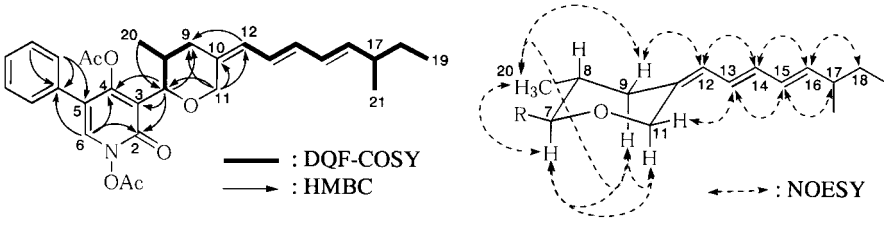

Figure 1. 2D-NMR experiments of **2**.

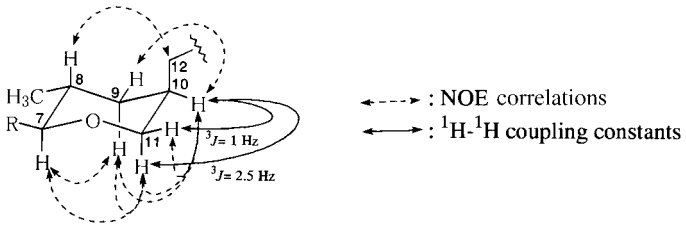

Figure 2. Key NOE correlations and ^{1}H–^{1}H coupling constants of **3**.

and HMBC correlations from H-11 (δ 4.77 and 3.83) to C-7 (δ 79.4), C-9 (δ 43.0) and C-10 (δ 137.1). The HMBC correlations from H-11 to C-10 (δ 137.1) and C-12 (δ 125.1), and from H-12 to C-9 (δ 43.0) indicated that the octadienylidene moiety was connected with the pyranyl moiety at C-10. The presence of a mono substituted benzene ring was also indicated from the characteristic ^{13}C and ^{1}H NMR signals, and HMQC and HMBC correlations. The structure of 1,4-diacetoxy-2(1*H*)-pyridinone moiety was made up by the remaining five sp^2 carbons (^{13}C: δ 157.3, 157.1, 134.9, 124.4 and 117.6), three oxygens, one nitrogen and two acetyl groups. The structure was confirmed by HMBC correlations from H-7 to C-3 (δ 124.4), C-2 (δ 157.3) and C-4 (δ 157.1), and from H-6 (δ 7.87) to C-2 and C-4, leading the plane structure of **2**.

The relative stereochemistry at C-7 and C-8 was determined to be *trans* on the basis of the ^{1}H–^{1}H coupling constant, $^3J_{\text{H-7,H-8}}$=9.9 Hz, and NOE correlation, CH$_3$-20/H-7. The configuration of the double bond at C-10 was assigned as *Z* form from NOE correlations, H-9eq/H-12, and H-11eq/H-13. The configuration of the two double bonds at C-13 and C-15 was determined to be *E* form, based on the large coupling constants, $^3J_{\text{H-13,H-14}}$=14.2 Hz and $^3J_{\text{H-15,H-16}}$=14.8 Hz, and NOE correlations H-12/H-14, H-13/H-15, H-14/H-16, and H-15/H-17 (Fig. 1). The gross structure of **2** derived from the above results, demonstrated that the structure of **1** was [5*Z*(*E*,*E*)]-1,4-dihydroxy-5-phenyl-3-[tetrahydro-3-methyl-5-(6-methyl-2,4-octadienylidene)-2*H*-pyran-2-yl]-2(1*H*)-pyridinone.

1.4. Hexahydro TMC-69 (3)

The molecular formula of **3** ($C_{26}H_{37}NO_4$, HRFAB-MS: *m*/*z* found 444.2173 $(M+Na)^+$, calcd 444.2151) with the absence of the triene absorption in the UV spectrum suggested that **3** was the hexahydro derivative of **1**. In the ^{13}C NMR spectrum, one methine carbon C-10 (δ 34.0) and five methylene carbons C-12–C-16 (δ 30.9, 30.0, 27.1, 27.8 and 36.9) in **3** were observed in place of the corresponding olefinic carbons in **1**. The structure of **3** was thus determined to be a C-10 and C-12–C-16-hexahydro derivative of **1**. The relative configuration at C-10 was deduced from the ^{1}H–^{1}H coupling ($^3J_{\text{H-10,H-11ax}}$=2.5 Hz and $^3J_{\text{H-10,H-11eq}}$=1 Hz) and NOE (H-8/H-12, H-10/H-9ax, and H-10/H-11ax) correlations as shown in Fig. 2.

1.5. Absolute structure

The absolute configuration of the tetrahydropyranyl moiety was determined by degradation studies of **1**, followed by the application of modified Mosher's method.[2] TMC-69 (**1**) was methylated with trimethylsilyldiazomethane to afford dimethyl TMC-69 (**4**). Ozonolysis of **4** gave a 10-oxotetrahydropyranyl derivative **5**, which was reduced with sodium borohydride to yield a 10-hydroxytetrahydropyranyl derivative **6**. The hydroxyl group at C-10 of **6** was determined to be located at equatorial position based on the large vicinal coupling constants, $^3J_{\text{H-10,H-9ax}}$=12 Hz and $^3J_{\text{H-10,H-11ax}}$=10.3 Hz. Finally, the 10-hydroxyl group of **6** was converted to (*S*)-(−)- and (*R*)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl (MTPA) esters to give **7** and **8**, respectively.

Distribution of the positive and negative δ values of the MTPA esters was in agreement with 10*S* stereochemistry (Fig. 3). From the above result along with the relative stereochemistry of the tetrahydropyranyl moiety, the absolute stereochemistry of **1**, **2** and **3** was established to be 7*R*, 8*R* and 7*R*, 8*R* and 7*R*, 8*R*, 10*R*, respectively.

TMC-69 (**1**) was thus determined to be [2*R*-*trans*,5*Z*(*E*,*E*)]-1,4-dihydroxy-5-phenyl-3-[tetrahydro-3-methyl-5-(6-methyl-2,4-octadienylidene)-2*H*-pyran-2-yl]-2(1*H*)-pyridinone. The stereochemistry at C-17 has not yet been established.

Several fungal metabolites, possessing phenyl-2(1*H*)-pyridinone skeleton, namely tenellin and bassianin as yellow pigments,[3,4] ilicicolin H as an antifungal antibiotic,[5] sambutoxin as a mycotoxin,[6] etc. have been reported. Among them, a tetrahydropyranyl moiety is only found in sambutoxin. Furthermore, 2(1*H*)-pyridinone skeleton with the tetrahydropyranyl moiety is seen in an antifungal antibiotic, funiculosin.[7] Both have the same substitution,

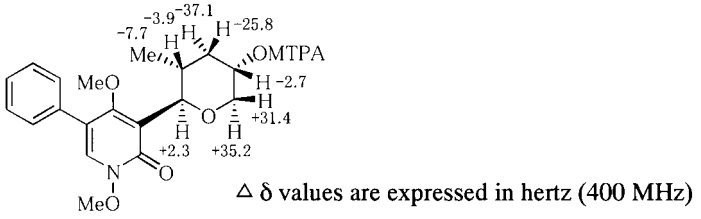

Figure 3. ^{1}H chemical shift differences ($\Delta\delta=\delta_7-\delta_8$) between **7** and **8**.

5-methyl-6-(1,3,5-trimethylhept-1-enyl)-, on the tetrahydropyranyl moiety. None of the known metabolites, however, shows the same substitution pattern on the tetrahydropyranyl moiety of TMC-69. Thus, TMC-69 is classified to be a new type of the 2(1*H*)-pyridinone antibiotic.

1.6. Biological activity

TMC-69 (**1**), and the derivatives **2** and **3** showed cytotoxicity against several tumor cell lines, human colon carcinoma HCT-116 (IC_{50} (μM); **1**: 6.8, **2**: 6.0, **3**: 0.8), B16 mouse melanoma (IC_{50} (μM); **1**: 6.6, **2**: 7.6, **3**: 1.9) and murine lymphoid neoplasm $P388D_1$ (IC_{50} (μM); **1**: 6.6, **2**: 6.8, **3**: 0.9). These results indicated that the free *N*-hydroxyl group and the triene moiety were not essential for cytotoxicity. TMC-69 derivatives **3**, exhibited significant in vivo antitumor activity in B16 melanoma or P388 leukemia bearing mice.[1] Moreover, **3** inhibited cdc25A phosphatase activity.[1] Although several related compounds having the 2(1*H*)-pyridinone skeleton have been reported, only TMC-69 derivatives showed promising antitumor activity as far as we know. Structure–activity relationships based on the synthetic study of TMC-69 will be reported in a near future.

2. Experimental

2.1. General methods

Optical rotations were determined using the sodium D line on a Horiba model SEPA-200 high sensitive polarimeter. UV spectrum of **1** and **3** were measured on a Shimadzu model UV-2200A spectrophotometer. UV spectrum of **2** was obtained on Hewlett–Packard HP-1100 diode array detector. IR spectra were recorded on a JASCO model 100 infrared spectrophotometer. The samples were prepared and mounted as KBr micropellets. FAB-MS spectra were obtained on a JEOL JMS HX-100 spectrometer and ESI-MS spectra were obtained using MStation 700 tandem type mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX-400 (400 MHz) and Varian Jemini 2000 (300 MHz) NMR spectrometer at 30°C. The chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard. Wakogel C-200 (Wako Pure Chemical Industries Ltd.) and Chromatorex DM1020 (Fuji Silysia Chemical Ltd.) were used for silica gel and amino silica gel column chromatography, respectively.

2.1.1. Isolation of TMC-69 (1). The fermentation broth (6.3 L) of *Chrysosporium* sp. TC 1068 was extracted with 1-butanol (2.7 L). The solvent layer (1.7 L) was separated and concentrated in vacuo to dryness. The residue was extracted twice with acetone (200 mL) to give a crude syrup (3.85 g). This syrup was applied onto a column of Sephadex LH-20 (40 i.d.×640 mm) and eluted with CH_2Cl_2/MeOH (1:1). The appropriate fractions, determined by TLC analysis, were combined and evaporated under reduced pressure to yield a crude solid (568 mg). A portion of this solid (10 mg) was further purified by preparative HPLC (column: YMC D-ODS-5-B, 30×250 mm; eluent: 75% CH_3CN–10 mM phosphate buffer (pH 3.5); flow rate: 25 mL min^{-1}; detection: UV at 254 nm; retention time: 33 min). This preparative HPLC was repeated 55 times for the remaining crude solid, and combined active eluates were concentrated and extracted with 1-butanol. The butanol extract was washed with water, concentrated and lyophilized to afford a pure TMC-69 (**1**, 43.3 mg) as colorless amorphous powder; UV (MeOH) λ_{max} 287 (4.63), 275 (4.76), 262 (4.69) and 245^{sh} (4.54) nm (log ε); IR (KBr) ν_{max} 3230, 2960, 2925, 2875, 1640, 1580, 1550, 1455, 1220, 1050, 990, 780 and 700 cm^{-1}; FAB-MS *m/z* 422 $(M+H)^+$, 444 $(M+Na)^+$; ESI-MS *m/z* 422 $(M+H)^+$, 334, 308, 282, 256, 216, 179 and 161. HRFAB-MS *m/z* 444.2173 $(M+Na)^+$, (calcd for $C_{26}H_{31}NO_4Na$ 444.2151); The ^1H and ^{13}C NMR data are given in Table 1.

2.1.2. Diacetyl TMC-69 (2). Compound **1** (70 mg) and acetic anhydride (1.8 mL) in dry pyridine (7.0 mL) were stirred at room temperature for 30 min. The reaction mixture was diluted with ethyl acetate (100 mL), washed with water, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting crude syrup was purified by preparative HPLC (column: YMC D-ODS-5B, 30 i.d.×250 mm; elution: 75% aqueous acetonitrile; flow rate: 25 mL min^{-1}; detection: UV at 254 nm; retention time: 55.3 min) followed by Sephadex LH-20 column chromatography with acetone to afford 18.8 mg of **2** as colorless amorphous powder; UV (75% aqueous acetonitrile) λ_{max} 284, 274, 265 nm; IR (KBr) ν_{max} 3430, 2960, 2925, 2875, 1810, 1775, 1670, 1540, 1365, 1160, 990 and 700 cm^{-1}; FAB-MS *m/z* 506 $(M+H)^+$, 528 $(M+Na)^+$; HRFAB-MS *m/z* 528.2373 $(M+Na)^+$, (calcd for $C_{30}H_{35}NO_6Na$ 528.2362); The ^1H and ^{13}C NMR data are given in Table 1.

2.1.3. Hexahydro TMC-69 (3). Compound **1** (100.5 mg) in methanol (2.0 mL) was reduced with hydrogen over 50% wet-10% palladium charcoal (20.0 mg) for 1 h. The reaction mixture was filtered and concentrated under reduced pressure. The resulting syrup was purified by preparative HPLC (column: YMC D-ODS-5-B, 30 i.d.×250 mm; elution: 85% aqueous acetonitrile; flow rate: 25 mL min^{-1}; detection: UV at 254 nm; retention time: 55.3 min) followed by Sephadex LH-20 column chromatography with CH_2Cl_2/MeOH (1:1) to give 6.5 mg of **3**. Colorless, amorphous powder; $[\alpha]_D^{25}=+143.4°$(*c* 0.480, MeOH); UV (MeOH) λ_{max} 300 (3.65), 241 (4.38), 210 (4.36) nm (log ε); IR (KBr) ν_{max} 3180, 2960, 2925, 2855, 1640, 1580, 1560, 1500, 1455, 1380, 1220, 1050, 980, 870, 775 and 700 cm^{-1}; FAB-MS *m/z* 428 $(M+H)^+$, 450 $(M+Na)^+$; HRESI-MS *m/z* 426.2621 $(M-H)^-$, (calcd for $C_{26}H_{36}NO_4$ 426.2644); The ^1H and ^{13}C NMR data are given in Table 1.

2.1.4. Methylation of 1. To a solution of **1** (655 mg) in CH_2Cl_2 (100 mL) and MeOH (20 mL) was added excess diazomethane in diethyl ether. The mixture was stirred at 0°C for 30 min and then at room temperature for 3 h. After removing the solvent, the residue was purified on amino silica gel column with EtOAc/*n*-hexane (1:1), followed by silica gel column with EtOAc/*n*-hexane (1:4, 1:2, 1:1; stepwise) to yield dimethyl TMC-69 (**4**, 120 mg, 17% yield). ESI-MS *m/z* 450 $(M+H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.50 (H-6, 1H, s), 7.33–7.45 (Ph, 5H, m), 6.30 (H-13, 1H, dd, 14, 11.2), 6.15 (H-14, 1H, dd, 14, 10.3), 6.05 (H-15, 1H, 14.7, 10.3), 5.87 (H-12, 1H, d, 11.2), 5.58 (H-16, 1H, dd, 14.7, 7.7), 4.85 (H-11eq, 1H, brd, 13), 4.68 (H-7, 1H, d,

10.1), 4.10 (N–OCH_3, 3H, s), 4.00 (H-11ax, 1H, d, 13), 3.35 (OCH_3, 3H, s), 2.46 (H-9eq, 1H, dd, 14, 4.2), 2.70 (H-8, 1H, m), 2.0–2.2 (H-9ax and H-17, 2H, m), 1.32 (H-18, 2H, m), 1.01 (H-21, 3H, d, 6.8), 0.85 (H-19, 3H, t, 7.3), 0.82 (H-20, 3H, d, 6.6).

2.1.5. Preparation of 6 from 4. Dry O_3 was bubbled into the solution of **4** (80 mg, 0.18 mmol) in MeOH (5 mL) at −78°C, until the starting material was disappeared on TLC (SiO_2, EtOAc/*n*-hexane (1:1)). To the reaction mixture, Me_2S was added (200 μL, 2.7 mmol) and then the mixture was stirred at room temperature for 1 h. To the resulting mixture, $NaBH_4$ was added (27 mg, 0.72 mmol) and then the mixture was stirred at 0°C for 30 min. After adding acetone (1 mL) to the reaction mixture, water was added and the mixture was extracted with CH_2Cl_2. The organic phase was washed with water, saturated NaCl, and dried over anhydrous Na_2SO_4. The solvent was concentrated in vacuo and the residual oil was purified on preparative TLC (SiO_2, 6% MeOH–$CHCl_3$) to yield a colorless oil of **6** (9 mg, 22% yield). ESI-MS *m/z* 346 $(M+H)^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.51 (H-6, 1H, s), 7.35–7.45 (Ph, 5H, m), 4.46 (H-7, 1H, d, 10), 4.15 (H-11eq, 1H, ddd, 10.3, 5, 2), 4.10 (N–OCH_3, 3H, s), 3.95 (H-10, 1H, m), 3.36 (OCH_3, 3H, s), 3.24 (H-11ax, 1H, t, 10.3), 2.75 (H-8, 1H, m), 2.24 (H-9eq, 1H, m), 1.25 (H-9ax, 1H, q, 12), 0.82 (H-20, 3H, d, 6.8).

2.1.6. (*S*)-(−)-MTPA ester of 6. To a stirred solution of **6** (2.3 mg, 0.0067 mmol) in dry CH_2Cl_2 (0.5 mL), (*R*)-(−)- α-methoxy-α-trifluoromethylphenylacetic chloride (MTPACl; 12 mg, 0.05 mmol), triethylamine (12 mg, 0.1 mmol) and 4-dimethylaminopyridine (DMAP; catalytic amount) was added and the mixture was stirred at room temperature for 7 h. The reaction mixture was directly purified on preparative TLC (SiO_2, 5% MeOH–$CHCl_3$) to yield 2 mg of (*S*)-(−)-MTPA ester, **7** (53% yield). FAB-MS *m/z* 562 $(M+H)^+$; HRFAB-MS *m/z* 562.2050 $(M+H)^+$, (calcd for $C_{29}H_{31}NO_7F_3$ 562.2053); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.504 (H-6, 1H, s), 7.4–7.5 (Ph×2, 10H, m), 5.227 (H-10, 1H, m), 4.520 (H-7, 1H, d, 11), 4.224 (H-11eq, 1H, ddd, 10.5, 5.0, 2.0), 4.099 (N–OCH_3, 3H, s), 3.541 (OCH_3, 3H, s), 3.436 (H-11ax, 1H, t, 10.5), 3.364 (OCH_3, 3H, s), 2.823 (H-8, 1H, m), 2.305 (H-9eq, 1H, m), 1.375 (H-9ax, q, 12), 0.823 (H-20, 3H, d, 6.6).

2.1.7. (*R*)-(+)-MTPA ester of 6. To a stirred solution of **6** (3.0 mg, 0.0087 mmol) in dry CH_2Cl_2 (0.5 mL), (*S*)-(+)-MTPACl (12 mg, 0.05 mmol), triethylamine (12 mg, 0.1 mmol) and DMAP (catalytic amount) was added and the mixture was stirred at room temperature for 7 h. The reaction mixture was directly purified on preparative TLC (SiO_2, 5% MeOH–$CHCl_3$) to yield 3.9 mg of (*R*)-(+)-MTPA ester, **8** (80% yield). FAB-MS *m/z* 562 $(M+H)^+$; HRFAB-MS *m/z* 562.2059 $(M+H)^+$, (calcd for $C_{29}H_{31}NO_7F_3$ 562.2053); ^1H NMR ($CDCl_3$, 400 MHz) δ 7.503 (H-6, 1H, s), 7.4–7.5 (Ph×2, 10H, m), 5.234 (H-10, 1H, m), 4.514 (H-7, 1H, d, 11), 4.145 (H-11eq, 1H, ddd, 10.5, 5.0, 2.0), 4.098 (N–OCH_3, 3H, s), 3.557 (OCH_3, 3H, s), 3.362 (OCH_3, 3H, s), 3.348 (H-11ax, 1H, t, 10.5), 2.833 (H-8, 1H, m), 2.370 (H-9eq, 1H, m), 1.468 (H-9ax, q, 12), 0.843 (H-20, 3H, d, 6.6).

2.2. In vitro cytotoxic activity

The cells used for assay were cultured in the following medium; HCT-116: complete McCoy's 5A supplemented with 10% fetal bovine serum, B16: complete D-MEM supplemented with 10% fetal bovine serum, $P388D_1$: complete RPMI-1640 supplemented with 5% fetal bovine serum.

In vitro cytotoxic activity was tested in 96-well microtiter plates of which well containing 1×10^4 each cell lines in 135 μL medium. The test samples were dissolved in 10% DMSO. The serially diluted DMSO solution (15 μL) was added to each well of plates. After addition, the cells were incubated at 37°C for 72 h in a humidified 5% CO_2 atmosphere. In vitro cytotoxic activity was evaluated by the microculture tetrazolium (MTT) assay method for each cell and by the colorimetrical determination method at 540 nm.

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