New Diterpenic Altrosides of the Fungus *Acremonium striatisporum* Isolated from a Sea Cucumber

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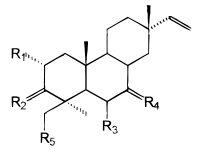
Two new diterpenic glycosides, virescenosides M (**1**) and N (**2**), have been isolated from a marine strain of *Acremonium striatisporum* KMM 4401 associated with the holothurian *Eupentacta fraudatrix*. Their structures were determined on the basis of MS and NMR data as β-D-altropyranosido-19-7-oxo-isopimara-8,15-diene-2α,3β-diol (**1**) and β-D-altropyranosido-19-isopimara-7,15-diene-2α,3β,6β-triol (**2**). Three other altrosides (**3**−**5**), identified as virescenosides A, B, and C from the terrestrial strain *Acremonium luzulae*, were also isolated. The cytotoxic activity of the virescenosides was examined.

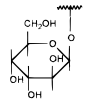
Marine fungi are potentially prolific sources of chemically interesting and biologically active secondary metabolites for the development of new pharmaceutical agents.[1−4] In our search for secondary metabolites from marine fungal isolates with cytotoxicity and/or novel chemical structure, we have investigated the fungus *Acremonium striatisporum* (Onions et G. L. Barron) W. Gams isolated from the holothurian *Eupentacta fraudatrix*. We report herein the isolation and structure of two new (**1**, **2**) and three known (**3**−**5**) diterpenic glycosides from this fungus.

The fungus was cultured for 21 days on rice medium[5] specially modified by us. The $CHCl_3$−MeOH (2:1, v/v) extract of the culture of *A. striatisporum* was fractionated by Si gel column chromatography followed by reversed-phase HPLC to yield individual compounds **1**−**5**.

The structures of new compounds **1** and **2** were established by interpretation of spectral data (NMR and HRMALDIMS), as well as by comparison of their spectra with those of related compounds.

The molecular formula of virescenoside M (**1**) was determined as $C_{26}H_{40}O_9$ on the basis of HRMALDIMS and ^{13}C NMR spectra. A close inspection of the ^1H and ^{13}C NMR spectral data (Table 1) of **1** revealed the presence of three quaternary methyls; seven methylenes, including two oxygen-bearing ones; seven oxygenated methines, including one methine linked to an anomeric carbon; one tertiary and three C−C-bonded saturated quaternary carbons, and a monosubstituted double bond. The remaining functionality, corresponding to the carbon signals at 198.7, 163.5, and 128.4 ppm, suggested the presence of the tetrasubstituted enone chromophore. The UV spectrum showed a λ_{max} at 248 nm (log ϵ 3.7) consistent with the enone system in structure **1**. Based on these data, a diterpene monoside with a tricyclic aglycon structure was indicated for **1**. A direct comparison of ^1H and ^{13}C NMR spectra of **1** with those of the virescenosides A−C, glycosides obtained earlier from the terrestrial fungus *Acremonium luzulae*,[6−9] suggested that virescenoside M has the structure of an isopimaradienic altroside. The ^{13}C NMR spectrum of **1a** obtained upon acid hydrolysis of **1** contained signals for a monosubstituted double bond at 145.6 and 111.6 ppm. The proton signals of a typical ABX system of a vinyl group at 5.65 ppm (1H, dd, 18.2, 10.1 Hz) and 4.90 (2H, m) in the





β-D-altropyranosyl-

	R_1	R_2	R_3	R_4	R_5	
1	OH	H, β-OH	H	O	β-D-altropyranosyl-	$\Delta^{8,9}$
1a	OH	H, β-OH	H	O	OH	$\Delta^{8,9}$
2	OH	H, β-OH	β-OH	H, H	β-D-altropyranosyl-	$\Delta^{7,8}$
3	OH	H, β-OH	H	H, H	β-D-altropyranosyl-	$\Delta^{7,8}$
3a,b	OH	H, β-OH	H	H	OH	$\Delta^{7,8}$, $\Delta^{8,9}$
3c,d	OAc	H, β-OAc	H	H, H	OAc	$\Delta^{7,8}$, $\Delta^{8,9}$
4	H	H, β-OH	H	H, H	β-D-altropyranosyl-	$\Delta^{7,8}$
4a,b	H	H, β-OH	H	H, H	OH	$\Delta^{7,8}$, $\Delta^{8,9}$
4c,d	H	H, β-OAc	H	H, H	OAc	$\Delta^{7,8}$, $\Delta^{8,9}$
5	H	O	H	H, H	β-D-altropyranosyl-	$\Delta^{7,8}$
5a,b	H	O	H	H, H	OH	$\Delta^{7,8}$, $\Delta^{8,9}$
5c,d	H	O	H	H, H	OAc	$\Delta^{7,8}$, $\Delta^{8,9}$

spectrum of **1a** indicated the C-15,C-16 position of this double bond.[10−13] Furthermore, the position of the C-17 methyl group (1.02 ppm) and of the exo-vinyl group at C-13 were argued by $^1H-^1H$ NOE measurements on **1a** shown in the Figure 1. The stereochemistry at C-13 was suggested to be 13(*S*) on the basis of the similarity of C-15−C-17 chemical shifts in the spectrum of **1a** with those of the $\Delta^{8(9)}$-isopimaradiene and isovirescenols B and C.[12,14] In addition, compound **1a** shows the characteristic Cotton effects at 328 (positive), 258 (negative), and 210 (positive) nm, which were in good agreement with those for methyl 7-oxo-13-epi-pimara-8,15-dien-18-oate.[13]

Correlations observed in the double resonance NMR experiments on **1a** indicated the presence of an isolated spin-system corresponding sequence $-CH_2-CHOH-CHOH$ (C-1−C-2−C-3). The relative stereochemistry of protons on C-2 and C-3 was defined based on the $^1H-^1H$ coupling constant ($J = 9.7$ Hz) and assigned as axial. The signals of

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Table 1. NMR Data for Compounds **1**, **1a**, and **2** (*J* in Hz)

	1		**1a**		**2**	
atom	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	43.4 t		42.0 t	1e: 2.20 dd (4.6, 16.8) 1a: 1.29 d (16.8)	46.8 t	
2	68.0 d	4.28 m	68.8 d	3.98 m	68.6 d	4.30 m
3	83.2 d	4.40−4.55	84.4 d	3.20 d (9.7)	84.7 d	4.40−4.55
4	40.4 s		42.8 s		43.9 s	
5	50.2 d	1.92 dd (15.0, 3.5)	49.8 d	1.82 dd (14.3, 3.6)	48.2 d	1.51 d (4.9)
6	33.8 t	6e: 2.92 dd (18.0, 3.5) 6a: 3.30 dd (18.0, 15.6)	34.9 t	6e: 2.56 dd (17.4, 3.6) 6a: 2.25 dd (17.4, 14.3)	72.5 d	4.61 dd (1.7; 4.9)
7	198.7 s		198.0 s		132.8 d	5.51 d (1.7)
8	128.4 s		129.0 s		140.8 s	
9	163.5 s		163.5 s		46.6 d	
10	40.7 s		40.4 s		34.5 s	
11	23.4 t		23.3 t		19.4 t	
12	34.2 t		33.7 t[a]		34.9 t	
13	34.5 s		34.4 s		37.5 s	
14	37.3 t		33.6 t[a]		46.6 t	
15	145.9 d	5.72 dd (18.0, 11.2)	145.6 d	5.65 dd (18.2, 10.1)	147.8 d	5.73 d (18.2, 11.0)
16	111.3 t	5.00 m	111.6 t	4.90 m	110.9 t	4.94 m
17	27.2 q	0.95 s	27.9 q	1.02 s	26.5 q	0.98 s
18	23.2 q	1.39 s	22.6 q	1.27 s	24.9 q	1.54 s
19	71.9 t	19a: 3.49 d (10.2) 19b: 4.19 d (10.2)	64.9 t	19a: 3.46 d (11.0) 19b: 4.18 d (11.0)	72.7 t	19a: 3.57 d (10.2) 19b: 4.15 d (10.2)
20	19.0 q	1.31 s	19.7 q	1.14 s	16.3 q	1.05 s
1^1	101.0 d	5.46 d (1.6)			101.3 d	5.54 d (1.6)
2^1	72.0 d	4.57 dd (1.6, 4.5)			72.0 d	4.39 m
3^1	72.4 d	4.74 m			72.3 d	
4^1	68.0 d	4.79 dd (3.5, 9.0)			68.2 d	
5^1	76.3 d	4.37 m			76.9 d	
6^1	63.6 t	4.40−4.55			63.8 t	

[a] Values may be reversed.

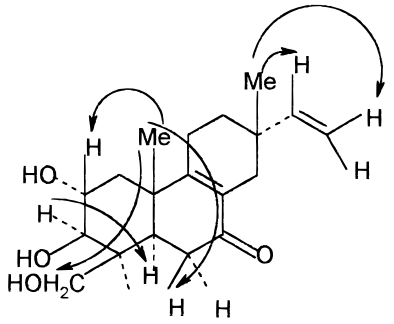

Figure 1. The results of $^1H-^1H$ NOE for **1a**.

the ABX system at 1.82 (1H, dd, 14.3, 3.6 Hz), 2.56 (1H, dd, 17.4, 3.6 Hz), and 2.25 ppm (1H, dd, 17.4, 14.3 Hz) were consistent with the presence of sequence >CH−CH_2−CO− (C-5−C-6−C-7) and indicated the 8-en-7-one position for the tetrasubstituted enone chromophore in **1a**. This fact was substantiated by the characteristic fragment (*m*/*z* 148) in EIMS obtained after cleavage of **1a** at C-9,C-10 and C-5,C-6.[15] The ^1H NMR spectrum of **1a** showed two signals corresponding to an AB system coupling at 3.46 and 4.18 ppm (each 1H, each d, 11 Hz), which were consistent with the presence of CH_2OH group linked to a quaternary sp^3 C carbon. The position and stereochemistry of the methyl and hydroxymethyl groups at C-4 and methyl group at C-10 were established based on NOE data. Furthermore, NOE correlations between H-2a and H_3-20 and between H-3a and H-5a indicated a trans ring fusion between rings A and B. All these data are consistent with a $\Delta^{8,15}$-isopimaradienic skeleton with equatorial hydroxyl groups at C-2 and C-3, an axial hydroxymethyl group at C-4, and a 7-keto group conjugated with Δ^8-unsaturation for **1a**. The carbon signals of **1a** were assigned by comparison with those of isovirescenol A,[12] lanost-8-en-7-one,[16] and selective decoupling experiments. The ^1H and ^{13}C NMR data (Table 1) for the aglycon moiety of **1** agreed with those of **1a** except for the C-19 atom signal, which appeared at lower field (+7 ppm) in **1** than the corresponding signal in **1a**. This indicated that the sugar moiety was linked at C-19.

A comparison of the ^{13}C NMR spectrum of **1** with published data for α- and β-D-altropyranoses and α-methyl-D-altropyranoside, as well as a good coincidence of carbon signals due to the glycosidic moiety with those of virescenoside A together with magnitudes of H-1−H-2 (1.6 Hz) and C-1−H-1 (156 Hz) spin-coupling constants in NMR spectra of **1**,[9,17−20] elucidated the presence of a β-D-altropyranoside unit in **1**. On the basis of all the above data, the structure of virescenoside M was established as β-D-altropyranosido-19-7-oxo-isopimara-8,15-diene-2α,3β-diol.

Virescenoside N (**2**) gave in HRMALDIMS a quasimolecular ion at *m*/*z* 521.2726 [M + Na]. These data, coupled with ^{13}C NMR spectral data, established a molecular formula $C_{26}H_{42}O_9$ for **2**. The general features of the ^1H and ^{13}C NMR spectra (Table 1) of **2** closely resembled those of virescenoside M and virescenoside A[9] with the exception of proton and carbon signals corresponding to one additional secondary hydroxyl group (δ_C 72.5, δ_H 4.61 ppm) in the aglycon moiety. Furthermore, the signals of the trisubstituted double bond in the NMR spectra of **2** appeared at lower field than those in virescenosides C,[7,9] D, and H[8] with Δ^7-unsaturation. Correlations observed in the double resonance NMR experiments on **2** indicated the presence of an isolated spin system corresponding to the sequence >CH−CHOH−CH═, and thus the additional oxymethine signal at 72.5 ppm was assigned to C-6. The small coupling constant of the vinylic H-7 signal at 5.51 ppm (1H, d, 1.7 Hz) indicated that **2** contained an allylic secondary alcohol function with an axial configuration. On the basis of the above data the structure of virescenoside N was proposed as β-D-altropyranosido-19-isopimara-7,15-diene-2α,3β,6β-triol.

Besides the new virescenosides M (**1**) and N (**2**), three other known diterpenic altrosides (**3-5**) have also been isolated from the fungus *A. striatisporum*. These glycosides were identified as virescenosides A, B, and C based on LRFABMS and comparison of their NMR data with literature values.[6,7,9] Furthermore, NMR data for the genuine aglycons (**3a**−**5a**), $\Delta^{8,9}$-isomers (**3b**−**5b**), and their acetylated derivatives (**3c,d**−**5c,d**) also matched literature values.[7,11,12,21]

Virescenosides A, B, C, M, and N showed cytotoxic effect on developing eggs of the sea urchin *Strongylocentrotus intermedius* (MIC_{50} = 2.7−20 μM), and their activity decreased in the order C > M > B > A > N. It was shown that these glycosides exhibited cytotoxic action against tumor cells of Ehrlich carcinoma (IC_{50} = 10−100 μM) in vitro.

A detailed study of the structure−activity relationship of virescenosides A−C, M, and N and their genuine aglycons is currently in progress. Virescenosides A−C, M, and N are the first biologically active metabolites to be isolated from marine fungi associated with echinoderms.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded in both $CDCl_3$ and pyridine on a Bruker WM-250 spectrometer operating at 250 and 62.9 MHz with TMS as internal standard. EIMS were measured on a LKB-9000S spectrometer (ionizing energy 70 eV). Positive-ion FABMS data were obtained on a Varian MAT 95 spectrometer. HRMALDIMS analyses were carried out with a Bruker Biflex time-of-flight mass spectrometer equipped with a UV-nitrogen laser (337 nm). CD spectra were obtained with a JASCO model J-500. IR spectra were determined on a Specord M 82 (Carl Zeiss, Jena) in $CHCl_3$, and UV spectra were recorded on a Specord UV−vis spectrometer in MeOH. Optical rotations were measured on a Perkin-Elmer 141 polarimeter, and the melting points were obtained with a Boetius hot-stage apparatus.

Cultivation of *A. striatisporum*. The culture of *A. striatisporum* KMM 4401 was isolated from superficial mycobiota of the sea cucumber *E. fraudatrix* collected from the Sea of Japan (Kitovoe Rebro Bay, depth 10 m). The fungus was grown stationary at 22 °C for 21 days in 10 Erlenmeyer flasks (500 mL), each containing 20 g of rice, 20 mg of yeast extract, 10 mg of KH_2PO_4, and 40 mL of seawater.

Extraction and Isolation. At the end of the incubation period, the mycelium and medium were homogenized and then thrice extracted with a mixture of $CHCl_3$−MeOH (2:1, v/v, ca. 2 L). After evaporation of the solvent, the residual material (4 g) was layered on a Si gel L (40−100 μm, Chemapol, former Czechoslovakia) column (6 × 40 cm), which was eluted first with $CHCl_3$ (500 mL) followed by step gradient from 5% to 20% MeOH in $CHCl_3$ (total volume 2 L). Fractions of 10 mL were collected and combined on the basis of TLC (Si gel, $CHCl_3$−MeOH, 3:1, v/v). Fractions containing the desired compounds were further purified by reversed-phase HPLC on a Silasorb-ODS column (10 μm, 9.6 × 200 mm, 220 nm, 2 mL/min), eluting with step gradient from 52% to 75% MeOH in H_2O to yield **1** (22 mg), **2** (14 mg), **3** (1.2 g), **4** (230 mg), and **5** (125 mg).

***β*-D-Altropyranosido-19-7-oxo-isopimara-8,15-diene-2α,3*β*-diol (1):** colorless plates (MeOH); mp 143−146°; $[\alpha]^{20}_D$ +29° (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ϵ) 248 (3.9) nm; ^{1}H and ^{13}C NMR spectra (C_5D_5N), see Table 1; HRMALDIMS *m/z* 519.2589 (calcd for $C_{26}H_{40}O_9Na$, 519.2570).

***β*-D-Altropyranosido-19-isopimara-7,15-diene-2α,3*β*,6*β*-triol (2):** colorless amorphous solid; $[\alpha]^{20}_D$ −18° (*c* 0.25, MeOH); HRMALDIMS *m/z* 521.2726 (calcd for $C_{26}H_{42}O_9Na$, 521.2726); ^{1}H and ^{13}C NMR spectra (C_5D_5N), see Table 1.

***β*-D-Altropyranosido-19-isopimara-7,15-diene-2α,3*β*-diol (3); *β*-D-altropyranosido-19-isopimara-7,15-diene-3*β*-ol (4); and *β*-D-altropyranosido-19-3-oxo-isopimara-7,15-diene (5):** ^{1}H and ^{13}C NMR spectra data obtained for compounds **3**−**5** in agreement with published data[6,7,9] for virescenosides A−C.

Acidic Hydrolysis of Compound 1. A solution of compound **1** (20 mg) in 0.1 M TFA (2 mL) was heated in a stoppered reaction vial for 30 min. The water layer was extracted with $CHCl_3$. The residue obtained after evaporation of the extract was chromatographed on a Si gel column (1.5 × 25 cm), eluting first with $CHCl_3$ and finally with a solvent system of $CHCl_3$−MeOH (40:1), to yield 14 mg of **1a**.

7-Oxo-isopimara-8,15-diene-2α,3*β*,19*β*-triol (1a): colorless amorphous solid; $[\alpha]^{20}_D$ +31° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 251 (4.08) nm; CD (4.79 × 10^{-5} M, MeOH) $\Delta\epsilon_{328}$ +0.88, $\Delta\epsilon_{258}$ −0.26, and $\Delta\epsilon_{210}$ +2.46; IR ($CHCl_3$) ν_{max} 3615, 3578, 3080 1655, 1617, 910 cm^{-1}; ^{1}H and ^{13}C NMR spectra ($CDCl_3$), see Table 1; EIMS *m/z* 334 $[M]^+$ (14), 316 (5), 298 (4), 285 (5), 279 (13), 186 (5), 148 (71), 82 (100).

Isolation and Identification of Compounds 3a−d to 5a−d. The mixtures of genuine aglycons (**3a**−**5a**) and their $\Delta^{8,9}$-isomers (**3b**−**5b**) obtained after acidic hydrolysis of virescenosides A−C were separated by HPLC on a Silasorb ODS column (10 μm, 4.6 × 250 mm) using a step gradient from 80% to 85% MeOH in H_2O to yield the individual **3a**,**b**−**5a**,**b** compounds. The acetylation of **3a**,**b**−**5a**,**b** with Ac_2O and pyridine afforded the acetates **3c**,**d**−**5c**,**d**. ^{1}H and ^{13}C NMR data obtained for compounds **3a**−**d** to **5a**−**d** were identical with those reported earlier.[7,11,12,21]

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