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ANTITUMOR PRINCIPLES IN MOSSES: THE FIRST ISOLATION AND IDENTIFICATION OF MAYTANSINOIDS, INCLUDING A NOVEL 15-METHOXYANSAMITOCIN P-3

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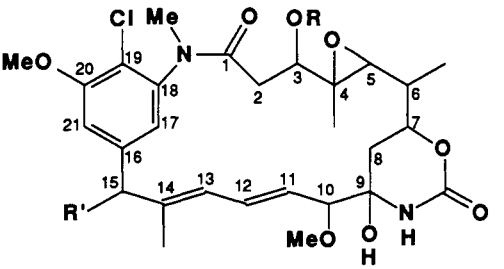
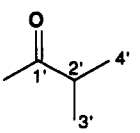
ABSTRACT.—A novel 15-methoxyansamitocin P-3 [**2**], in company with three known maytansinoids **1**, **3**, and **4**, was isolated for the first time from two Japanese mosses, *Isothecium subdiversiforme* and *Thamnobryum sandei*. All are potent cytotoxic compounds.

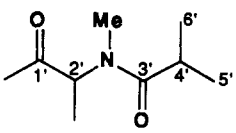
During our study on the isolation of the components with biological activity in mosses (1), we searched for antitumor components utilizing the screening of mosses by in vitro cytotoxicity tests against mouse P-388 lymphocytic leukemia cells. Among a large number of mosses examined, crude MeOH extracts of two mosses, *Isothecium subdiversiforme* Broth. (Lembophyllaceae) and *Thamnobryum sandei* (Besch.) Iwatsuki (Neckeraceae), had activity (50% cell growth inhibitory concentration, IC_{50} 2–5 μg/ml). These mosses are seen commonly in the southern half of Japan. Every sample of *I. subdiversiforme* collected from six locations in Japan revealed cytotoxic activity. Moreover, a moss extract that was concentrated to 100 times more in vitro activity showed a moderate degree of in vivo activity against mouse P-388 cells (prolonged survival interval T/C 149%, day 1–5, ip, dose 40 mg/kg).

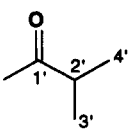
The results of activity tests prompted us to carry out isolation of the active cytotoxic principles from the mosses. As a result, we confirmed the components as four maytansinoids, including a novel 15-methoxy derivative of ansamitocin P-3.

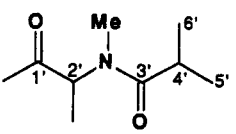
Maytansinoids are ansamacrolides that were isolated first by Kupchan *et al.* (2) in 1972 from an African shrub *Maytenus serrata* in very low yield. They are potent cytotoxic substances with excellent activity in mouse P-388 leukemia in vivo tests (2–



1 R=  , R′=H

3 R=  , R′=H

2 R=  , R′=OMe

4 R=  , R′=OMe

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4). The subsequent plant screening program operated by NCI revealed the existence of a number of biologically active maytansinoid derivatives. Besides the plant origin, Asai *et al.* (5) reported in 1979 the production of ansamitocins with maytansinoid skeleton by a microorganism of *Nocardia* species as the potent antitumor components.

From mosses in which maytansinoids had not previously been reported, we have isolated and identified four maytansinoids that exhibit a potent cytotoxicity.

RESULTS AND DISCUSSION

ISOLATION AND IDENTIFICATION FROM *I. SUBDIVERSIFORME*.—Extraction with Et_2O of whole moss (20.3 kg) collected at Amagi in Izu, Shizuoka Prefecture, Japan, gave crude extract (183 g, IC_{50} against P-388 cells 3μg/ml). Extensive cc treatments and preparative hplc separations led to isolation of four compounds, **1**–**4**, with potent cytotoxicity. The isolated compounds were present only in trace quantitities in yields estimated to be 50 μg or less. As only trace amounts were obtained, the structural analyses were performed principally using 400 MHz ^{1}H-nmr spectra. The compounds revealed similar nmr spectra, which suggested that they had analogous structures (Table 1). Compound **4** was chosen for 2D-COSY and homodecoupling measurement, because the peaks separated best among them, and the analysis seemed to be

TABLE 1. 400-MHz ^{1}H-nmr Data of *Isothecium subdiversiforme* Components.[a]

Proton	Maytansinoid components			
	1	**2**	**3**	**4**
2a	2.21 dd (14,3)	2.20 dd (14,3)	2.19 dd (14,3)	2.19 dd (14,3)
2b	2.57 dd (14,12)	2.51 dd (14,12)	2.62 dd (14,12)	2.56 dd (14,12)
3	4.82 dd (12,3)	4.80 dd (12,3)	4.79 dd (12,3)	4.77 dd (12,3)
4-Me	0.83 s	0.80 s	0.81 s	0.79 s
5	2.96 d (11)	2.95 d (10.1)	3.03 d (9.6)	3.03 d (9.6)
6-Me	1.30 d (6.3)	1.29 d (6.32)	1.30 d (6.94)	1.30 d (6.27)
7	4.28 m	4.27 m	4.30 m	4.29 m
10	3.50 d (8.8)	3.52 d (9.2)	3.51 d (11.3)	3.53 d (9.1)
11	5.46 dd (15,9)	5.55 dd (15,9)	5.65 d (16,9)	5.73 dd (15,9)
12	6.45 dd (15,11)	6.49 dd (15,11)	6.44 dd (15,11)	6.48 dd (15,11)
13	6.17 bd (12)	6.32 bd (9.9)	6.79 bd (11.5)	7.00 bd (11)
14-Me	1.70 bs	1.58 bs	1.65 bs	1.54 bs
15a	3.52 d (13.1)	4.71 bs	3.67 bd (13.0)	4.87 s
15b	3.21 d (14)		3.10 bd (12.5)	
17	6.84 d (1.8)	6.77 d (1.5)	6.66 d (1.8)	6.55 d (1.5)
21	6.89 d (1.8)	7.25	6.82 d (1.8)	7.24 d (1.5)
10-OMe	3.36 s	3.38 s	3.35 s	3.37 s
15-OMe		3.40 s		3.38 s
20-OMe	4.00 s	4.02 s	3.99 s	4.01 s
18-NMe	3.16 s	3.16 s	3.18 s	3.18 s
2′	2.60 m	2.56 sep (7)	5.39 m	5.38 m
2′-Me			1.30 d (6.38)	1.29 d (6.18)
2′-NMe			2.90 s	2.90 s
3′	1.21 d (6.8)	1.21 d (6.76)		
4′	1.29 d (7.2)	1.28 d (7.24)	2.78 sep (6.7)	2.79 sep (7)
5′			1.06 d (6.7)	1.08 d (6.53)
6′			1.13 d (6.85)	1.13 d (6.86)
9-NH	6.20 bs	6.20 bs	6.21 b	6.22 bs
9-OH	2.97 bd	2.99 d (2.1)	3.31 bs	3.31 bs
6,8	1.25, 1.5, 1.7	1.25, 1.5, 1.7	1.25, 1.45, 1.55	1.25, 1.45, 1.55

[a]Chemical shifts (δ) are expressed in ppm from internal TMS, and coupling constants (*J*) are expressed in Hz. Spectra were recorded in $CDCl_3$ solution on a Bruker AM-400 spectrometer.

facile (Figure 1). The analysis of the spectra of **4** gave six independent partial structures (Figure 2), and an arrangement of the partial structures led to a suspicion that the substance **4** might be a maytansinoid. The precise comparison of the chemical shifts and coupling constants with those of trewiasine, isolated from *Trewia nudiflora* (6), resulted in a complete coincidence. Compound **3** was considered to be maytanbutine, isolated from *Maytenus buchanii* (2), based on the similarity of the spectrum with that of **4** except that a methoxy group at C-15 in **4** is substituted by a hydrogen. The spectrum of compound **1** differed from that of **3** only in the signals of the C-3 substituent; the methylated alanyl group was replaced by the isobutanoyl group. The substance **1** has previously been reported as ansamitocin P-3, obtained from a fermentation broth of a *Nocardia* species (5). The comparison of its ^{1}H-nmr spectrum with that of the authentic sample gave complete coincidence of the spectra. As to the structure of **2**, the precise comparison of the ^{1}H-nmr spectrum with that of **1** disclosed that **2** differed only at the C-15 carbon atom, which carried a methoxy group and a oxygenated methine proton at C-15. This was exemplified by similar shifts of protons in **4** when compared with that of **3**, such as low-field shifts of C-11, C-13, and C-21 protons, and up-field shifts of C-17 and

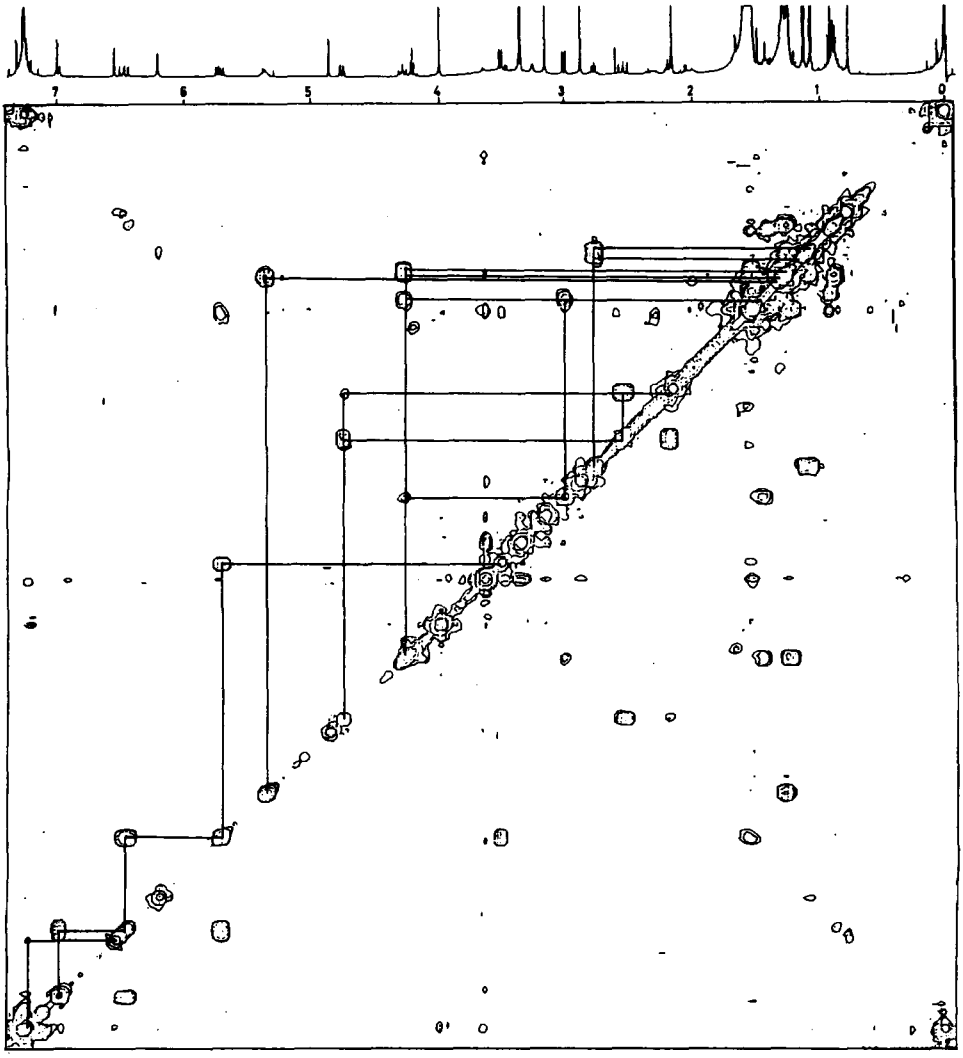

FIGURE 1. 2D-COSY ^{1}H-^{1}H spectrum of compound **4**. The spectrum was obtained after 7 days' scan.

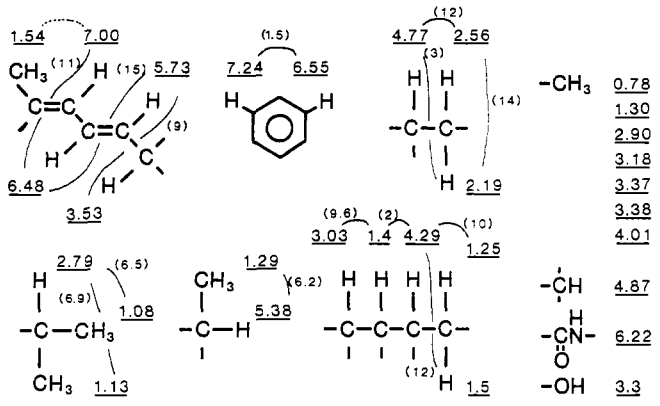

FIGURE 2. ^{1}H-nmr assignment of partial structures of compound **4**. Chemical shifts δ are expressed in ppm, and coupling constants (J) are expressed in Hz.

14-Me protons. The mass spectrum of **2** (m/z $[M]^+$ 664) revealed a fragmentation pattern similar to that of **1** (m/z $[M]^+$ 634) with 30 mass unit differences, consistent with the substitution of a methoxy group in **2**. Based on this evidence, **2** was identified as 15-methoxyansamitocin P-3, which has not been previously reported.

As Wani *et al.* (7) isolated colubrinol, 15-hydroxymaytanbutine, a possibility could not be eliminated completely that 15-methoxy compounds **2** and **4** were produced during the course of separation by use of MeOH repeatedly as solvent or eluent.

ISOLATION AND IDENTIFICATION FROM *T. SANDEI*.—Extraction was performed as for *I. subdiversiforme*. Crude extract (25.2 g) of the moss (6.1 kg) collected in Izu (voucher specimen at Amagi) was first partitioned in liquid-liquid phases. Subsequent cc treatments followed by hplc gave four pure compounds. Analysis by 400 MHz ^{1}H nmr showed that two of the compounds were identical with **1** (ansamitocin P-3) and **2** (15-methoxyansamitocin P-3). ^{1}H-nmr analysis of the minor components provided only information on methyl signals after 51,000 times FID accumulation. However, the positions of methyl signal shifts of these two compounds coincided with the methyl signals of **3** (maytanbutine) and **4** (trewiasine) so that the two compounds are probably **3** and **4**.

We have established the presence of highly cytotoxic maytansinoids in two specific mosses, *I. subdiversiforme* and *T. sandei*. The isolation of the compounds, present in only trace amounts, was guided by the highly potent activity, and the identification by ^{1}H-nmr spectroscopy demonstrated the active principles as the four maytansinoids, three of them already known and one a novel derivative.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Hplc was performed with an instrument with uv detector. High-field 400-MHz ^{1}H-nmr spectra were recorded with a Bruker AM-400 instrument; $CDCl_3$ solutions were used with TMS as an internal standard. Low resolution ms were obtained with a Hitachi M-80A instrument.

CYTOTOXICITY TESTS IN VITRO.—Leukemia P-388 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 μM 2-hydroxyethyl disulfide, and kanamycin (100 μg/ml), and the culture medium was controlled to a cell density of 5×10^4 cells/ml. The weighed samples were added, followed by incubation in a CO_2 incubator at 37°. After 48 h, 0.25% of trypsin was added and then incubated for 5 min at 37° to deaggregate cells. Cells were counted in a Model ZB1 Coulter counter. A 50% cell growth inhibitory concentration (IC_{50}) was determined from calculated cell growth inhibitory rates at several concentrations.

I. SUBDIVERSIFORME.—*Extraction procedures*.—The whole green moss, after washing with H_2O (14.9 kg), was frozen in liquid N_2 and crushed to a powder while frozen. The powdered moss was extracted

twice with Et_2O (2 × 120 liters) for 7 days. A crude extract (118 g) was obtained by removal of the solvent (IC_{50} against P-388 cells 3 μg/ml).

Column chromatographic separations.—The crude extract (118 g) was subjected to repeated cc to concentrate the activity. The first separation was performed on Si gel (Wakogel C-200, 1.8 kg) eluted with CH_2Cl_2 and CH_2Cl_2-MeOH (14:1). Fractions that showed IC_{50} 2.1–0.7 μg/ml were collected to give 28.8 g of a concentrate. The next cc was also conducted on Si gel (Wakogel C-200, 1.5 kg) eluted with C_6H_{14}-Et_2O (1:2) (8 liters), C_6H_{14}-EtOAc (1:2) (6 liters), C_6H_{14}-EtOAc (1:3) (7 liters), Et_2O (3 liters), and then EtOAc (5 liters). The active fractions (IC_{50} 0.2–0.5 μg/ml) were eluted with Et_2O to afford 6.77 g of the second concentrate. This fraction was chromatographed on Si gel [Wakogel C-300, 1.0 kg; elution with CH_2Cl_2-MeOH (19:1)], and fractions with IC_{50} 0.03–0.07 μg/ml were collected to give 1.31 g of the residue. Another moss sample (5.4 kg) was subjected to the same extraction and separation treatments to afford 1.17 g of a concentrate with IC_{50} 0.03–0.09 μg/ml and combined with the first preparation. The combined concentrate was chromatographed on Si gel (Wakogel C-300, 200 g) and eluted in a gradient mode from CH_2Cl_2 to CH_2Cl_2-MeOH (9:1), and the active fractions were collected to give 128 mg with IC_{50} 0.003 μg/ml. Separation on a Sephadex LH-20 column eluted with CH_2Cl_2 afforded 24.0 mg of the active portion with IC_{50} 3.8×10^{-4} μg/ml.

Hplc separations.—The active portion 24.0 mg was then separated by repeated hplc treatments. Separation on a TSK silica 150 column [size 7.8 × 300 mm, eluent CH_2Cl_2-MeOH (98:2), flow rate 1.0 ml/min] gave two active fractions with retention times at 22–24 min (fraction A, 1.5 mg) and at 24–26 min (fraction B, 1.4 mg). The treatment of fraction B on a TSK ODS 120T column (size 7.8 × 300 mm, eluent 35% H_2O/MeOH, flow rate 1.0 ml/min) afforded the two pure active components **3** and **4** with retention times at 62–68 min and 73–80 min, respectively, as white amorphous product after lyophilization. The material from fraction A was rechromatographed on a TSK ODS 120T column (size 7.8 × 300 mm, eluent 35% H_2O/MeOH, flow rate 0.7 ml/min), and components **1** and **2** with retention times at 75–80 min and 80–85 min, respectively, were obtained. Final purification on a TSK ODS 80TM column (size 4.6 × 150 mm, eluent 45% H_2O/MeOH, flow rate 0.3 ml/min) gave the pure compounds **1** and **2**. The amounts of the products were estimated to be 18 μg each for **1** and **2** and 50 μg each for **3** and **4** from 400-MHz ^1H nmr by comparison of integrated area of methyl signals with that of a signal from a weighed standard sample of dimethyl pentadecandioate.

The 400-MHz ^1H-nmr spectra were obtained after FID accumulation of 20,232 times for **1**, 19,448 times for **2**, 15,268 times for **3**, and 53,096 times for **4** (Table 1). 2D-COSY of **4** was obtained after 7 days' scanning (Figure 1).

T. SANDEI.—*Extraction procedures.*—The whole of green moss, after washing with H_2O (6.1 kg), was treated similarly to *I. subdiversiforme*. The crude extract (22.4 g, IC_{50} 2 μg/ml) was subjected to liquid-liquid partition between C_6H_{14} (2 liters) and 90% MeOH/10% H_2O (2 liters). The aqueous MeOH layer was washed three times with 0.7-liter portions of C_6H_{14} and condensed to 0.5 liters under reduced pressure, followed by extraction with CH_2Cl_2 (3 × 1 liter). The solvent was removed after drying over Na_2SO_4 to give 9.6 g of the residue with IC_{50} 0.62 μg/ml.

Column chromatographic separations.—The extract (9.6 g) was subjected to repeated chromatography. The first chromatography on Si gel column (Wakogel C-200, 200 g) was eluted in a gradient mode with C_6H_{14}-EtOAc-MeOH (9:1:0, 0:1:0, 0:9:1, 0:0:1). Fractions which showed IC_{50} 1.2–0.4 μg/ml were collected to give a concentrate (1.97 g). Further concentration was obtained on a Si gel column (Wakogel C-300, 150 g, eluent $CHCl_3$/MeOH in a gradient mode) to afford 135 mg of the concentrate with IC_{50} 0.0016 μg/ml. A chromatography on a Sephadex LH-20 column eluted with $CHCl_3$ gave 10.1 mg of an active portion, IC_{50} 4×10^{-4} μg/ml.

Hplc separations.—The concentrate (10.1 mg) was treated further with hplc on a TSK silica 150 column (size 7.8 × 300 mm, eluent 1% MeOH/CH_2Cl_2, flow rate 1.0 ml/min) to yield a fraction with a retention time at 28–35 min. Following treatment on a TSK ODS 120T column (size 7.8 × 300 mm, eluent 35% H_2O/MeOH, flow rate 0.8 ml/min) separated the three pure compounds **1**, **2**, and **4** with retention times at 60–64 min, 64–68 min, and 86–91 min, respectively. A component eluted at 72–80 min was further purified on a TSK ODS 120T column (size 7.8 × 300 mm, eluent 35% H_2O/MeOH, flow rate 0.6 ml/min) to give compound **3** with a retention time at 94–100 min.

The 400-MHz ^1H-nmr spectra were obtained after FID accumulation of 36,800 times for **1**, 40,800 times for **2**, 30,400 times for **3**, and 51,000 times for **4**.

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