This method of quantifying suspended solids is about 50 times as sensitive as the current weight determination method, and can reach the sensitivity range of protein determination[2] if the micro-assay is employed. It is apparently non-specific at high pH and can be applied, albeit with caution, to particles of diverse chemical composition. It is rapid, does not require sophisticated instruments and can be used in the field after a short exposure of the particles to formaldehyde. Once the ratio between the dye-binding capacity of a given particle-assemblage and their mass (the 'B-value') has been established, the method could be applied to many situations in microbiology, limnology, biotechnology or industry where sensitive quantification is required.

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Ansamitocin P-3, a maytansinoid, from *Claopodium crispifolium* and *Anomodon attenuatus* or associated actinomycetes[1, 2]

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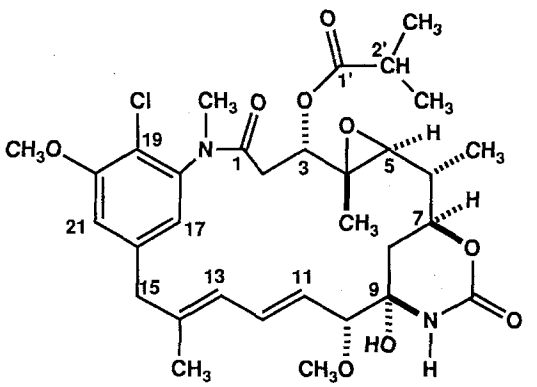
Summary. Guided by cytotoxicity, ansamitocin P-3, a maytansinoid, was isolated in very low yield from two members of the moss family Thuidiaceae, *Claopodium crispifolium* (Hook.) Ren. & Card. and *Anomodon attenuatus* (Hedw.) Hueb. Ansamitocin P-3 showed potent cytotoxicity against the human solid tumor cell lines A-549, HT-29. A possible basis for the occurrence of this compound in mosses is discussed.
Key words. Ansamitocin P-3; maytansinoids; *Claopodium crispifolium*; *Anomodon attenuatus*; Thuidiaceae; mosses; cytotoxicity; antitumor activity.

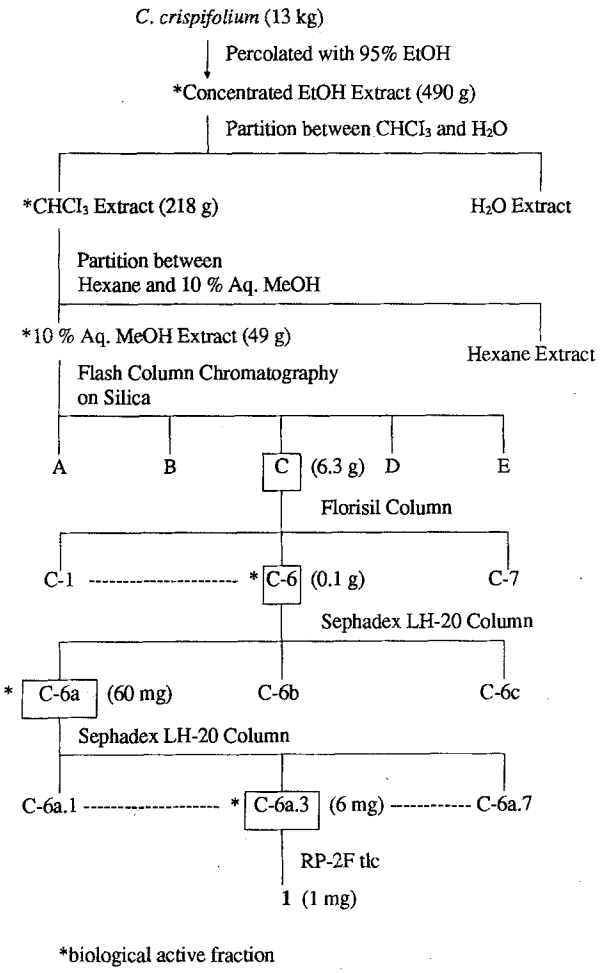
Previous screening for antitumor activity among the bryophytes (mosses, liverworts and hornworts) was established by cooperation between the US Department of Agriculture and the National Cancer Institute[4]. Among those species tested, two thuidiaceous mosses, *Claopodium crispifolium* (Hook.) Ren. & Card. and *Anomodon attenuatus* (Hedw.) Hueb. exhibited significant activity against P-388 lymphocytic leukemia in mice and both 9PS (murine lymphocytic leukemia) and 9KB (human nasopharyngeal carcinoma) cell culture systems and were, therefore, selected for further investigation to isolate the compounds responsible for the biological activity. In this communication, we report the cytotoxicity bioassay-directed isolation and identification of the active constituent, ansamitocin P-3 (**1**), from the active 10% aqueous methanol extracts of these two mosses.

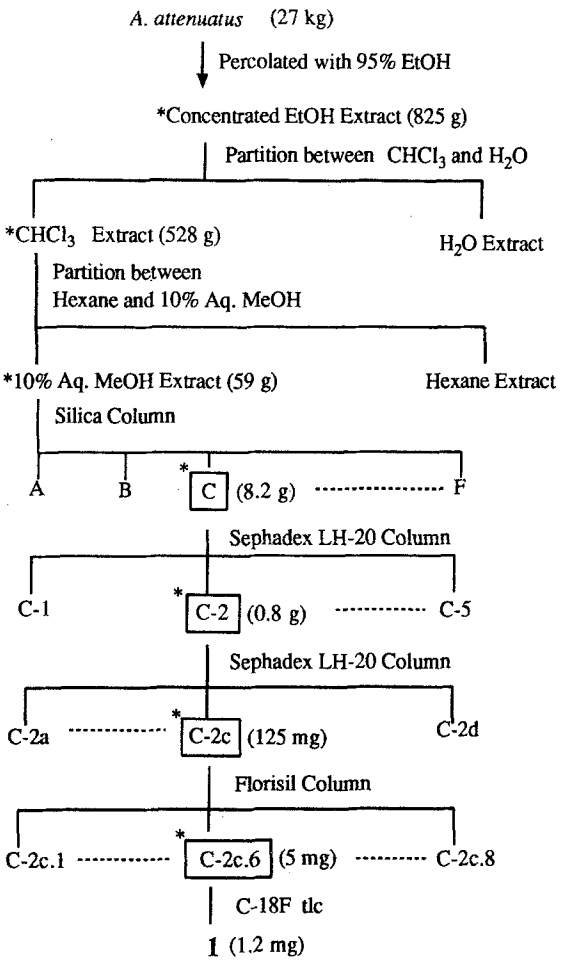
C. crispifolium was collected from rocks on steep slopes in a Douglas fir forest in Oregon during May, 1981. The bioassay-guided fractionation of *C. crispifolium* is illustrated in the scheme. The whole air dried moss (13 kg) was ground and slowly percolated with 95% ethanol. The concentrated extract was partitioned between water and chloroform, and the concentrated chloroform extract was further partitioned between hexane and 10% aqueous methanol. The 10% aqueous methanol extract (49 g) showed cytotoxicity against 9PS and 9KB cells in culture at $ED_{50} = 3 \times 10^{-1}$ μg/ml and antitumor activity against the P-388 system in mice at %T/C = 147–



Ansamitocin P-3

Fractionation procedures of *C. crispifolium* and *A. attenuatus*.





200 mg/kg [5]. This active crude extract was then subjected to chromatography on a silica gel (1.5 kg, E. Merck, 230–400 mesh) flash column (10 × 100 cm) eluted with 20 l of 2% and 12 l of 5% methanol in chloroform; 500-ml fractions were collected and pooled into five major fractions (A–E). The activity was concentrated in fraction C which was eluted with 2% methanol in chloroform between volumes 12–18.5 l. Fraction C (6.3 g) was further chromatographed on a Florisil column (400 g, Analtech, 60–100 mesh, 5 × 75 cm) with increasing methanol in chloroform (4 l each); 25-ml fractions were collected. Fractions 330–380 eluted with 20% methanol in chloroform were pooled and concentrated in vacuo to give the active fraction C-6 (110 mg, 9 PS $ED_{50} < 10^{-4}$ µg/ml). Further purification was performed on a Sephadex LH-20 column (20 g, Pharmacia, 1 × 100 cm) using dichlormethane-methanol (5:1) as an eluent. The active material was concentrated between volumes 30–110 ml (fraction C-6a, 60 mg). Chromatography of this fraction on a Sephadex LH-20 column (30 g, 1 × 100 cm) with dichloromethane-hexane (5:1) gave the active fraction C-6a.3 (6 mg, HT-29 $ED_{50} = 2 \times 10^{-6}$ µg/ml), eluted between volumes 270–320 ml. Final purification was achieved by chromatography on a RP-2F reversed phase TLC plate (EM Science, 0.25 mm) using tetrahydrofuran-water (1:1) as a developing solvent to yield about 1 mg of the active compound **1** (1×10^{-5}% yield).

A. attenuatus was collected from rocks and trees of very steep slopes in the forest of West Virginia in September, 1985. As shown in the scheme, extraction of the air dried moss (27 kg) was performed as that of *C. crispifolium* to give the active 10% aqueous methanol extract (59 g, ED_{50}, 9PS = 5×10^{-1}, 9KB = 7×10^{-1}, HT-29 = 2×10^{-1} and A-549 = 3×10^{-1} µg/ml; %T/C of P-388 in mice = 151–200 mg/kg). Silica gel column chromatography (2 kg, 10 × 100 cm) eluted with 20 l of chloroform, 30 l of 2% and 20 l of 5% methanol in chloroform gave 6 major fractions (A–F). The active fraction C (8.2 g, HT-29 $ED_{50} = 2 \times 10^{-2}$ µg/ml), eluted by 2% methanol in chloroform between volumes 28–39.5 l, was further purified (2 g at a time) by a Sephadex LH-20 column (250 g, 4 × 100 cm). Elution (20 ml-fractions) was begun with 1.5 l of dichloromethane followed by 2 l of 20% methanol in chloroform and 1 l of methanol. Fraction C-2 (0.8 g), eluted between volumes 1380–2120 ml, was rechromatographed on a Sephadex LH-20 column (150 g, 2 × 100 cm) using 1.5 l of

dichloromethane-hexane (1:1) as eluent. Fractions (20 ml each) were collected and combined into four fractions. The eluting volume (860–1220 ml) provided fraction C-2c (125 mg) which had activity against HT-29 at $ED_{50} < 10^{-3}$ µg/ml. Using Florisil column chromatography (60 g, 2 × 70 cm) with 2 l of chloroform and increasing methanol in chloroform (0.6 l each) as eluant concentrated the activity in a 5 mg fraction (C-2c.6) which was eluted by 20% methanol in chloroform. This active fraction was finally purified by a C-18F reversed phase TLC plate (Whatman 0.20 mm) developing with a mixture solvent of acetonitrile, methanol and water (1:6:3) to obtain about 1.2 mg of compound **1** (4×10^{-6}% yield).

Compound **1** was optically active ($[\alpha]_D^{20} = -75°$, C = 0.1, $CHCl_3$). The 470 MHz ^1H *nmr* spectral analysis (table) and spin decoupling experiments of **1** indicated the presence of six molecular fragments, three methyl groups connecting to heteroatoms, one tertiary methyl group and two protons which were exchangeable with D_2O. The FAB mass spectrum of **1** using DTT/DTE as a matrix showed protonated molecular ion peaks at m/z 635 (26%), and m/z 637 (12%) with the relative intensity indicating the presence of one chlorine atom. The *uv* spectrum of **1** showed maximum absorption at 232, 240 (sh), 252, 280 and 288 nm; and the *ir* spectrum showed carbonyl absorptions at 1730, 1700, 1650 cm^{-1} as well as C-C double bond absorption at 1560 cm^{-1}. These data are typical of the maytansinoid group[8,9]. Comparison of the *uv*, *ir*, ^1H-*nmr* spectral data of **1** with literature data[6,8,9] and direct comparison of **1** with the authentic sample clearly confirmed that **1** is identical to ansamitocin P-3. The absolute configurations of maytansinoids were established to be 3S, 4S, 5S, 6R, 7S, 9S and 10R[8,9]. Further biological evaluation of ansamitocin P-3 showed potent cytotoxicity against the human solid tumor cell line systems A-549 (lung carcinoma), HT-29 (colon adenocarcinoma) and MCF-7 (breast adenocarcinoma) at $ED_{50} = 4 \times 10^{-7}$, 4×10^{-7}, 2×10^{-6} µg/ml, respectively. The correlation of this cytotoxicity to the dose response of the crude active fractions confirmed that ansamitocin P-3 was responsible for antitumor activity in these two thuidiaceous mosses, *C. crispifolium* and *A. attenuatus*. Recently, Sakai et al.[7] reported the first occurrence of maytansinoids including a new derivative, 15-methoxyansamitocin P-3, from the Japanese mosses *Isothecium sudiversiforme* (Lembophyllaceae) and *Thamnobryum sandei* (Neckeraceae). Ansamitocin P-3 is a member of the group of maytansinoids[8,9] which were previously isolated from the culture broth of *Nocardia* sp.

The variation in bioactivity (9KB, $ED_{50} = 2 \times 10^{0} - 6 \times 10^{-2}$ µg/ml and P-388 %T/C = 123–156) among nine samples of *C. crispifolium* collected from various locations in California and Oregon[10] suggested that other organisms might account for the bioactivity of the moss. The co-occurrence of ansamitocin P-3 in mosses in very low yield and in the actinomycete *Nocardia* might suggest a possible association between the active mosses and the maytansinoid-producing microorganisms. The actinomycetes are described as prokaryotic bacteria having the ability to form branching hyphae. Mycelial-like substances were frequently observed underneath moss carpets when collecting the moss samples during rainy periods and it was impractical to clean the moss samples absolutely free of foreign materials including these mycelia. Although, moss-actinorhizal relationships are not known, many species of bacteria and fungi also grow among mosses and in some cases their associations are specific[11–14]. Spiess et al.[11] have hypothesized that moss-associated bacteria induced moss to produce a particular substance(s) and facilitated the moss growth. However, we cannot rule out the possibility that the moss itself produces maytansinoids as an alleopathic response to the microorganisms[6]. To clarify the source of maytansinoids precisely, further research is currently underway to isolate and identify the maytansinoid-producing microorganism(s) from the moss samples.

470 MHz ^1H *nmr* data of **1**[a].

Proton	δ, J	Proton	δ, J
2a	2.20 dd(3.0, 13.8)	11	5.46 dd(9.0, 15.5)
2b	2.56 dd(12.0, 13.8)	12	6.44 dd(11.2, 15.5)
3	4.82 dd(3.0, 12.0)	13	6.16 bd(11.2)
4-CH_3	0.82 s	14-CH_3	1.70 bs
5	2.96 d(9.7)	15a	3.20 d(12.8)
6-CH_3	1.29 d(7.1)	15b	3.52 d(12.8)
6	1.45 m(7.1, 9.7, 11.0)	17	6.88 d(1.5)
7	4.27 bt(11.0)	18-NCH_3	3.16 s
8a	1.23 dd(11.0, 13.7)	20-OCH_3	3.99 s
8b	1.64 bd(13.7)	21	6.84 d(1.5)
9-OH	3.00 s	2′	2.60 m(6.8, 7.1)
9-NHCO-	6.21 bs	2′-CH_3	1.21 d(6.8)
10	3.50 d(9.0)	2′-CH_3	1.28 d(7.1)
10-OCH_3	3.36 s		

[a] Data were recorded in $CDCl_3$ solution (δ in ppm, J in Hz), using TMS as an internal reference on a Nicolet NT-470 spectrometer.

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Corrigendum

Due to a regrettable technical error parts of the multi-author review (Phylogeny and Function of the Pineal/Experientia 45/10) contribution, 'The pineal and melatonin: Regulators of circadian function in lower vertebrates' by H. Underwood, were misplaced in the final text. We are therefore reprinting the corrected article in its entirety. When citing, please refer to both publications:

Experientia 45 (1989) 914–922; 46 (1990) 120–128.

The pineal and melatonin: Regulators of circadian function in lower vertebrates

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Summary. The pineal has been identified as a major circadian pacemaker within the circadian system of a number of lower vertebrates although other pacemaking sites have been implicated as well. The rhythmic synthesis and secretion of the pineal hormone, melatonin, is suggested as the mechanism by which the pineal controls circadian oscillators located elsewhere. Both light and temperature cycles can entrain the pineal melatonin rhythm. The pineal, therefore, acts as a photo and thermoendocrine transducer which functions to synchronize internal cycle with cycles in the environment. A model is presented which portrays the pineal as a major component of a 'multioscillator' circadian system and which suggests how these multiple circadian clocks are coupled to each other and to cycles of light and temperature in the external world.
Key words. Pineal; melatonin; circadian rhythm.

Organisms, from unicellulars to vertebrates, are structured in time as well as in space. Many, if not most, biochemical, physiological and behavioral parameters exhibited by organisms show daily fluctuations. Significantly, most of these daily rhythms will persist under constant conditions, with periods near 24 h in length, showing that they are driven by an internal daily or 'circadian clock'. Under natural conditions these rhythms are 'entrained' by environmental stimuli (such as light and temperature) so that the 'right' events occur at the 'right time of day'.

The use of the term 'circadian clock' does not mean that a single discrete 'clock' is responsible for driving all of an organism's daily rhythms. In recent years it has become appreciated that multicellular organisms are multi-oscillator in nature; that is, more than one circadian clock may exist within a single organism. Normally, however, all of an organism's multiple circadian rhythms exhibit fixed phase relationships with each other under both entrained and freerunning conditions. Accordingly, when multiple clocks exist within an individual organism, they must be coupled together in some manner.

Several areas have been implicated as being important to circadian organization in vertebrates; these include the pineal organ, the suprachiasmatic nuclei (SCN) of the hypothalamus, and the lateral eyes. Interesting similarities, as well as significant differences, seem to exist between species in the relative roles that these areas play within an animal's circadian system.

Among lower vertebrates most of the studies to date have focused on the role of the pineal organ. Embryologically, the pineal arises as an evagination of the roof of the