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Occurrence and non-detectability of maytansinoids in individual plants of the genera *Maytenus* and *Putterlickia*

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Dedicated to Meinhart H. Zenk on his 70th birthday

Abstract

Individual plants belonging to different species of the family Celastraceae collected from their natural habitats in South Africa (*Putterlickia verrucosa* (E. Meyer ex Sonder) Szyszyl., *Putterlickia pyracantha* (L.) Szyszyl., *Putterlickia retrospinosa* van Wyk and Mostert) and Brazil (*Maytenus ilicifolia* Mart. ex Reiss., *Maytenus evonymoides* Reiss., *Maytenus aquifolia* Mart.) were investigated for the presence of maytansinoids and of maytansine, an ansamycin of high cytotoxic activity. Maytansinoids were not detectable in plants grown in Brazil. Analysis of plants growing in South Africa, however, showed clearly that maytansinoids were present in some individual plants but were not detectable in others. Molecular biological analysis of a *Putterlickia verrucosa* cell culture gave no evidence for the presence of the aminohydroxybenzoate synthase gene which is unique to the biosynthesis of aminohydroxybenzoate, a precursor of the ansamycins including maytansinoids. Moreover, this gene was not detectable in DNA extracted from the aerial parts of *Putterlickia* plants. In contrast, observations indicate that this gene may be present in microbes of the rhizosphere of *Putterlickia* plants. Our observations are discussed with respect to the possibility that the roots of *Putterlickia* plants may be associated with microorganisms which are responsible for the biosynthesis of maytansine or maytansinoids.

Keywords: *Putterlickia verrucosa*; *Putterlickia* species; *Maytenus* species; Maytansine; Maytansinoids; Aminohydroxybenzoate synthase gene; Microbes; Rhizosphere

1. Introduction

Ansamycins (Lancini, 1986) are a group of compounds comprising rifamycins, naphthomycins, geldanamycins, streptovaricins and maytansinoids. These metabolites consist of an aliphatic ansa chain which is linked to either a benzenic or naphthalenic chromophore. Ansamycins often exhibit antibiotic activity. Some maytansinoids are highly cytotoxic (Komoda and Kishi, 1980; Reider and Roland, 1984; Smith and Powell, 1984) and have been used in clinical trials (Issell and Crooke, 1978) as well as in experimental systems designed to exploit their potent antitumor activity (Chari et al., 1992; Okamoto et al., 1992; Liu et al., 1996).

The biosynthesis of ansamycins in general (August et al., 1998; Schupp et al., 1998; Chen et al., 1999) and of maytansinoids in particular (Yu et al., 2002) is currentlyunder intense investigation. The process is initiated by aminohydroxybenzoic acid (AHBA) as the starter unit (Hatano et al., 1982; Kim et al., 1998), which is chain-extended by acetate, propionate or glucose-derived chain extension units (Hatano et al., 1985) on a type I polyketide synthase to give the ansa chain. Various post-PKS

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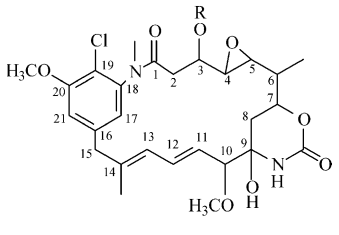
modifying enzymes then introduce additional substituents to yield the biologically active end-products.

Maytansine (**1**) (Fig. 1) was the first maytansinoid isolated. In their work, Kupchan and coworkers (1972, 1977) used *Maytenus ovatus* Loes, *Maytenus serrata* (Hochst. ex A. Rich) R. Wilczak as well as *Putterlickia verrucosa* as a source. While the latter plants are indigenous to Africa, *Maytenus ilicifolia* occurs in South America (Brazil, Uruguay, Paraguay, Argentina) and has also been reported to contain maytansinoids (Ahmed et al., 1981). Plants of the genus *Maytenus* and *Putterlickia* belong to the family Celastraceae. Maytansinoid compounds are also present in *Colubrina texensis* Gray (Rhamnaceae) (Wani et al., 1973) and *Trevia nudiflora* L. (Euphorbiaceae) (Powell et al., 1982).

Notably, maytansinoids have also been isolated from four different species of mosses, namely *Claopodium crispifolium* (Hook) Ren. & Card. and *Anomodon attenuatus* (Hedw.) Hueb (Thuidiaceae) (Suwanborirux et al., 1990), *Isothecium subdiversiforme* Broth. (Lembopyllaceae) and *Thamnobryum sandei* (Besch.) Iwatsuki (Neckeraceae) (Sakai et al., 1988). Two of these mosses occur in Oregon (USA) (*Anomodon* and *Claopodium*) whereas two are indigenous to Japan (*Isothecium* and *Thamnobrium*). The four moss species belong to three different families. Remarkably, maytansinoids occur also in bacteria (Higashide et al., 1977; Asai et al., 1978). *Actinosynnema pretiosum* (formerly *Nocardia* sp. No. C-15003), which has been isolated from an undetermined *Carex* species, produces maytansinoids which are called ansamitocins.

The naturally occurring maytansinoids differ mainly by the presence or absence of a substituent at carbon 15 or in the structure of the acyl function attached to the C-3 hydroxyl group. An acid component may also be linked to both the aryl amide nitrogen and the 3-hydroxyl group, thus forming a second ring system.

Natural products are often used as chemotaxonomic markers in order to investigate evolutionary relationships between plant taxa (Hegnauer, 1962–1992). Occurrence of structurally related or identical natural products in different taxa is generally thought to indicate that these taxa are evolutionarily related. The occurrence of maytansinoid compounds in such diverse taxa as bacteria, mosses and higher plants excludes the possibility that the presence of maytansinoids can be taken as an indication of any close evolutionary link between these taxa. It seems possible, however, that during evolution a horizontal gene transfer occurred between different, taxonomically unrelated species. This might explain why maytansinoids are distantly distributed between pro- and eucaryotes. Alternatively, it cannot be excluded that microbial colonization by maytansinoid-producing microorganisms is responsible for the occurrence of maytansinoids in the diverse plant taxa. The fact that the molecular biological basis of maytansinoid biosynthesis is presently being explored (Yu et al., 2002), provides new tools to address these questions. The data reported herein show that the first alternative (horizontal gene transfer) is less likely whereas the second possibility (microbial colonization) may be the more likely explanation for the occurrence of maytansinoids in such diverse taxa.



1. R= $COCH(CH_3)N(CH_3)COCH_3$
2. R= $COCH(CH_3)N(CH_3)COCH_2CH_3$
3. R= $COCH(CH_3)N(CH_3)COCH(CH_3)_2$

Fig. 1. Structure of maytansine (**1**), maytanprine (**2**), and maytanbutine (**3**).

2. Results

2.1. Plant material

Individual plants growing in South Africa, in Brazil and in the greenhouse of the Institut für Pharmazeutische Biologie in Bonn were collected and analyzed separately for the presence of maytansinoids. Herbarium specimens taken from South African plants were identified by A. v. W. and deposited in the H. G. W. J. Schweickerdt Herbarium of the Botanical Institute in Pretoria and in the herbarium of the Institute in Bonn, Germany. Brazilian plants were identified by A. M. P. and Dr. Rita Maria de Carvalho-Okano and herbarium specimens deposited in the herbarium in Bonn. Plants collected in the field were characterized with respect to their habitat and geographical position following the "Quarter Degree Grid Reference System" (QDGRS) as outlined in the Experimental Section. Most of the plants described in detail were collected from their natural habitat in South Africa. Some plants indigenous to South Africa, however, were analyzed after collection from locations outside their natural habitats. These were one *Putterlickia verrucosa* plant growing in the Botanical garden in Pretoria (South Africa) (plant A, Table 1) and plants growing in the greenhouse of the Institut für Pharmazeutische Biologie in Bonn. Maytansine was isolated by a published procedure (Nettleton et al., 1981). The amount of maytansine present in an extract was determined quantitatively using HPLC calibrated with an authentic sample of maytansine. The minimum amount of maytansine detectable by HPLC was 10 ng. The recovery yield for an authentic sample of maytansine (20 μg) added to a plant extract devoid of

Table 1
Description of geographical location, habitat and occurrence of maytansinoids in individual *Putterlickia verrucosa* (E. Meyer ex Sond.) Szyszyl. plants. Limit of detection of maytansine in extracts of wood stems: 0.05 mg/kg of powdered wood. The QDGRS system is described in the Experimental Section. n.d. = not detectable. In the bioassay the heliozoon *Actinophris sol* was employed

Designation of plants	Locality	Biotope (elevation) (pH of soil)	QDGRS	Date of collection	Amount of maytansine (mg/kg wood)	Additional maytansinoids likely to be present	Bioassay (*Actinophris sol*) positive or negative
Putterlickia verrucosa							
A	Pretoria, Botanical Garden	Semi shade to sunny; (1000 m); (5.9)	2528 CC	03.04. 97	0.8	Yes	Pos.
B	KwaZulu-Natal, Pietermaritzburg district	Shade, small trail (300 m) (6.1)	2930 DC	08.04.97	n.d.	n.d.	Neg.
C	KwaZulu-Natal, Pietermaritzburg district	Semi shade, riverside (300 m) (4.8)	2930 DC	08.04.97	n.d.	n.d.	Neg.
D	KwaZulu-Natal, Pietermaritzburg district	Sunny, riverside (300 m) (4.9)	2930 DC	08.04.97	n.d.	n.d.	Neg.
E	KwaZulu-Natal, Hawaan Forest	Semi shade, dune forest (50 m) (6.3)	2931 CA	08.04.97	1.6	Yes	Pos.
F	KwaZulu-Natal, Amanzimtoti	Semi shade, forest (50 m) (4.8)	3030 BB	09.04.97	0.9	Yes	Pos.
J	Eastern Cape Bay	Sunny, bushfield (30 m) (5.2)	3227 DD	15.04.97	1.2	Yes	Pos.
U	KwaZulu-Natal, Hawaan Forest	Semi shade, dune forest (30 m) (6.6)	2931CA	22.05.98	n.d.	n.d.	Neg.
V	KwaZulu-Natal, Hawaan Forest (Greenhouse in Bonn)	Semi shade, dune forest (30 m) (6.7)	2931CA	22.05.98	0.7	Yes	Pos.
W	Kwazulu –Natal, Hawaan Forest (Greenhouse in Bonn)	Semi shade, dune forest (30 m) (6.6)	2931CA	22.05.98	n.d.	n.d.	Neg.

any maytansine was found to be 80%. The identity of a maytansine sample isolated by HPLC was checked by MALDI-TOF and ESI-MS-mass spectrometry. The resulting spectra were compared to those of an authentic sample. The antibiotic activity of a maytansine-containing fraction was additionally assayed qualitatively and quantitatively using *Penicillium avellaneum* UC 4376 as a test organism. The cytostatic activity of the isolated maytansine fraction was also checked by means of either the heliozoon *Actinophris sol* Ehrenberg or *Actinosphaerium eichhorni* Ehrenberg. These protozoons have axopods containing microtubules which react to maytansine by disintegration of microtubules and retraction of the axopods. This was visualized microscopically. This technique is suitable to distinguish between microtubule stabilizing cytostatics (such as paclitaxel) and microtubule degrading cytostatics (such as maytansine or vinblastine) (Fig. 2). In the present experiments in every case microtubule degrading activity was observed when the presence of maytansine was indicated by mass spectrometry.

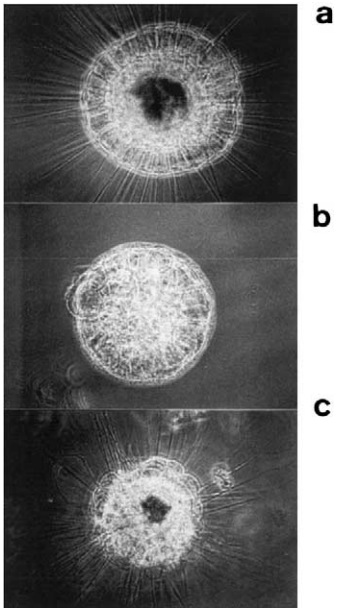


Fig. 2. The heliozoon *Actinosphaerium eichhorni* (a) and its reaction to a solution (1 μM) of maytansine (b). The same changes are observed in the presence of vinblastine, vincristine or vindesine. When paclitaxel is applied axopods appear in a more irregular arrangement (c) compared to (a).

2.2. Distribution of maytansine within a Putterlickia verrucosa plant

Ground wood (3.225 kg) of plant E (*Putterlickia verrucosa*) (Table 1) obtained from stems and twigs was analyzed and found to contain 1.6 mg of maytansine per kg of wood. Signals in the MALDI-TOF mass spectrum suggested also the presence of maytanprine (**2**) (706 + 1 + 1; H^+ adduct; 728 + 1 + 1: Na^+ adduct) and maytanbutine (**3**) (742 + 1 + 1; Na^+-adduct). No authentic samples of these compounds were at hand,

however. Thus, only maytansine could be definitely proven to be present.

By the same criteria the presence of the same maytansinoid compounds was observed when the root system of the plant was worked up. The amounts present matched those of the branches on a weight per weight basis. When the bark and the xylem of a branch were analyzed separately no antibiotic activity against *Penicillium avellaneum* was found in the extract of the bark, the activity resided in the central cylinder (xylem) of the stems.

Young twigs and leaves did not seem to contain maytansinoids: We obtained leaves and twigs of *Maytenus ovatus* through the courtesy of Dr. John M. Cassady, The Ohio State University. This material was derived from the same *Maytenus ovatus* plant from the stems of which Kupchan et al. (1972) had isolated maytansine for the first time. We found that this material (340 g) did not contain detectable amounts of maytansine.

2.3. Analysis of individual South African plants

Results on South African plants are listed in Tables 1 and 2. The fact that there are no reports on the occurrence of maytansinoids in *Putterlickia retrospinosa* may be due to the fact that this species had not been described until 1987 (van Wyk and Mostert, 1987). This plant is endemic to the sandstone region of Southern Natal Pondoland. Two individual plants (G and X) (Table 2) were worked up. In both biotest systems (*Penicillium avellaneum* and *Actinophris sol*) a positive response to extracts of the plants was observed. MALDI-TOF MS suggested the presence of maytansine in the case of plant X and of maytansinoids other than maytansine (mass 722 + 1 + 1 and 744 + 1 + 1) in both plant G and plant X. This constitutes the first report of maytansinoids in *Putterlickia retrospinosa*.

Similar observations were made with *Putterlickia pyracantha* which occurs in the coastal forest of the Eastern Cape area. One individual plant (K) was worked up and found to contain maytansine in a rather low concentration (Table 2). By contrast, a *Putterlickia pyracantha* plant which was kept for many years in the greenhouse in Bonn was apparently devoid of maytansinoids. This parallels the situation in *Putterlickia verrucosa* (Table 1). The data show that there are individual plants which definitely do contain maytansine (plants A, E, F, J, V, X, K) whereas in extracts of other plants (B, C, D, U, W) no maytansine is found.

The plants listed in Table 1, except plant A, were collected from their natural habitat extending along the coast of the Indian Ocean South East of the Drakensberg mountain range. One *Putterlickia verrucosa* plant (plant A, Table 1) growing outside of this habitat in the Botanical garden in Pretoria was also analyzed and was clearly found to contain 0.8 mg per kg of maytansine. This plant had been raised in the Botanical garden from seeds (Dr. Robert Archer, National Botanical Institute, Pretoria, personal communication). Cuttings of a length of 20–30 cm of the terminal twigs of this particular plant had been taken in 1993, immediately brought to Bonn, Germany, by airplane and the cuttings rooted in the greenhouse. In 1998 when these plants (not listed in Table 1) were analyzed they had a height of 2 m, flowered and produced fruits. Two individual plants were investigated but in no case was any trace of maytansinoids detectable.

Plants V and W (Table 1) were young plants (20–30 cm height) growing in the Hawaan Forest in South Africa in the vicinity of plant U, an adult plant. Both the young plants V and W were brought intact to Germany in 1998 together with their root system and the soil of their African biotope. These plants were kept in the greenhouse in Bonn and were analyzed for maytansine in 2001. At this time the plants had a height of 100 cm.While plant V clearly contained maytansine (Table 1), this compound was not detectable in plant W which has the same "history".

Table 2
Description of geographical location, habitat of plants and possible occurrence of maytansinoids in *Putterlickia retrospinosa* van Wyk and Mostert and *Putterlickia pyracantha* (L.) Szyszyl. Limit of detection of maytansine in extracts of wood stems: 0.05 mg/kg of powdered wood. The QDGRS system is described in the Experimental section. n.d. = not detectable. In the bioassay the heliozoon *Actinophris sol* was employed except in the case of plant K where *Actinosphaerium eichhorni* was used

Designation of plant	Locality	Biotope (elevation) (pH of soil)	QDGRS	Date of collection	Amount of maytansine (mg/kg wood)	Additional maytansinoids likely to be present	Bioassay (with heliozoon) positive or negative
Putterlickia retrospinosa							
G	KwaZulu-Natal Port Edward	Semi shade shrub island in grass land (350 m) (4.8)	3130 AA	13.04. 97	n.d.	yes	pos.
X	KwaZulu-Natal	Semi shade tree island in grass land (300 m) (5.0)	3031 CC	23.05.98	3.1	yes	pos.
Putterlickia pyracantha							
K	Eastern Cape Beacon Bay	Slope of a dune (550 m) (5.2)	3227 DD	15.04.97	0.5	n.d.	pos.

From the experiments no pattern could be deduced which would enable us to predict which individual plants will contain maytansine or maytansinoids. These observations raised doubts as to the capacity of the *Putterlickia verrucosa* plant to produce maytansinoids by itself. It was concluded that microorganisms associated with the plants might be responsible for the occurrence of maytansinoids within the plants.

2.4. Analysis of individual Brazilian plants

Celastraceae collected in Brazil and belonging to the following species were analyzed for the presence of maytansinoids: *Maytenus ilicifolia* (7 plants), *Maytenus aquifolia* (14 plants), *Maytenus evonymoides* (2 plants), and one undetermined *Maytenus* species (1 plant). Among these *Maytenus ilicifolia* is the only Brazilian species reported to contain maytansinoids, such as maytansine (**1**), maytanprine (**2**) and maytanbutine (**3**) (Ahmed et al., 1981). In a preliminary screening samples from 24 individual plants belonging to the above-mentioned species were collected from different biotopes in Brazil. These biotopes included natural habitats of the plants in the provinces of Botucatu, Contenda, Araucaria, Sao Jose dos Pintas as well as the plantation of the university UNAERP in Ribeirão Preto. Extracts of these plant samples were checked for their antibiotic activity using the maytansine sensitive *Penicillium avellaneum* as the test organism. Growth of this fungus was not inhibited by 19 different plant extracts while in some samples the result was unclear. Therefore five plants listed in Table 3 and collected from different sites were analyzed in more detail. Wooden slices of the stems were placed directly onto test agar containing spores of *Penicillium avellaneum* and the agar plate incubated. No inhibitory activity was observed. Extracts of these plants were also checked by bioautography and by MALDI-TOF mass spectrometry without any indication that maytansine (**1**) was present.

Although a total of seven individual *Maytenus ilicifolia* plants were analyzed no maytansine, maytanbutine or maytanprinewas detected in any one of these plants. This contrasts with the report by Ahmed et al. (1981) who stated that these compounds were detectable by co-chromatography (HPLC) with authentic samples.

In summary, we were unable to detect maytansinoids in any one of the plants collected in Brazil.

2.5. Chemical and genetic analysis of a cell culture of Putterlickia verrucosa

In order to investigate the possible microbial colonization of the *Putterlickia verrucosa* plants (Table 1) a callus culture was established. Callus cultures are believed to be sterile and therefore production of maytansinoids by these cultures would have been evidence that the plant cells rather than any microorganisms are responsible for the synthesis of maytansinoids. However, we were never able to detect any maytansinoids in these cultured cells. It was, of course, not possible to decide whether the plant cells do not have the genetic capacity to produce maytansinoids or are simply unable to do so under the culture conditions. We therefore decided to check the cultured cells for the presence of genes known to be involved in bacterial maytansinoid biosynthesis (Yu et al., 2002). Two techniques, Southern blot experiments and polymerase chain reaction with sequencing were employed. For the Southern blot experiments, probes derived from genes involved in an early step (AHBA synthase, *asm 24* and *asm 43*) and a late step (amide synthase, *asm 9*) (Yu et al., 2002) in maytansinoid biosynthesis were chosen. While both probes hybridized to DNA from *Actinosynnema pretiosum*, the maytansinoid producing bacterium, no reaction was observed with DNA from the *Putterlickia verrucosa* cell suspension culture. This may indicate that genes involved in maytansinoid biosynthesis are not present, but it cannot be excluded that these negative results are due to a low homology between the bacterial and putative plant genes responsible for maytansinoid biosynthesis.

Before experiments were started employing the polymerase chain reaction, the codon usage of the *Putterlickia verrucosa* plant was elucidated by sequencing two DAHP synthase (E.C. 4.1.2.15) genes cloned from the

Table 3
Description of habitat and geographical location of Brazilian *Maytenus* plants investigated for the presence of maytansinoids. The QDGRS system is described in the Experimental section

Designation of plants	Locality	Biotope (elevation) (pH of soil)	QDGRS	Date of collection
Maytenus aquifolia				
MaB	Botucatu district, Sao Paulo Reserva da Mata Atlantica	Atlantic rain forest (600 m) (7.2)	4822 DC	20.04.99
14	Plantation of the university UNAERP in Ribeirão Preto	Sunny (1000 m) (5.6)	4721AA	17.06.99
Maytenus evonymoides				
32	Municipio des São José dos Pinhais, Curritiba, Parana	Atlantic rain forest (2000 m) (4.1)	5025 DA	11.06.99
Maytenus ilicifolia				
35	Araucaria district, Ana Christina, Parana	Riverside (1500 m) (4.5)	5025 DA	11.06.99
36	Araucaria Contenda	Roadside (1500 m) (5.5)	5025 CD	11.06.99

cultured cells (see Experimental section). The codon preference deduced from these sequences was then used to design oligonucleotides homologuous to the AHBA synthase genes in *Actinosynnema pretiosum* (Yu et al., 2002). The oligonucleotides were employed in different combinations with DNA from the cell culture as template. Fifteen DNA sequences of the expected size were found and sequenced, but none was homologous to any of the known AHBA synthase genes of *Actinosynnema pretiosum*. While oligonucleotides for this first set of PCR experiments were derived from randomly picked sequences of the AHBA synthase genes of *Actinosynnema pretiosum*, oligonucleotides for a second set of PCR experiments were targeted to highly conserved sequences of the hitherto known (Kim et al., 1998; Chen et al., 1999; Mao et al., 1999; Yu et al., 2002) AHBA synthase genes present in different bacteria. A total of 5 forward and 5 reverse primers were designed and employed in 21 different combinations. Each combination was tested with different annealing temperatures, with and without DMSO included in the reaction mixture. Only one DNA fragment of the expected size was observed and sequenced but showed no homology to any known AHBA synthase gene. Thus, no indication was found that such a gene is present in *Putterlickia verrucosa*.

2.6. Further search for microbial genes involved in maytansinoid biosynthesis

The observations reported above would be consistent with the assumption that a microbe, a bacterium or a fungus, is responsible for the occurrence of maytansinoids in Celastraceae plants. In an attempt to find evidence for such a notion we incubated hairy roots collected from plant E (Table 1) in liquid ISP_2-Medium. It was hoped that the microorganisms adhering to the root would grow and multiply under these conditions.

Aliquots of the culture which comprised many different microbial organisms were centrifuged 24, 48 and 72 h after inoculation and the DNA extracted from the pellet. Polymerase chain reaction with oligonucleotides 1f and 2r (see Experimental section) targeted to the AHBA synthase gene resulted in the appearance of a 400 bp DNA band which was sequenced and found to exhibit a GC-content (68.9%) typical of *Actinomycetes*. This sequence had 79.2% homology to the AHBA synthase gene from *Amycolatopsis mediterranei* (Kim et al., 1998) and 81.7 and 80.8% homology, respectively, to the two AHBA synthase genes present in *Actinosynnema pretiosum* (Yu et al., 2002). This sequence was only detectable 48 or 72 h but not 24 h after inoculation.

AHBA synthase contains a typical phosphate binding motif with a conserved aspartate (Asp-159) (Kim et al., 1998). The oligonucleotide primers selected for our PCR experiment were designed in such a way that this motif, but not the active site lysine (Lys-188) (Kim et al., 1998), which is also present in the gene, would be detectable. Sequencing of the 400 bp PCR fragment showed that this was indeed observed.

The 400 bp sequence was not detected in DNA extracts from the branches of plant E. These experiments were repeated with branches and root hairs from plant F which has also been shown to contain maytansinoids (Table 1). Essentially the same observations were made.

Sequences homologuous to the known AHBA synthase genes were observed in five out of ten experiments. The fact that these sequences were not observed in every single case may be due to the experimental design in which DNA was not extracted from a single defined microorganism but from a mixture of microbes.

All attempts, however, to isolate from these plants a microorganism which produces maytansine (**1**) in culture have so far met with failure.

3. Discussion

No evidence was found that maytansinoids are present in 24 individual *Maytenus* plants (*Maytenus aquifolia*—14 plants, *Maytenus evonymoides*—2 plants, *Maytenus ilicifolia*—7 plants, and one undetermined species) collected in Brazil. Among the species tested maytansinoids (maytansine, maytanprine, maytanbutine) were only reported to occur in *Maytenus ilicifolia* (Ahmed et al., 1981). We were unable, however, to detect maytansinoids in *Maytenus ilicifolia* plants. The previous report is based on HPLC analysis and co-chromatography using authentic samples. Strangely, the reported peaks were detected after aqueous extraction of plant material, whereas maytansinoids are lipophilic compounds. In contrast to Ahmed et al. (1981) we used not water but ethanol for the extraction of our plant material. It was possible to detect the same peaks reported previously, but after changing the chromatographic conditions, the peak coinciding with maytansine no longer co-chromatographed with an authentic sample. We therefore question the validity of the identification by Ahmed et al. (1981) of their peaks as maytansinoid compounds. Up to now it has not been shown conclusively that *Maytenus ilicifolia* contains maytansinoids.

Maytansine was also reported to be present in a callus culture (Misawa et al., 1983) obtained from *Putterlickia verrucosa*. A fraction inhibitory against KB cells was obtained which contained a compound that co-chromatographed (TLC, HPLC) with an authentic sample and which showed a UV-spectrum "similar" to that of authentic material. This contrasts with conclusions drawn by Kutney et al. (1981) after analysis of a *Maytenus buchananii* cell culture. While the intact plant

contains maytansine the cultured cells seemed to be devoid of this material. The culture, however, produced cytotoxic triterpene quinone methides, tingenone and 22β-hydroxytingenone. One wonders if compounds of this type were also present in the *Putterlickia verrucosa* cell culture reported by Misawa et al. (1983) and were responsible for the observed inhibitory activity against KB cells. The results of Kutney et al. (1981) are in line with our observations that cell cultures of Celastraceae do not contain detectable amounts of maytansoids. Moreover, we tried to demonstrate the presence of a key gene involved in maytansine biosynthesis in our *Putterlickia* cell culture. This was an obvious approach because two gene clusters have been detected in *Actinosynnema pretiosum* (Yu et al., 2002) which jointly are responsible for the production of maytansinoids (ansamitocins). It was hoped that the information on the bacterial clusters would facilitate detection of corresponding genes in the cell culture DNA. The attempts to identify a gene essential for maytansine biosynthesis were, however, unsuccessful. Thus, we were unable to discover any evidence for the formation of maytansine by cultured *Putterlickia verrucosa* plant cells.

Maytansinoids were clearly detected, however, in two *Putterlickia retrospinosa*, one out of two *Putterlickia pyracantha* and some (A, E, F, J, V) but not all (B, C, D, U, W) *Putterlickia verrucosa* plants. One *Putterlickia verrucosa* plant growing in its African soil in the greenhouse in Bonn (plant V) contained maytansine whereas plants raised from cuttings of plant A seemed to be devoid of maytansine.

The highest amount of maytansine detected in *Putterlickia verrucosa* plant material was reported by Kupchan et al. (1977) to be 13.2 mg/kg. By contrast, we found much lower quantities (Tables 1 and 2) and some plants seemed to be completely devoid of maytansine. The authors of this manuscript are aware that it is impossible to prove that a certain type of natural product (e.g. maytansinoids) does not occur in a particular plant or plant species. The occasional inability to detect maytansinoids in *Putterlickia* plants on the other hand had also been observed by the late Dr. Morris Kupchan and his associates.[1]

[1] In a message to one of us (C. B. P.) Professor A. van Wyk from the Department of Botany in Pretoria reports on a conversation with Dr. Michael Wells, the botanist who collected the plant material for Dr. Morris Kupchan: "Mike tells me he had sent several consignments of *Putterlickia verrucosa* to the USA. THEY JUST COULD NOT FIND ANY MAYTANSINE in all but one of these collections. On the one occasion he collected material of *P. verrucosa* near Hluhluwe in northern KwaZulu-Natal and sent it to the USA. Surprisingly, they found traces of maytansine. However, this was, according to Mike, the only success, out of very many collections. All the MANY other collections he subsequently made from all over the region did not yield any evidence of the compound. They were baffled and exhausted. Their only explanation was that the substance is probably associated with some kind of infective organism".

In principle, the occurrence or non-detectability of secondary metabolites in individual plants of one particular species is a phenomenon known for chemotypes (Hegnauer, 1962–1992). Our experimental approach excludes this possibility, however, because some of the plants grown in our greenhouse in Bonn were raised from cuttings of a maytansinoid-containing plant in the Botanical Garden in Pretoria (plant A, Table 1). Thus, these plants are members of a clone. They were vegetatively propagated and therefore are genetically identical. In spite of this, in green house grown plants maytansinoids are not detectable whereas plant A (the "mother" plant) contains maytansine in an amount exceeding the limit of detection by a factor of 16.

The plants analyzed in this work were collected from different habitats. Thus, it may be argued that the quantitative variability of maytansinoid content is due to different climatic conditions or different stages of development of the plants investigated. We believe that this is not the case, however, because plants V and W (Table 1) are plants of the same biotope, identical developmental stage but different content of maytansine. Moreover, the AHBA synthase genes of maytansinoid biosynthesis which we have cloned and sequenced from the bacterium *Actinosynnema pretiosum* (Yu et al., 2002) were not detectable in DNA-extracts of a *Putterlickia verrucosa* plant and callus culture. We therefore believe that microorganisms may be responsible for the occurrence of maytansinoids within the *Putterlickia* plants. Endophytic fungi are well known to contribute to the spectrum of secondary metabolites found in higher plants (Redlin and Carris, 1996; Strobel and Long, 1998). Endophytic bacteria, however, have also been identified (Hallman et al., 1997; Sturz et al., 2000): They comprise over 129 Gram-negative and Gram-positive species representing over 54 genera. The major bacterial taxa colonizing higher plants belong to the former Pseudomonas group (*Pseudomonas*, *Burkholderia*, *Phyllobacterium*) and Enterobacteriaceae (*Enterobacter*, *Erwinia*, *Klebsiella*). Occasionally, representatives of the genus *Streptomyces* have also been reported (Shoda, 2000; Mundt and Hinkle, 1976). We favour the idea that endophytic bacteria rather than fungi may be responsible for the occurrence of maytansinoids in *Putterlickia* because the different types of ansamycins isolated up to now (Lancini, 1986) are well known bacterial rather than fungal metabolites.

Similar conclusions were drawn by Spjut et al. (1988) who analyzed different samples of the moss *Claopodium crispifolium*, a lower plant which had been shown to contain the maytansinoid compound ansamitocin P3 (Suwanborirux et al., 1990). The cytotoxicity of extracts of moss samples collected in Oregon (USA) from different sites was checked using a KB cell system. A significant variation in cytotoxicity was observed and attributed to variations in ansamitocin P3 content. As in

the present paper, it was assumed that associated microorganisms (cyanobacteria) may be responsible for the occurrence of maytansinoids in the moss.

Maytansinoids are present within a *Putterlickia retrospinosa* and a *Putterlickia verrucosa* plant in the central cylinder of the older branches and roots, but in our experience not in the younger twigs and leaves. These observations may indicate that the microbial (bacterial) colonization of the plant proceeds via the root system. This is in agreement with our observation that a maytansinoid-producing microorganism may be associated with roots of *Putterlickia* plants (*vide supra*). It is also in agreement with the observation that alder trees (*Alnus glutinosa* (L.) Gaertn.) harbour *Actinomycetes* in root nodules producing an antibiotic, alnumycin (Bieber et al., 1998). This example shows that bacterial colonization of roots of a higher plant is possible and that the hosted bacterium is able to produce an antibiotic. Often bacteria are considered to be symbionts, which live in higher plants and play an ecological role as they ward off pathogenic fungi attacking higher plants (Broadbent et al., 1971; Weller, 1988). One such example is *Streptomyces hygroscopicus* var. *geldanus*, a bacterium that produces the ansamycin antibiotic, geldanamycin, and which is able to protect a pea plant (*Pisum sativum* L.) from fungal attack by *Rhizoctonia solani* (Rothrock and Gottlieb, 1984).

The fact that maytansinoids accumulate also in the seeds of *Maytenus rothiana* (Walp) Labreau-Callen and *Trevia nudiflora* could be explained by the assumption that bacteria are subject to movement within plants (Hallman et al., 1997; Sturz et al., 2000).

4. Experimental

4.1. Collection of plant material

Plants belonging to the genus *Putterlickia* (Celastraceae) were collected in South Africa and identified by A. v. W. Herbarium specimens were deposited in the H. G. W. J. Schweickerdt-Herbarium of the Botanical Institute in Pretoria and in the herbarium of the Institut für Pharmazeutische Biologie, Bonn, Germany. The plants and their biotopes were described, soil samples taken, the pH determined and the geographical location of the plants described according to the Quarter Degree Grid Reference System (QDGRS). In this system latitude and longitude are given in degree and the rectangular field between both and the next higher coordinates divided into four fields of equal size. These fields are designated A, B (upper fields) and C, D (lower fields). Each field (A to D) is then subdivided into four smaller fields (A to D) in the same way and the location of the plant described by its position in the large and the small field. In the QDGRS system first the latitude and then the longitude are given followed by the large and the small field. As an example the location of Bonn (Germany) is given as 5007 AA.

The *Putterlickia* plants grown in the greenhouse in Bonn were derived from cuttings (20–30 cm length) taken from a plant (plant A, Table 1) in the Botanical Garden in Pretoria. The plants were rooted in the greenhouse in Bonn and worked up four years thereafter. Two small plants (V and W, Table 1) were brought intact with their African soil to Bonn and also kept in the greenhouse.

The plants collected in Brazil were taken from their natural habitat or from the plantation of the university UNAERP in Ribeirão Preto and immediately extracted. The plants had been identified by A. M. P. and Dr. Rita Maria de Carvalho-Okano. Herbarium specimens of *Maytenus evonymoides*, *Maytenus alaternoides* and *Maytenus ilicifolia* were deposited in the herbarium of the institute in Bonn. Since all analyses of Brazilian plants were negative only those plants and their habitats are given which were analyzed in more detail (Table 3).

4.2. Determination of maytansinoids

Maytansinoids were extracted from plants after the wood was powdered and the extract was fractionated following Nettleton's method (Nettleton et al., 1981). The identity of a fraction co-eluting with an authentic sample of maytansine was also checked by re-chromatography in a different solvent system containing (12:1 v/v) CH_2Cl_2 and a mixture of isopropanol and H_2O (95:5 v/v). Maytansinoids were also analyzed by mass spectrometry and by bioassays as described below.

4.3. Antibiotic activity using Penicillium avellaneum UC 4376

This fungus was previously used by Hanka and Barnett (1974) to test for the presence of maytansine. Extracts were applied to a silica gel chromatography plate which was developed in $CHCl_3$/MeOH (95:5 v/v). After evaporation of the solvent the plate was covered with a thin layer of a warm spore suspension of *Penicillium avellaneum* in the medium described by Hanka and Barnett (1974). The plate was kept at room temperature while the layer cooled down. Subsequently the plate was incubated for 24 h or longer at 42 °C.

4.4. Test for microtubule interacting activity using heliozoons

The heliozoons *Actinophris sol* and *Actinosphaerium eichhorni* were maintained as described by Hauser (1986). The limit of detection was 100 μg/ml (*Actinophris sol*) for maytansine. It was later found that *Actinosphaerium eichhorni* was even more sensitive to

maytansine. The test solution was dissolved in $CHCl_3$ and evaporated on a glass microscope slide. The residue was dissolved in aqueous DMSO (0.1%) (50 μl) and 50 μl of a suspension of the heliozoon was added. Disintegration of microtubules containing axopods was observed microscopically. Aqueous DMSO (0.1%) alone did not affect the microtubules.

A similar test in which the regeneration of cilia of *Tetrahymena pyriformis* W was observed in the presence of a cytostatic agent has been described by Tanida et al. (1979).

4.5. Southern blot experiments

For the generation of probes designed to detect either the AHBA synthase genes (*asm 24* or *asm 43*) or the amide synthase gene (*asm 9*) (Yu et al., 2002) in *Putterlickia verrucosa* cell suspension cultures, PCR reactions were employed. For the probe targeted to the AHBA synthase genes, DNA from *Actinosynnema pretiosum* and a PCR labeling approach was used. The PCR was carried out with the PCR DIG Probe Synthesis Kit of Roche Diagnostics (Mannheim, Germany). The following oligonucleotides (AHBA1f and AHBA2r) were used: (+) GCS GTS ACS AAC GGS ACS CAGG and (−) CSG TCA TSA GCT TSC CGT TCT GG where S stands for G or C. To obtain the amide synthase gene *asm 9*, plasmid pHGF 7579 (Yu et al., 2002) was cut with NdeI and KpnI and the resulting 800 bp fragment isolated by electrophoresis. This fragment was used as a template for random primed DNA labeling with the Klenow fragment. The probe was labeled using the DIG High Prime Kit (Roche Diagnostics, Mannheim, Germany). Southern blot experiments were carried out at low stringency according to the DIG Applications Manual (Roche Diagnostics, Mannheim, Germany).

4.6. Callus culture of Putterlickia verrucosa

A twig of a green house grown plant was removed and surface sterilized as previously described (Zenk et al., 1975). Callus was initiated and maintained on agar medium containing Phytagel (4 g/l) and the components of Gamborg's medium (Gamborg et al., 1968) modified in the following way: (mg/l); $NaH_2PO_4 \times 2\ H_2O$, 172.0; $CaCl_2 \times 2\ H_2O$, 150.0; $(NH_4)_2SO_4$, 134.0; $(NH_4)H_2PO_4$, 230.0; NH_4NO_3, 320.0; $MgSO_4 \times 7\ H_2O$, 247.0; KNO_3, 2530.0; $FeSO_4 \times 7\ H_2O$, 27.85; $Na_2EDTA \times 2\ H_2O$, 37.25; KJ, 0.75; $MnSO_4 \times 4\ H_2O$, 13.2; H_3BO_3, 3.0; $ZnSO_4 \times 7\ H_2O$, 2.0; $Na_2MoO_4 \times 2\ H_2O$, 0.25; $CuSO_4 \times 5\ H_2O$, 0.039; $CoCl_2 \times 6\ H_2O$, 0.025; nicotinic acid, 1.0; thiamine dichloride, 10.0; pyridoxal ×HCl, 1.0; *myo*-inositol, 100.0; kinetin, 1.0; 3,6-dichloro-*o*-anisic acid, 1.0; and (g/l) sucrose, 30.0. The pH was adjusted to 5.5.

4.7. Search by PCR for an AHBA synthase gene in a Putterlickia verrucosa callus culture

First, the codon usage of the *Putterlickia* plant was investigated by PCR using oligonucleotides

(+) ATG GCT GGW CAA TTW GCT AAA C, 22mer;
(−) GAA YTC WAC RTG AGC WCC ATC, 21mer;
(−) CGC ATR TTY TCA GCW CCC AT, 20mer;
(−) AGA TCT AAG AAG GTT GAG AG, 20mer

designed to target highly conserved regions of the two DAHP synthase genes present in higher plants (Görlach et al., 1993). The resulting DNA fragments were isolated by electrophoresis and sequenced. The codon usage was employed to design oligonucleotides consisting of sequences targeted to the known bacterial AHBA synthase genes, however, the third position of the codons was adjusted to the relatively low GC-content observed in *Putterlickia*. The following "wobbled" oligonucleotides were employed:

(+) GAR CAR GGW CAR TGG TGG, 18mer;
(+) GGW ACY GAR GTN ATC GTN CC, 20mer;
(+) GTW CCR GTW GAR GTW GA, 17mer;
(−) CCW GCC ATR TGN ACY GGC AT, 20mer;
(−) TTW CCR TTY TGR AAR CTR AA, 20mer;
(−) ACW GCW CCW CCY TCW CC, 17mer;
(+) CAA GAY GCW GCW CAY GC, 17mer;
(−) GCR AAC ATW GCC ATR TA, 17mer.

W stands for A or T, Y for C or T, S for C or G, R for A or G, N = any nucleotide.

These oligonucleotides were used in various combinations in PCR reactions. 15 DNA fragments of the expected size were isolated and sequenced. None was found to exhibit any homology to the known bacterial AHBA synthase genes or enzymes.

These experiments were repeated with another set of oligonucleotides also adjusted to the codon usage of *Putterlickia verrucosa*:

(+) ACH AAY GGD ACW CAY GC, 17mer;
(+) CCW GCW TTY ACH TTY AT, 17 mer;
(+) ATM ATG CCW GTB CAY ATG GC, 20mer;
(−) WGC RTG WGC WGC WTC YTG, 18mer;
(+) CAR GAT GCW GCW CAY GC, 17mer;
(−) TTH CCR TY TGR AAW GAR AA, 20mer;
(−) GCD GTC ATM AGY TTH CCR TT, 20mer;
(+) TCW TTY CAR AAY GGD AA, 17mer;
(−) ADY CRV WWR TTW GWH CC, 17mer;
(−) WGA RAA YTC RTT MAD YC, 17mer;

M stands for A or C,; R for A or G; W for A or T; Y for C or T; H for A, C or T; D for A, G or T; B stands for G, C or T.

As opposed to the former set of oligonucleotides these were targeted to highly conserved regions of the AHBA synthase gene. No DNA fragment of the expected sequence was observed.

4.8. Search for an AHBA synthase gene in the rhizosphere of plants E and F

A sample of hairy roots and its adhering soil of plants E or F (*Putterlickia verrucosa*) was incubated in ISP2-medium (30 ml) at 28 °C on a rotary shaker (250 rpm). The incubation was terminated by centrifugation after 24, 72 or 120 h. The pellet was used as a source for DNA which was submitted to PCR using oligonucleotides 1f and 2r (vide supra). A 400 bp band was isolated by electrophoresis and sequenced.

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